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Scientific Sessions
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Session Type Options:

- Continuing Education
- Scientific
- Engagement
- Education-Career Advancement
- Historical Highlights
- Informational
- Regional Interest

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Preface

This issue is devoted to the abstracts of the presentations for the Continuing Education courses and Scientific Sessions of the 65th Annual Meeting of the Society of Toxicology, at the San Diego Convention Center, San Diego, California, March 22–25, 2026.

An alphabetical Author Index, cross-referencing the corresponding abstract number(s), begins on page 582.

The issue also contains a Keyword Index (by subject or chemical) of all the presentations, beginning on page 603.

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CE Continuing Education Courses	R Roundtable Sessions
EC Education-Career Development Sessions	RI Regional Interest Sessions
IS Informational Sessions	S Symposium Sessions
PL Platform Sessions	W Workshop Sessions
PS Poster Sessions	

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A. Borrel. Sciome, Research Triangle Park, NC.

New approach methods (NAMs), such as quantitative structure-activity relationship (QSAR) models and read-across (RAX) approaches, are intended to rationalize and reduce the need for further experimental testing on animals. Both these approaches rely on the principle of molecular similarity for assessing property profiles of untested chemicals. Software tools to visualize chemical structures and their experimental and predicted properties employing principles of molecular similarity can make the process of reliably assessing the profiles of untested compounds efficient. This course will explore the concept of chemical similarity and chemical space and their application for toxicity assessment. The first speaker will introduce the concept of similarity, covering both supervised and unsupervised methods. The speaker will discuss the context of toxicity studies, detailing common errors and best practices for effectively using similarity approaches. The second speaker will introduce OrbiTox and ChemMaps.com, presenting them as two powerful tools designed for exploring chemical space. The speaker will explain how these platforms provide access to toxicity data and QSAR models, facilitating the reporting of analogue profiles to support decision-making. By the conclusion of this course, participants will possess a better understanding of chemical similarity and chemical space in relation to toxicity assessment. They will be equipped with the necessary expertise to efficiently navigate ChemMaps and OrbiTox for effectively exploring chemical structure and property landscapes. This training aims to empower attendees with practical skills and insights, fostering informed decision-making and enhancing proficiency in utilizing cutting-edge tools for chemical analysis and toxicological assessment. Learning Objectives: (1) Clearly explain the concepts of chemical similarity, chemical space, and their significance in toxicology assessment. (2) Understand the advantages and limitations of current chemical similarity approaches. (3) Develop proficiency in navigating ChemMaps.com and OrbiTox tools for property profiling. (4) Explore and understand limitations of relationships between chemical structures and toxicology properties.

1002 AM02: Advancing Developmental Immunotoxicology: Human-Relevant Models, Adverse Outcome Pathways, and Translational Tools

F. Sillé. Johns Hopkins University, Baltimore, MD.

The developing immune system is particularly vulnerable to environmental exposures during critical windows from prenatal development through adolescence. These exposures can lead to persistent immune dysfunction, including increased risk of allergy, autoimmunity, infection susceptibility, and chronic inflammation. However, traditional in vivo testing methods for developmental immunotoxicity (DIT) are resource-intensive, lack human relevance, and fail to capture nuanced mechanisms across key developmental windows. This course offers an in-depth and timely look at emerging new approach methodologies (NAMs) that address these issues and have the potential to replace or reduce reliance on animal testing for protection of the developing immune system. Participants will strengthen existing knowledge on how in vitro platforms, including stem cell-derived hematopoietic assays and immune organoids, are being developed and optimized to model early immune development. Novel technologies, such as bone-marrow-on-a-chip and lymphoid organoids, provide physiologically relevant systems to assess developmental immune outcomes, offering higher resolution and mechanistic insight. Molecular frameworks such as adverse outcome pathways (AOPs) are also gaining traction in the field of DIT and serve as important organizational tools for integrating mechanistic knowledge and guiding regulatory strategy. This course will highlight critical components necessary to strengthen existing knowledge of NAMs into validated tools for hazard characterization and identification and regulatory use. These include the development of reliable functional endpoints, the identification and use of appropriate reference compounds, and consensus criteria for assay readiness. Attendees will also gain insights into the evolving regulatory landscape that increasingly emphasizes human relevance, transparency, and scientific confidence in NAMs for health risk decision-making. The course will include a brief concluding panel discussion, with an interactive Q&A with all speakers and the audience on remaining challenges, harmonization, and how the field should advance beyond 2026. By bringing together international leaders in toxicology, immunology, and regulatory science, this course aims to build a shared understanding of where the field stands today and where strategic investment is needed to ensure that DIT NAMs are accepted and implemented to improve public health protection. Learning Objectives: (1) Describe critical developmental windows and molecular targets relevant to immunotoxicity. (2) Compare and evaluate various human-relevant in vitro models used in DIT. (3) Understand the development and use of AOPs in immunotoxicity assessment. (4) Identify appropriate reference chemicals and validation strategies for DIT NAMs. (5) Explore regulatory challenges and scientific pathways for integrating DIT NAMs into risk assessment frameworks. Target Audience: Toxicologists, regulatory scientists, risk assessors, developmental biologists, immunologists, and industry stakeholders engaged in safety assessment and NAM implementation.

1003 AM03: Applications of Machine Learning and Artificial Intelligence Approaches to Advance Toxicology and Environmental Health Sciences

Z. Lin. University of Florida, Gainesville, FL.

Machine learning and artificial intelligence (AI) approaches have significantly contributed to progress in many scientific disciplines, including toxicology, risk assessment, and environmental health. A number of machine learning models have been published to predict the absorption, distribution, metabolism, excretion, and toxicity (ADMET) of chemicals; the degree of environmental pollution, such as air quality and water quality; and the human health outcomes due to environmental chemical exposures. Development of machine learning models to study toxicology and environmental health issues requires multidisciplinary expertise, including mechanistic toxicology, pharmacokinetics, environmental health, computer science, programming, and machine learning algorithms. However, there are not many researchers who have all the relevant expertise. It is common and essential for computational scientists and mechanistic toxicology and environmental health scientists to collaborate to complete machine learning-based projects. However, computational scientists often find it challenging to grasp the ADMET mechanisms of xenobiotics, just as mechanistic toxicologists may struggle to comprehend and apply machine learning models. Additional teaching materials and training opportunities are needed to address this challenge. To address this need and further leverage machine learning approaches to advance toxicology and environmental health, a group of scientists recently completed a dedicated textbook entitled Machine Learning and Artificial Intelligence in Toxicology and Environmental Health. This book introduces the fundamental concepts and principles of widely used machine learning and AI techniques relevant to toxicology and environmental health. It provides practical examples of the applications of machine learning and AI methods to predict ADMET properties of chemicals, air quality, water quality, food safety, antimicrobial risk assessment, and chemical risk assessment. It also includes case studies, sample code, and interactive computer exercises to support the practical implementation of these methods in toxicology and environmental health. This course aims to facilitate the dissemination of the scientific knowledge of this textbook to the field of toxicology and environmental health. Note that this book will only be used to relay and support the science of this course, and it is not a requirement or recommendation of speakers for attendees to purchase. This course will start with a brief overview of the book and an introduction on advances in applying machine learning and AI in the regulatory landscape, followed by individual presentations. The first presentation will introduce how to develop and apply machine learning-based quantitative structure-activity relationship (QSAR) models to predict ADME properties of chemicals. The second presentation will introduce how to build machine learning-empowered physiologically based pharmacokinetic (PBPK) models for chemicals and nanoparticles. The third presentation will demonstrate how to integrate machine learning with cell painting to predict bioactivity based on in vitro assays. The fourth presentation will discuss how to develop machine learning-based QSAR models to predict developmental toxicity. The fifth presentation will explore how machine learning models function in human health risk assessment. The sixth presentation will discuss how to apply generative AI for research translation in toxicology and environmental health and the ethical considerations. Most of these presentations will cover both a theoretical introduction to model development and practical examples to demonstrate each application. This course will introduce both theory and many practical example applications of machine learning approaches in different areas of toxicology. It is expected that this course will be beneficial for a diverse population of SOT attendees, including experimental toxicologists and computational toxicologists at different career stages (e.g., students, postdocs, and senior scientists) across various sectors, including academia, industry, and regulatory agencies.

1004 AM04: Biotherapeutics Impurities: Current Practices in Risk Assessment and Analytical Evaluation

V. Kale. Bristol Myers Squibb, New Brunswick, NJ.

Impurities in biotherapeutics include residual host cell proteins (HCPs), high and low molecular weight species, Protein A, host cell DNA and RNA, lipids, aggregates, process-related contaminants, and product-related variants. Due to their intricate nature, potential impurities in biotherapeutics can have a significant potential impact on safety and efficacy profiles. This course will offer a comprehensive overview of risk assessment of impurities in biotherapeutics. The introductory talk will provide an overview of impurities in biologic products, emphasizing the complex nature of these contaminants and the critical importance of understanding analytical methods, testing strategy, and regulatory expectations. This talk will establish a foundational understanding for analytical considerations, impurity risk assessment, and mitigation strategies. One of the speakers will focus on highlighting sophisticated techniques, like high-performance liquid chromatography (HPLC), mass spectrometry, and capillary electrophoresis, essential for detecting and quantifying impurities from various sources, such as host cells and raw materials. This presentation will outline the current state of analytical technology and methods for impurity testing, including challenges and limitations, as well as advancements that are driving continuous improvements in impurity testing. This course will further discuss the framework for assessing biologic impurities of concern with emphasis on the significance of controlling HCPs as critical quality attributes through comprehensive risk assess-

ments. To understand current industry practices in risk assessment of HCPs, the findings of an IQ DruSafe Impurities Safety Working Group industry-wide survey will be discussed in this course. This survey highlights the current challenges, strategies, and regulatory interactions associated with HCPs in biopharmaceutical development and commercialization. Concluding the session, interactive case studies will engage the audience in practical impurity assessment scenarios using polling tools, fostering dialogue and sharing learning among participants. Overall, this course will provide the audience with a clear understanding of the types of impurities encountered with biopharmaceuticals, fit-for-purpose analytical methods, and the conduct of risk assessment for those impurities.

CE 1005 AM05: Chemical Emergencies: Emergency Response, Toxicology, and Exposure Considerations

R. Nocco. Consultant, Danville, CA.

Chemical emergencies may have adverse impacts on public health as evidenced by landmark incidents: the 1984 Bhopal disaster, the 1995 Tokyo subway attack, and the 2023 East Palestine train derailment. Toxicology plays an essential role in successfully managing the associated risks of these emergencies, which could include outcomes from terrorist (CBNR), military, transportation, and chemical plant incidents. This course will cover (1) the evolution and history of public health emergency exposure guidelines; (2) an overview of current guidelines; (3) public health exposure values; (4) the application of toxicology disciplines to emergency response decisions; (5) the role of toxicologists in the incident management system (NIMS); (6) remedies and treatment; (7) exposure measurements; and (8) risk assessment and risk tolerance criteria. Learning Outcomes: Upon completion, the participant will be able to (1) Develop an understanding of chemical emergencies. (2) Discuss the benefits and limitations of exposure guidelines. (3) Explain how public health exposure guidelines differ from occupational exposure guidelines (OELs). (4) Describe how PH exposure values are developed. (5) Apply toxicology guides in ER situations and in proactive ER planning and process safety.

CE 1006 AM06: Systematic Methods for AOP Development and Application: New Technologies and Tools

B. Meek. University of Ottawa, Ottawa, ON, Canada.

Adverse outcome pathways (AOPs) provide convenient integrating organizational constructs for assembling and evaluating mechanistic information at different levels of biological organization in a form designed to support a range of regulatory applications. These include the development of integrated approaches to testing and assessment (IATA) and chemical specific assessment to inform predictive inference and mode-of-action (MOA) analysis. There has been much experience gained in the last thirteen years, since the introduction of the AOP development program by the Organisation for Economic Co-operation and Development (OECD). Guidance and an associated handbook supports developers in the description and evaluation of AOPs via a publicly available knowledgebase. The program also includes formal peer engagement in the form of coaching for AOP development, external scientific review, and endorsement by parent OECD committees on testing and assessment. Increasing experience in AOP development and application contributes to the continuing evolution of the methodology to meet regulatory needs. This course addresses aspects of the application of systematic review tools in the development and application of AOPs. Areas of evolution include the more systematic consideration of supporting evidence, extension of qualitative weight of evidence considerations to quantitation of key event relationships, the integration of emerging data such as 'omics, and increasing experience in application. This course builds on training developed within the OECD program on best practices of documentation and assessment to support regulatory implementation, focusing particularly on the consideration of systematic workflows and tools in AOP development and practical examples of application. Practical exercises include the application of topic modeling in AOP development. The course is designed to promote critical evaluation of data and familiarity with emerging approaches supporting transparent, systematic AOP development for regulatory and research applications. Following a brief introduction of the course outline and learning objectives, the first presentation will update attendees on how the available guidance and tools for AOP development have evolved to reflect experience with increasing and expanding content and biological space. The second presentation will emphasize the importance of transparently and effectively documenting evidence collection and evaluation by sharing tools and best practices with prospective AOP authors and users. The third presentation outlines methodology adopted to systematically consider available data in the development of an AOP as a basis for an integrated approach to testing and assessment by the European Food Safety Authority, while the fourth addresses the application of 'omics in the context of AOPs. The fifth presentation considers relevant approaches to systematic evidence mapping in AOP development, and the final presentation addresses case studies to assess biological plausibility in hazard assessment integrating systematic review methods in AOP development and consideration. A hands-on exercise addresses the application of topic modeling in AOP development.

CE 1007 PM07: Evolution of OECD GL 497; Integration of NAMs for Skin Sensitisation Hazard and Point of Departure for Application in Risk Assessment

E. Reinke. Inotiv, Research Triangle Park, NC.

Developing approaches for assessing skin sensitization potential and potency of chemicals and ingredients has been an area of active research over many years. Regulatory restrictions on animal use in Europe, increased emphasis on nonanimal approaches globally, and understanding of the mechanisms by which skin sensitization occur has accelerated their development and acceptance. This has resulted in a variety of nonanimal new approach methods (NAMs) aligned with key events described in the adverse outcome pathway (AOP) for skin sensitization; many of which have been validated and evaluated for inclusion into the Organisation for Economic Co-operation and Development (OECD) test guideline program. Generally, these methods are not adequately predictive alone; therefore, these methods have been combined in a series of defined approaches (OECD GL 497) that span use cases for hazard classification, sub-potency classification, and, most recently, point of departure (POD) determination for risk assessment. This course is intended to provide attendees with a broad overview of the advances in skin sensitization testing and assessment since the initial publication of OECD GL 497. The first talk will discuss the OECD test guideline program using the development of the skin sensitization test guidelines, anchored to the mechanisms of skin sensitization as an example. This will be followed by a walkthrough of the updates to OECD GL 497, the evaluation undertaken for these updates, and their potential applications and advantages beyond the original guideline. With these changes, there is interest in developing best practices for how to use these additional data sources. The next talk will discuss the concept of best practices and their application to skin sensitization testing and assessment and the lessons learned. This will be followed by a talk on next-generation risk assessment and the tools available for deriving a POD for a skin sensitization risk assessment. This will culminate in a live demonstration and training in how to conduct a risk assessment through the use of freely available tools, such as SARA-ICE, for deriving a skin sensitization POD. The demonstration will showcase how the model can be applied and utilized in integrated frameworks to ensure a thorough assessment of skin sensitization risk for safety assessment, ultimately leading to safer consumer products and improved regulatory compliance. Upon completion, attendees will have a better understanding of how the OECD GL process for validation and implementation is conducted for NAMs and how this process resulted in the updated international guidance on skin sensitization testing and its application to a risk assessment. Attendees will be able to identify where to find and how to apply best practices for skin sensitization testing and assessment.

CE 1008 PM08: Lost in Translation? Navigating Species Selection for Immunotoxicological Assessment in Preclinical Studies

M. Piche. Charles River Laboratories, Senneville, QC, Canada.

Selecting the right species for immunotoxicological assessment in nonclinical studies is essential for ensuring the relevance, reliability, and translatability of immune safety data to human health. However, significant interspecies differences in immune system structure and function present an ongoing challenge to accurately predicting human outcomes. Understanding these differences—and how they affect study design, interpretation, and risk assessment—is critical, especially as biologics, immunomodulators, and other immune-targeting therapies become increasingly common. This course will explore the complexities of species selection for immunotoxicology studies by examining the immunological similarities and differences between commonly used preclinical species—rats, minipigs, dogs, and nonhuman primates—and humans. Each course will focus on the comparative immunology of one species, followed by real-world examples that demonstrate how species-specific immune traits have influenced study outcomes, clinical translation, or mitigation strategies. Speakers will also highlight formulation-specific challenges (e.g., excipient-related immune reactions) and areas where alternative models are gaining interest. The course will offer a practical framework to help participants integrate immunobiology, therapeutic modality, and study design considerations into species selection decisions. The discussion will be enriched by perspectives from health authorities, providing regulatory context around model selection and expectations for translational relevance. These insights will help illustrate how a mechanistic understanding of interspecies differences can inform study design and support scientifically grounded, human-relevant risk assessment. Participants will leave with practical tools and insights to design more scientifically justified, translationally relevant immunotoxicity studies, while learning to anticipate potential gaps or artifacts in translation, leaving with actionable strategies for designing and interpreting immunotoxicity studies with greater confidence. Course Goals: (1) Equip scientists with tools to select the most immunologically and translationally relevant species for biologics and small molecule safety assessment. (2) Highlight where standard models (rat, dog, NHP, minipig) succeed or fall short in predicting human immune responses. (3) Share best practices and real-world case studies for evaluating biologics-related immunotoxicity, including formulation-specific reactions and alternative models. (4) Promote fit-for-purpose study designs that increase human relevance and regulatory confidence.

B. Riffle. BASF Agricultural Solutions, Wake Forest, NC.

Assessing the risks of chronic toxicity and carcinogenicity from long-term exposure to chemicals is a critical part of the regulatory approval process of various chemistries. To evaluate the potential for chronic toxicity and carcinogenicity resulting from exposure to chemicals, regulatory authorities typically require the conduct of long-term rodent bioassays, such as the OECD guideline studies for chronic toxicity, carcinogenicity, and/or combined chronic/carcinogenicity studies. New approach methodologies may provide a less resource intensive and a more rapid means to assess chronic toxicity and carcinogenicity of chemicals with equivalent or better accuracy and resultant human health protection. In some regulatory jurisdictions, regulators have the flexibility to consider a scientific weight of evidence (WOE) based approach that satisfies toxicological data requirements without conducting the lifetime rodent bioassays. Depending on regional legislation, agricultural, industrial chemical, and pharmaceutical registrants can submit scientific rationales addressing the toxicological data requirements for regulatory assessment, which allow for the integration of available information to make data need determinations that are health protective and scientifically sound. A workgroup convened under the Rethinking Carcinogenicity Assessment for Agricultural Project (ReCAAP) to develop a framework building off the ICH S1 WOE, to help structure a WOE based estimation of a point of departure that would be health protective against chronic risk for agricultural safety evaluation, including effects related to carcinogenicity. This course aims to provide an example of implementing the ReCAAP approach for assessing the potential for carcinogenicity to support data requirements for chemical safety assessment. Examining a practical example will provide insight to better understand how to use the available framework and guidelines in a fit-for-purpose manner. The course is not meant to offer technical lectures on WOE methods or the selection of uncertainty factors for risk assessments. Rather, the lectures will be focused on experiences of ReCAAP applications, including a practical example, in decision-making for researchers and the regulated community to consider while developing a WOE-based chemical safety assessment. The first set of speakers will introduce the ReCAAP framework and regulatory implications of rodent bioassays in the risk assessment of a variety of chemicals. Activities related to applying a WOE-based approach to carcinogenicity assessment will be discussed. The next speaker will present the workflow of the ReCAAP framework to discuss the process, key factors considered, and how to address uncertainties with this approach. After the break, the next speaker will provide a presentation on the fundamentals of read-across, followed by a short demonstration of the framework functionality survey to determine which chemicals this framework will work best. The course will conclude with a hands-on evaluation of a case study followed by panel discussion. Participants will be asked to conclude whether one or both of the long-term rodent cancer bioassays should be waived based on the available data set.

1010 PM10: This Is Real: How AI Can Actually be Used in Hazard and Risk Assessments

A. Rooney. NIEHS, Research Triangle Park, NC.

While application of artificial intelligence (AI) approaches has tremendous potential for decision-making and efficiencies in toxicology, questions remain as to reliable and transparent use of AI for hazard and risk assessment. This course will highlight actual use and innovative tools that enhance workflows in conducting hazard and risk assessments. Traditional approaches to risk assessment often involve resource intensive manual processes that can be time and resource intensive, prone to human error, and challenging to scale effectively, particularly with the rapid growth in relevant scientific research. This course will illustrate how AI technologies can significantly streamline these complex activities, while maintaining transparency. By automating routine tasks such as identifying relevant studies, extracting critical data, and systematically analyzing extensive datasets, AI tools offer meaningful improvements. These advancements increase efficiency, scalability, and the robustness of evaluations while simultaneously ensuring accuracy, transparency, and reproducibility—critical factors for maintaining trust and credibility in public health and environmental decision-making. Recent advancements in AI and informatics offer transformative solutions for researchers, regulatory agencies, and public health organizations tasked with assessing human health risks from chemical exposures. Sophisticated computational tools and analytical techniques provide researchers and regulators with enhanced capabilities to process, interpret, and manage the large and complex scientific datasets that underpin modern risk assessments. These methods support more informed, timely, and effective decision-making, allowing regulatory agencies to respond proactively to emerging health threats. However, decision-making on hazard and risk require certainty and transparency in the process such that evidence, workflow, and the basis of scientific judgments are clearly presented. Thoughtfully integrated AI technologies, guided by the key principles of transparency and responsible use of AI methods, not only enhance assessment speed but also bolster the consistency, rigor, and reproducibility of outcomes. Currently, regulatory organizations around the world are actively exploring, testing, and integrating these technologies within their existing processes. This course seeks to facilitate an interactive and collaborative discussion among participants, including regulators, academics, and industry professionals, on the practical implementation of AI within established

risk assessment frameworks. Discussions will highlight real examples of AI in existing workflow, opportunities for progress, clearly define emerging challenges, and encourage the exchange of best practices to optimize successful integration. Goals: (1) Clearly presenting AI capabilities and recognizing practical limitations in public health, environmental safety, and regulatory contexts. (2) Demonstrating cutting-edge informatics solutions that automate and enhance data acquisition, organization, and analysis, particularly in systematic reviews, significantly reducing manual labor and minimizing error. (3) Providing real-world case studies showcasing specific instances where AI has notably improved hazard identification and risk evaluation processes, thereby offering attendees valuable insights into both successes and practical lessons learned. (4) Facilitating dynamic and interactive discussions among stakeholders—industry professionals, regulatory authorities, researchers, and academics—to exchange experiences, share best practices, and discuss strategies to overcome the challenges in integrating AI-driven methodologies in risk assessments. (5) Discuss the importance of developing and applying standards for use of AI in decision-making and each step in the hazard or risk assessment process. (6) Bridging theory with practice by presenting tangible examples of AI currently employed within regulatory environments, designed to inspire broader acceptance and implementation. The session has three segments: (1) foundational insights into key AI methodologies and obstacles encountered in their practical application; (2) demonstrations of state-of-the-art informatics tools that significantly enhance systematic review procedures and handling of expansive datasets; and (3) detailed, real-world case studies exemplifying successful AI integration into regulatory assessment processes. By the conclusion of the course, attendees will possess a comprehensive understanding of how these innovations are effectively reshaping the landscape of risk assessments. They will appreciate the significant enhancements in accuracy, reliability, and efficiency achievable through these methods and recognize how these improvements directly support robust public health protection and environmental stewardship.

1011 Developmental PFAS Toxicity: Leveraging Zebrafish as a Model for Phenotypic, 'Omics, and Organ-Level Studies

S. Dasgupta. Clemson University, Clemson, SC.

Per- and polyfluoroalkyl substances (PFAS) have garnered increasing attention over the past decade due to their widespread environmental persistence and their potential to disrupt biological systems. These synthetic compounds, found in everything from firefighting foams to food packaging, have been dubbed “forever chemicals” for their resistance to degradation in both nature and the human body. As PFAS continue to accumulate in water sources, soil, and living organisms, the urgency to understand their toxicological impacts has never been greater. One of the critical questions that we face is how PFAS exposure during development affects gene expression and organogenesis. Zebrafish (*Danio rerio*) is a powerful model with conserved organ systems and genetic tractability, offering a unique *in vivo* platform to assess toxicity at the molecular, cellular, and organ levels—often with high-throughput efficiency. Within this model, bulk and single-cell ‘omics approaches following whole embryonal exposures can be used to predict pathogenesis in developing organs. Owing to these advantages, zebrafish has been widely used to screen PFAS for phenotypic and genomic responses, as well as examine impacts on organogenesis. The objective of this globally relevant Symposium is to bring together zebrafish investigators that are actively conducting research on prediction and assessment of PFAS toxicity, with focus on gene expression studies and organogenesis. Following a brief introduction, the first speaker will talk about using single cell-sequencing data to study impacts of PFOS (a legacy PFAS) on organogenesis, focused on the pancreas and liver. The second speaker will talk about developmental exposures to PFNA (a long chain PFAS) and their impacts on behavioral phenotypes and gene expression disruption within the eye and brain. The third speaker will talk about developmental PFAS exposures and lipidomic changes associated with mitochondrial disruption and neurotoxicity. The fourth speaker will talk about their work on phenotypic and transcriptomic screening of emerging PFAS that are ubiquitously found in Cape Fear River in North Carolina, with a focus on the kidney as a target organ. The final speaker will present their work on developmental screening of a library of 182 PFAS chemicals, with inflation of the swim bladder as a toxic endpoint. The session will conclude with a panel discussion.

1012 Impacts of PFAS on the Pancreas: Insights from Transcriptomics for Organ Development and Function

A. Timme-Laragy. University of Massachusetts Amherst, Amherst, MA.

Per- and polyfluoroalkyl substances (PFAS) are a superfamily of fluorinated, anthropogenic chemicals used in numerous commercial and consumer products. Epidemiological and animal studies have linked exposure to PFAS to numerous adverse health outcomes, including diabetes, pancreatic β -cell dysfunction and oxidative stress. We have shown that exposure to several PFAS during embryonic development in zebrafish can disrupt transcription of genes involved with key pancreatic functions, corresponding to smaller and mis-shaped islets of Langerhans, truncation of the exocrine pancreas, dysregulation of lipids and digestive enzyme production, and insulin biosynthesis defects. To gain additional mechanistic insight into how PFAS can exert toxicity during early life, we used single-cell RNA-sequencing (scRNA-

Seq) to identify cell and tissue-specific transcriptomic responses to the PFAS legacy member, perfluorooctanesulfonic acid (PFOS), from 3-72 hours post. Findings indicated numerous tissue-specific differences in processes including apoptosis, cell division, epigenetic changes, and hormone secretion, and also identified potential causative pathways that may underlie disruptions to pancreatic development and function. Overall, this work illustrates the enhanced mechanistic understandings of PFAS toxicity that can emerge through integration of transcriptomic and organ-level assessments.

S 1013 Perfluorononanoic Acid Exposure and Light Conditions: A Dual Impact on Developing Eye and Brain Transcriptome in Zebrafish

S. Dasgupta. Clemson University, Clemson, SC.

Perfluorononanoic acid (PFNA) is a synthetic long chain homologue of PFAS which is frequently detected in environment as an emerging contaminant with potential threats to living organisms. It has broad applications in industrial and consumer products including adhesives, water and paint repellent surfaces, non-stick coatings and lubricants. We have shown that developmental exposures (2 h post fertilization to 5 days post fertilization) to PFNA induce significant changes behavioral phenotypes, and these phenotypic changes are strongly dependent on light exposure; incubations in complete dark result in hyperactivity, whereas 14 hr light: 10 hr dark incubations reverse this effect. Surprisingly, our LC-MS data showed that tissue uptake of PFNA was 3 times higher in 14 hr light: 10 hr dark incubations. To assess the impacts of PFNA x light on development of the brain and eye- organs that process light cues- we dissected these organs from PFNA-exposed fish and conducted mRNA sequencing. Our data show significant transcriptomic disruption in both organs with common affected pathways regulating steroid biosynthesis, fatty acid metabolism and PPAR, MAPK and P53 signaling in both light and dark incubations. Follow-up studies with Nile Red staining also showed higher lipid accumulation in PFNA exposures. However, several signaling pathways, such as Notch signaling in brain, were uniquely disrupted in dark incubations and not 14 hr light: 10 hr dark incubations. Collectively, these results suggest that neurotoxic effects of PFNA may be driven by lipid and metabolic disruptions that can be partially protected by circadian rhythms.

S 1014 Lipidomics Links PFAS Neurotoxicity with Mitochondrial Damage, Oxidative Stress, and Dopamine Alterations in Developing Zebrafish

J. Freeman. Purdue University, West Lafayette, IN.

Exposure to per- and polyfluoroalkyl substances (PFAS) has been associated with neurodevelopmental and neurodegenerative disease risk, particularly with changes along dopaminergic and associated pathways. Furthermore, dopamine alterations can occur from impacts to mitochondrial dysfunction, increased oxidative stress, and lipid disruptions; all of which are also implicated in PFAS toxicity. To further investigate mechanisms of PFAS neurotoxicity, developing zebrafish were exposed to select PFAS during embryogenesis [1-72 hours post fertilization (hpf)] at concentrations ranging from 0.004 to 40 ppb (µg/L). At 72 hpf PFAS exposure was ceased and zebrafish either prepared for assessments or allowed to develop until 120 hpf without additional PFAS exposure (i.e., in water only) for assessment. Multiple outcomes were investigated for potential neurotoxicity including lipidomics, mitochondrial distribution and membrane potential, oxidative stress, and alterations associated with dopamine. Biological results were anchored to toxicokinetic profiles of each PFAS to enhance understanding of body burden and comparative translation to other species. Results revealed exposure to select PFAS (e.g., PFOA) resulted in lipid changes associated with mitochondrial damage, oxidative stress, and dopamine signaling. Additional assays supported decreased mitochondrial distribution and membrane potential, an increase in reactive oxygen species and lipid peroxidation, and decreased dopamine concentrations. Overall, findings indicate mechanisms of PFAS developmental neurotoxicity related to increased oxidative stress, lipid disruption, impaired mitochondrial integrity, and dopamine loss.

S 1015 Developmental and Organ Toxicity of Emerging PFAS Compounds

N. Jayasundara. Duke University, Durham, NC.

Per- and polyfluoroalkyl substances (PFAS) and their byproducts pose a significant threat to both human and ecological health. Despite extensive research efforts, the adverse outcomes and mechanisms of toxicity for many emerging PFAS compounds, such as perfluoro-2-methoxyacetic acid (PFMOAA), remain poorly characterized. In this study, we used the zebrafish (*Danio rerio*) model to evaluate the developmental toxicity and nephrotoxicity of PFMOAA and four other compounds ubiquitously found in the Cape Fear River (CFR) in North Carolina—a major drinking water source for millions of residents and a site heavily contaminated with PFAS compounds. In addition to the individual compounds, we also assessed the developmental neurotoxicity of water samples collected from CFR. Our preliminary findings reveal that zebrafish exposed from 6 hours post-fertilization to 6 days post-fertilization to both Cape Fear River water samples containing PFAS mixtures and exposure to individual PFAS

chemicals results in larval mitochondrial dysfunction, altered neurobehavior, and proximal tubular damage in the kidney. Transcriptomic analyses comparing signatures across five PFAS compounds further underscore distinct and common molecular responses. Together, these results emphasize the critical impact of early-life exposures and highlight the need for further research into sensitive developmental endpoints to better assess the risks associated with environmentally relevant PFAS compounds.

S 1016 Zebrafish Developmental Screening of a PFAS Chemical Library

D. Phelps. US EPA/Oak Ridge Institute for Science and Education, Durham, NC.

Per- and polyfluoroalkyl substances (PFAS) are present in environmental matrices as well as many consumer and industrial products. While some PFAS, notably perfluorooctanesulfonic acid (PFOS), are developmentally toxic in mammals, the vast majority of PFAS have not been evaluated for developmental toxicity potential. A concentration-response study of 182 unique PFAS using the zebrafish (*Danio rerio*) medium-throughput, developmental toxicity assay was conducted to investigate chemical toxicity profiles. Embryos were exposed to each PFAS (= 100 µM) beginning on the day of fertilization and maintained at 26°C. At 6 days post-fertilization (dpf), two independent observers blinded to treatment graded developmental landmarks for each larva (e.g., mortality, hatching, swim bladder inflation, edema, abnormal spine/tail or craniofacial structure). Thirty percent of the PFAS were developmentally toxic, with swim bladder non-inflation the most sensitive toxic endpoint. Edema was also commonly noted, but usually secondary to swim bladder non-inflation and at higher concentrations. Moreover, the pattern of developmental landmark malformations did not appear to be dependent on PFAS structural category. PFOS was developmentally toxic in the low micromolar range, however other chemicals were more potent, including perfluorooctanesulfonamide, N-methylperfluorooctane sulfonamide, ((perfluorooctyl)ethyl)phosphonic acid, perfluoro-3,6,9-trioxatridecanoic acid, and perfluorohexane sulfonamide. The developmental toxicity profile for these more potent PFAS is largely unexplored in mammals and other species. Based on these zebrafish developmental toxicity results, additional screening may be warranted to understand the toxicity profile of these chemicals in other species. *This work does not reflect the official policy of the US EPA.*

S 1017 Efforts Toward Building Aligned Physiologically Based Kinetic Models for Thyroid Hormone Homeostasis in Rodents and Humans: Can We Develop "Supermodels"?

B. Riffle. BASF Agricultural Solutions, Wake Forest, NC.

Thyroid hormone (TH) homeostasis is regulated via the hypothalamic pituitary thyroid axis through a coordinated feedback system which alters synthesis of thyroxine (T4) and triiodothyronine (T3), metabolism, clearance, and elimination. In rodents, reductions of T4 and T3 concentrations by various molecular initiating events can result in rodent thyroid tumors that are of limited relevance to humans. However, investigations are ongoing to clarify the human relevance of reductions in THs (especially in rodent offspring) related to impaired neurodevelopment. Physiologically based kinetic (PBK) models are models that simulate biokinetics of a substance within the body by considering the physiological characteristics of pharmacokinetics and pharmacodynamics. Several thyroid PBK models have been developed to attempt to evaluate species differences in internal TH concentrations, TH transport, clearance, presence in brain, and organ-specific TH distributions and to predict effects of thyroid toxicants on hormone homeostasis in humans and rodents. This session explores the potential of developing an aligned PBK model for prediction of effects on thyroid homeostasis in humans following exposure to rodent thyroid toxicants by introducing the regulatory landscape regarding thyroid toxicants, evaluating some of existing individual thyroid PBK models, and concluding with a regulatory perspective on their potential application within the European Union hazard assessment process. The session will begin by introducing regulatory considerations regarding the use of rodent TH data for informing human health outcomes in both a risk- and hazard-based approach and then describing a project within CroPLife Europe exploring the feasibility of integrating existing models and approaches into an overarching mechanistic mathematical model platform, referred to as the "Supermodel," with an aim to represent all biological processes encompassed in the complete thyroid adverse outcome pathway (AOP) network. This will be followed by a presentation of a spatial human PBK model of THs accounting for the contribution of plasma binding proteins, followed by a presentation of a recent example of predictions of the effects in rat and humans using a PBK model for TH homeostasis following exposure to a liver enzyme inducer. A mechanistic modeling approach with a focus on TH receptor occupancy in the brain will next be discussed. The session will end with a presentation of the European regulatory perspective on PBK models, including potential challenges, as well as an overview of European-related work in projects such as the Partnership for the Assessment of Risks from Chemicals (PARC).

S 1018 Development of an Aligned PBK Model (aka the Supermodel) for the Prediction of Effects on Thyroid Homeostasis in Humans Following Exposure to Rodent Thyroid Toxicants

B. Riffle. BASF Agricultural Solutions, Wake Forest, NC.

Most of the data generated to support regulatory decisions regarding hazard identification and risk assessment of potential TH toxicants is generated from animal models including the rodent. Some effects, such as thyroid tumors, are thought to be species-specific due to physiological differences. However, significant alterations in circulating THs are thought to be related to adverse impacts on the neurodevelopment of rodent offspring and more information is needed to assess the human relevance of these findings in rodents. Several thyroid PBK models have been developed by individual Crop Life Europe (CLE) member companies to attempt to evaluate species differences in internal substance concentrations, TH transport, clearance, presence in brain, organ-specific TH distributions and to predict effects of thyroid toxicants on hormone homeostasis in humans and rodents. This presentation will introduce the concept of an integrated singular mechanistic mathematical model (otherwise known as "The Supermodel") that represents all biological processes encompassed in the complete adverse outcome pathway (AOP) network, including quantifiable species-specific parameters linked to specific molecular initiating events (MIEs) and key events (KEs) of the underlying AOP is envisioned where MIEs related to thyroid toxicity (direct or indirect), KEs, and adverse outcomes (AOs) related to developmental neurotoxicity (DNT) are captured. Validation of such a model will be discussed including comparisons of model predictions to rodent and human data. This effort could not only support a potential distinction in classification for rodent thyroid toxicants under the European Chemicals Agency (ECHA) classification process but also assist in risk assessment (relevant for countries with a risk-based approach like the United States) by better predicting human outcomes.

S 1019 Tissue Distribution of Thyroid Hormones by Plasma Binding Proteins and Alteration by Endocrine-Disrupting Chemicals

Q. Zhang. Emory University Rollins School of Public Health, Atlanta, GA.

The thyroid hormones (THs), thyroxine (T4) and triiodothyronine (T3), are under homeostatic control by the hypothalamic-pituitary-thyroid axis and plasma TH binding proteins (THBPs), including thyroxine-binding globulin (TBG), transthyretin (TTR), and albumin (ALB). THBPs buffer free THs against transient perturbations and distribute THs to tissues. With a rigorously parameterized physiologically based kinetic (PBK) model of THs in humans, we explored the roles of different THBPs and how endocrine-disrupting chemicals (EDCs) may perturb TH homeostasis and tissue distributions. The model produces several novel findings. (1) The blood-tissue TH exchanges are fast and nearly at equilibrium especially for T4, providing intrinsic robustness against local metabolic perturbations that may be targeted by EDCs. (2) Continuous exposure to THBP-binding EDCs does not alter the steady-state levels of THs, while intermittent daily exposure to rapidly metabolized TBG-binding EDCs can cause much greater disruptions to plasma and tissue THs and their circadian rhythms. (3) Spatial gradients of TH transporters and metabolic enzymes may produce spatially heterogeneous TH tissue concentrations, which can be modulated by EDCs targeting these proteins. (4) Any of the 3 THBPs alone is sufficient to maintain the spatial patterns of TH tissue distributions, suggesting EDCs targeting THBPs will be ineffective at altering the spatial distribution of THs. In summary, the PBK model provides novel insights into TH kinetics and the homeostasis-disrupting effects of EDCs. Such insights into the spatial and temporal dynamics of THs and their interactions with EDCs are deemed a crucial component for the development of the "Supermodel".

S 1020 Toward a Modular Next-Generation Risk Assessment Ecosystem: A Case Study in Thyroid Disruption Modeling Across Species

S. Schaller. ESQlabs GmbH, Saterland, Germany.

Evaluating disruptions to thyroid hormones (TH), is challenging due to observed differences between human and animal responses. Nevertheless, chemical risk assessments continue to rely on animal studies, underscoring the need to mechanistically understand species-specific responses. This study presents a coupled physiologically based kinetic (PBK) quantitative systems toxicology (QST) platform for assessing disruption of TH regulation in both rats and humans, supporting the evaluation of chemicals by incorporating species specificity for key physiologic properties. An assessment use-case is presented using validated Uridine Diphosphate Glucuronosyltransferase (UDPGT) inducer Phenobarbital (PB) as a reference for assessing a new Pregnane X Receptor / Constitutive Androstane Receptor inducer (PI). The platform is built using the OSP Suite (PK-Sim and MoBi, version 12) and integrates PBK models of thyroid disruptors with QST modules for TH regulation and quantitative adverse outcome pathways (qAOP) modules for thyroid disruption. The QSP modules include pituitary synthesis of thyroid-stimulating hormone (TSH), feedback-controlled Triiodothyronine (T3) and Thyroxine (T4) synthesis, UDPGT-mediated clearance, circadian TSH regulation, and species-specific TH plasma protein binding (Albumin, Transthyretin (TTR), and Thyroxine-Binding Globulin (TBG)—absent in rats).

In rats, T3 clearance involves extensive glucuronidation and deiodination; in humans, T4 metabolism dominates, as well as distinct differences in transport proteins such as TBG. By integration of the PB and PI PBK models with the modular TD platform in an *in vitro* to *in vivo* extrapolation (IVIVE) approach, model assessment showed reduced risk for liver enzyme induction of PI vs PB. This PBK-QST platform exemplifies how a modular NGRA ecosystem enables the "Supermodel" concept. Modular PBK and QST/qAOP libraries allow rapid development, transparent reuse, and automated qualification of models across chemicals, species, and endpoints with shared pathways. Ultimately, this approach empowers regulatory engagement by delivering transparent, reproducible models, and traceable modeling workflows—critical for advancing NGRA as a reliable alternative to traditional toxicology.

S 1021 Modeling Developmental Toxicity via Thyroid Hormone Disruption: Dynamics in the Fetal Brain

J. Leedale. Syngenta Crop Protection LLC, Berkshire, United Kingdom. Sponsor: B. Riffle

Modeling developmental toxicity caused by thyroid hormone (TH) signalling disruption is a complex, multiscale challenge. It encompasses various temporal and spatial scales, from early-stage kinetics of TH-disrupting chemicals (TDCs) to whole-body TH regulation and local TH dynamics within specific tissues. When modelling potential toxicity in the developing brain, several factors must be considered: the effects of TDCs on circulating TH levels based on toxicokinetics and toxicodynamics; whether or not tissues with local regulation and significant biological barriers are resistant to external TH fluctuations; and, if foetal systemic TH levels are impacted, the potential impact on activity in the foetal brain. This study is focused on modelling TH dynamics in the foetal brain, aiming to understand how external TH fluctuations impact TH activity. Due to limited quantitative experimental data, a minimalist mathematical modelling approach has been adopted that incorporates the essential TH feedback mechanisms that maintain homeostasis. Multiple approaches were utilised for model building and validation, including novel neurosphere experiments; read-across from other *in vitro* and *in silico* models; sensitivity analysis and uncertainty quantification; and indirect parameterisation to recapitulate observed phenomena. The model was coupled to a pregnancy Physiologically Based Kinetic (PBK) model to fully explore the multiscale impact of TDCs on foetal brain activity, including mechanisms for placental transfer and regulation of circulating TH in rats. Further downstream applications of the dynamic foetal brain TH model such as specific localisation (e.g., astrocytes, neurons), and protein-mediated transport across the blood-brain barrier can be incorporated or adapted to increase physiological relevance. This model can be a crucial component for the development of the "Supermodel" by contributing to understanding the complex interplay of factors affecting TH signaling in foetal brain development and potential developmental toxicity caused by TDCs.

S 1022 Considerations When Developing Thyroid PBK Models for Regulatory Application: A Perspective from the European Union

P. Marx-Stoelting. Bundesinstitut für Risikobewertung (BfR), Berlin, Germany. Sponsor: B. Riffle

Many compounds affect the thyroid via distinct mechanisms. While a few directly interact with TH (T3, T4) synthesis via the thyroid peroxidase (TPO), many others trigger their effects indirectly via hepatic enzyme induction and increased clearance of T3 and T4. This indirect induction may be different in several species and findings e.g. in rodents may not fully be relevant for humans. Besides the differences in enzyme induction as differences in buffering T3/T4 hormone levels via TH binding globulins (TBG) accounts for pronounced species discrepancies. The intended talk will illustrate such differences upon examples from *in vivo* and *in vitro* studies and in doing so highlight challenges and benefits in the development of an aligned PBK model in addressing substance induced changes in T3/T4 hormone metabolism. The talk will also highlight some general regulatory requirements on PBK models and point out related EU work in projects such as Partnership for the Assessment of Risks from Chemicals (PARC). This perspective will emphasize the importance of aligning PBK models with regulatory needs and discuss how integrating them into a "Supermodel" framework can support hazard and risk assessment.

S 1023 Micro/Nanoplastics, Plastic-Associated Endocrine-Disrupting Chemicals, and Cardiovascular Disease

M. Campen. University of New Mexico, Albuquerque, NM.

Cardiovascular disease (CVD) is the leading cause of mortality and morbidity worldwide despite major advances in diagnoses and treatments in the past few decades. Plastics are fundamental in modern life and have recently been implicated in the etiology of CVD. Plastic-associated endocrine-disrupting chemicals (EDCs), including numerous base chemicals and phthalate plasticizers, are produced in high volume and are consistently associated with the increased CVD risk in large-scale cross-sectional and longitudinal human studies. As plastics age and degrade, they become progressively smaller and more highly oxidized on the surface, leading to the gener-

ation of ubiquitous micro/nanoplastics (MNPs). Emerging evidence demonstrates the accumulation of MNPs in human arteries and carotid artery plaque that may lead to increased cardiovascular events in exposed individuals. Although the associations between MNP and EDC exposures and CVD risk have been well-documented and attracted considerable public attention, the clinical impact and mechanistic insights of MNP/EDC exposures on cardiovascular health remain elusive. Thus, the following presentations provide epidemiological and clinical evidence for these associations and preclinical animal model and mechanism-based evidence for MNP/EDC-elicited CVD: (1) "Plastics: A Threat to Health Across the Lifespan and What We Can Do About It"; (2) "Effects of Plastic-Associated EDCs and Microplastics on Lipid Metabolism and Atherosclerosis"; (3) "Prevalence and Implications of Heightened Plastic Chemical Exposure on Cardiac Health"; (4) "Oral Microplastics Consumption and Atherogenesis"; and (5) "Micro/nanoplastics Impact in Clinical Vascular Disease: From Carotid to Aorta and Beyond." This Symposium brings epidemiologists, clinicians, and translational and basic scientists together to discuss the risks of environmental exposures to MNPs and EDCs on cardiovascular health and will address the critical knowledge gap by providing mechanistic insights explaining how MNP/EDC exposures cause CVD. The Symposium will facilitate collaborations and discussions among investigators in different disciplines targeting this emerging issue.

S 1024 Plastics: A Threat to Health Across the Lifespan and What We Can Do About It

L. Trasande. NYU Grossman School of Medicine, New York, NY. Sponsor: *M. Campen*

From cradle to grave and womb to tomb, plastics and related chemicals and micro- and nanoplastics present serious health concerns. Plastics contribute substantially to disease and associated social costs globally. In the United States, we estimated \$249 billion in health costs for plastic-attributable disease burden in 2018 alone. Globally, 13.5% of all CVD deaths (>350,000 deaths) in 2018 among people ages 55-64 could be attributed to plastic-associated phthalate exposure. The economic burden from the identified CVD deaths was estimated from \$510 billion to \$3.74 trillion. This presentation will present toxicologic and epidemiologic evidence with a focus on cardiometabolic outcomes, and identify US and global policy steps for action.

S 1025 Effects of Plastic-Associated EDCs and Microplastics on Lipid Homeostasis and Atherosclerosis Development

C. Zhou. University of California Riverside, Riverside, CA.

Several ubiquitous plastic-associated EDCs (e.g., base chemical bisphenol A [BPA], phthalates) have been associated with increased CVD risk and atherosclerosis development in humans. We identified many plastic-associated EDCs including BPA, its analogs, phthalate plasticizers, and FDA-approved phthalate substitutes as potent agonists for the nuclear receptor pregnane X receptor (PXR). PXR functions as a xenobiotic sensor regulating key genes involved in xenobiotic metabolism. We recently revealed the novel function of PXR in the regulation of lipid metabolism and atherosclerosis development, and demonstrated that several plastic-associated EDCs increase atherosclerosis and dyslipidemia in a PXR-dependent manner in suitable mouse models. For example, we identified BPA as a human PXR-specific agonist and chronic exposure to BPA led to increased atherosclerosis in the PXR-humanized mice. An understudied phthalate, dicyclohexyl phthalate (DCHP), can also activate PXR and cause dysregulated lipid homeostasis and increased atherosclerosis in mouse models. In addition to EDCs, we also found that exposure to microplastics led to significantly increased atherosclerosis in the LDL receptor-deficient mice. Single cell RNA sequencing analysis of the whole aorta revealed that microplastic exposure affected the proportions and cellular processes of key cell types involved in atherosclerosis development. These results reveal the atherogenic effects of plastic-associated EDCs and microplastics *in vivo* and provide mechanistic insights contributing to our understanding of EDC or EDC-carrying microplastic-elicited atherogenesis.

S 1026 Prevalence and Implications of Heightened Plastic Chemical Exposure on Cardiac Health

D. Guerrelli, and N. Posnack. Children's National Hospital, Washington, DC.

Phthalate chemicals are used to manufacture disposable plastic medical products, including blood storage bags and components of cardiopulmonary bypass circuits. As such, cardiac surgery patients may be inadvertently exposed to phthalate chemicals that are released from these plastic products. In this study, we quantified phthalate chemical exposure in patients undergoing cardiac surgery, examined associations to postoperative complications, and measured the direct effects of phthalates using experimental cardiac models. In pediatric cardiac surgery patients, the degree of phthalate exposure increased in the context of cardiopulmonary bypass with red blood cell (RBC) priming. Patients with heightened phthalate exposure more commonly experienced postoperative complications, including cardiac arrhythmias, low cardiac output, and hypotension. The cardiodepressive effects of phthalates

were validated using both animal and hiPSC-CM models, demonstrating dose (10-100 µg/ml) and time-dependent (15min-3 hour) slowing of heart rate, atrioventricular conduction, and conduction velocity. We demonstrate that phthalate chemical exposure can be (partially) mitigated by using "fresh" RBCs with a shorter storage duration, storing RBCs in alternative blood bags with reduced phthalate leaching, or washing RBCs to remove extracellular contaminants.

S 1027 Oral Microplastics Consumption and Atherogenesis

T. O'Toole. University of Louisville, Louisville, KY.

Small plastic particles of the micro- and nano- sizes are common environmental contaminants and human exposure to these particles (MNP) through inhalation, ingestion or absorption is inevitable. While MNP have now been identified in human vascular and cardiac tissues, and their abundance in these tissues has been correlated with adverse outcomes, their direct involvement in the genesis or progression of cardiovascular disease (CVD) is uncertain. To begin to address this issue, we examined CVD risk and atherosclerotic lesion development in chow-fed ApoE^{-/-} mice supplied with normal water (control) or that containing polystyrene (PS) beads (0.5mm; 1mg/ml) for 20 weeks. At this time we found that mice drinking the PS water had a statistically significant higher level of plasma glucose versus control (1.3-fold). Furthermore the average lesion area in the PS-exposed mice was greater than that in the control mice (0.81mm² vs. 0.57mm²; p=0.01). In related experiments, we supplied additional ApoE^{-/-} mice with a Western diet and normal water (control) or that containing a combination of polyethylene (0.065mm; 2.5ng/ml) and polyvinyl chloride (0.25mm; 200ng/ml) (PE+PVC) beads for 6weeks. These PE+PVC mice likewise demonstrated increased blood glucose (1.2-fold) and lesion area (0.16mm² vs. 0.11 mm²; p=0.02) versus the control mice. These results suggest that exposure to MNP may directly contribute to the development of overt CVD. The mechanistic possibilities and implications of these results will be discussed.

S 1028 Micro/nanoplastics Impact in Clinical Vascular Disease: From Carotid to Aorta and Beyond

R. Clark. University of New Mexico, Albuquerque, NM. Sponsor: *M. Campen*

Micro/nanoplastics (MNPs) in human tissues are recently recognized and the clinical effects have not yet been fully elucidated. Recent reports have associated carotid atherosclerotic plaque MNPs, immune cell deposition and poor long-term clinical outcomes. Investigations detailing MNPs in symptomatic carotid disease, aortic and peripheral artery disease (PAD) lesions are lacking. We examined whole carotid plaques as well as vessel tissue from aortic aneurysm, aortoiliac atherosclerotic occlusive disease and femoral artery plaques and compared them to age-matched control non-atherosclerotic arteries of all types from fresh cadaveric donors. Quantitative MNP concentrations were determined by pyrolysis-GC/MS and qualitative features by electron microscopy. Mean MNP concentrations in control carotid arteries were 57 µg/g. Plaques from subjects with asymptomatic carotid stenosis had significantly more MNPs with mean 895 µg/g (+16 fold, P<0.001) and those from symptomatic carotid stenosis had the highest MNP concentrations at 2888 µg/g (+51 fold, P<0.001). Mean MNPs in femoral plaques was 212 µg/g, compared to 1904 µg/g in atherosclerotic aortic tissue. Aneurysmal aortic tissue demonstrated 943 µg/g compared to 268 in control aortic tissue. Polyethylene and polypropylene were the most common polymers in all vessels and lesions. Single cell RNA sequencing of carotid lesions reveals alterations in pathways related to cellular stress (FKBP5), cytoskeletal organization (TPM1 and HIP1R) and anti-inflammation (CD163) in mesenchymal stem cells and macrophages without association with acute inflammatory markers TNF-alpha and IL-6. These data provide the first evaluation of the clinical effects of microplastics in carotid atherosclerotic plaques, aortic aneurysm tissue, aortic atherosclerotic plaque and femoral artery plaque and demonstrate a clear association with symptom status and specific vascular pathology in multiple vascular beds. The association of MNPs with altered gene expression in plaque mesenchymal stem cells (MSCs), which modulate immune signaling and plaque stability, suggests one possible mechanism by which MNPs influence atherosclerosis development and progression.

W 1029 Cancer Risk from Womb to Progeny Generations: Windows of Susceptibility and the Multigenerational Legacy of Environmental Exposures

X. Hu. Emory University, Atlanta, GA.

Early life environmental exposures are increasingly recognized as critical drivers of long-term health outcomes, including the risk of breast cancer and reproductive and developmental disorders. A growing body of human and mechanistic research shows that the health consequences of exposures can extend far beyond the directly exposed individual, impacting children, grandchildren, and even great-grandchildren through intergenerational and transgenerational transmissions. From metabolic reprogramming to persistent epigenetic modifications, ancestral exposures can shape disease susceptibility across lifespans and lineages, raising urgent questions

for regulatory policy and public health intervention. This session brings together a uniquely diverse panel of experts, which span epidemiology, mechanistic toxicology, epigenomics, public health advocacy, regulatory science, and the history of chemical industries, to examine how environmental exposures during sensitive developmental windows shape the health of future generations. Presenters will draw on landmark multigenerational cohorts, cutting-edge 'omics tools, and real-world case studies that elucidate sex-specific vulnerabilities, molecular mechanisms of inherited risk, and historical patterns of challenges in risk assessment and regulation. A special focus will be placed on the rising incidence of early onset breast cancer and emerging evidence linking both maternal and paternal exposures during pregnancy and fetal development to later-life cancer risk. This Workshop will begin with a brief introduction to the key principles and research tools used in developmental reprogramming and multigenerational inheritance. It will then feature five in-depth presentations: (1) epidemiology findings from a 60-year cohort tracking exposures, cancer, and reproductive outcomes; (2) epigenomic mechanisms linking ancestral exposures to developmental and cancer risk; (3) regulatory insights and gaps revealed through the study of endocrine-disruption mechanisms; (4) a funder's view on advocating transdisciplinary and community-based driven breast cancer prevention; and (5) what history can teach us about modern chemical regulation and cancer prevention. Panelists include a system biologist integrating multi-omic and transdisciplinary approaches to study early life exposure; a reproductive epidemiologist leading a multi-decade, multi-generational cohort; a molecular biologist discovering the epigenetic basis of inherited disease risk; a toxicologist instrumental in shaping national policy on endocrine-disrupting chemicals; a program director funding transdisciplinary and community-driven research; and a historian tracing the legacy of industry influence on environmental regulation. Together, their voices represent academic, governmental, community, and historical reflections on a complex and pressing issue. The session will conclude with a highly interactive panel discussion inviting the audience to engage on shared challenges, research gaps, and opportunities for coordinated action across sectors. By centering breast cancer and developmental health within a generational framework, this session seeks to reframe prevention as a multigenerational responsibility—and a scientific, regulatory, and societal imperative.

W 1030 Multigenerational Clues to the Rise in Early Onset Breast Cancer

B. Cohn. Public Health Institute, Berkeley, CA. Sponsor: X. Hu

The alarming rise in early-onset breast cancer demands a deeper understanding of its origins across time. The Child Health and Development Studies (CHDS), a landmark three-generation cohort, offers an unprecedented opportunity to trace how *in utero* and pregnancy exposures shape breast cancer risk not only in daughters, but also in granddaughters. Leveraging archived maternal and paternal serum samples collected during pregnancy in the 1960s, CHDS examines windows of susceptibility spanning gestation, fetal development, and even the ancestral germline. This presentation will highlight emerging findings from multi-omics approaches, including exposomics, metabolomics, and epigenomics. Results point to dynamic and sex-specific risk patterns, demonstrate the transmission of health risks along both maternal and paternal lineages, and underscore how environmental exposures can leave molecular imprints that persist into future generations. Together, these data provide powerful insights into the shifting landscape of breast cancer risk and inform a new paradigm for personalized prevention that begins long before diagnosis—and sometimes, even before birth.

W 1031 Epigenetic Memory: How Ancestral Exposures Echo Through Generations

J. LaSalle. University of California Davis, Davis, CA. Sponsor: X. Hu

DNA methylation in newborn blood provides a stable, high-resolution record of early-life environmental exposures, offering powerful insight into long-term disease risk. Unlike transient transcriptional changes, methylation marks capture durable epigenetic programming at the interface of genome and environment. This talk highlights the application of whole-genome bisulfite sequencing (WGBS) and the Comethyl network framework to integrate DNA methylation with exposure and health outcome data in both human and animal models. Methylation patterns in placental and newborn blood have been linked to prenatal exposures—including PCBs, phthalates, pesticides, bisphenol A, air pollution, and maternal smoking—all associated with increased cancer and neurodevelopmental risk. Cross-species analysis reveals convergence at key loci implicated in both autism spectrum disorder (ASD) and cancer. New findings also show that DNA methylation networks in F2 newborns are associated with ASD diagnoses and grandparental age and exposures, supporting the feasibility of using multigenerational bloodspots to trace exposome-metabolome-methylome connections to early-onset and heritable cancer risk.

W 1032 Born Vulnerable: What Dioxins and PFAS Teach Us About Chemical Regulation

L. Birnbaum. Duke University, Durham, NC.

Environmental exposures during critical windows of development can shape lifelong health trajectories and, in some cases, impact the health of generations yet unborn. Drawing on decades of research in toxicology and environmental health, the presentation will discuss how early-life exposures to endocrine-disrupting chemicals (EDCs) like dioxins, per- and polyfluoroalkyl substances (PFAS), phthalates, and bisphenol A can disrupt developmental programming during critical window of susceptibility, leading to long-term consequences for reproductive health, immune function, metabolism, and cancer risk. The talk will also draw lessons comparing regulatory efforts on dioxin-like chemicals and PFAS. Dioxins-like chemicals were unwanted byproducts but have been managed through a regulatory framework based on a single receptor. In contrast, PFAS present new challenges due to their complex mechanisms of toxicity and useful applications, which cause resistance to regulation. The comparison underscores the need for innovative regulatory approaches that move beyond chemical-by-chemical assessments.

W 1033 Catalyzing Solutions: A Funder's Perspective on Advancing Environmental Breast Cancer Research and Advocacy

S. Rasanayagam. California Breast Cancer Research Program, Oakland, CA. Sponsor: X. Hu

For over 25 years, the California Breast Cancer Research Program (CBCRP) has led a bold and community-centered approach to understanding and preventing breast cancer, particularly as it relates to environmental exposures. This talk will highlight CBCRP's role as a funder and convener of transdisciplinary research that bridges academia, government agencies, industry, and patient advocacy groups. By supporting investigator-initiated research alongside community-driven projects, CBCRP has advanced knowledge on environmental risk factors—including endocrine-disrupting chemicals, air pollution, and occupational exposures—and how these intersect with social determinants of health and systemic inequities. Key scientific accomplishments include cohort studies that have led to important insights on transgenerational impacts of exposure, collaborative studies identifying windows of susceptibility (including early life, adolescence and pregnancy), mechanistic insights from animal and human models, and innovative biomonitoring methods. The presentation will also discuss how CBCRP's investments have supported multigenerational research, led to policy-relevant findings, built sustained research-community partnerships, and helped shape national dialogues on breast cancer prevention and public health intervention.

W 1034 How to Sell a Poison: A Cautionary Tale for a Safer Tomorrow

E. Conis. University of California Berkeley, Berkeley, CA. Sponsor: X. Hu

To this day, many in the United States recall the 1950s as the height of the era of "better living through chemistry." But it was also the moment when much of the U.S. public became concerned that the growing number of synthetic chemicals in soil, water, air, food, and other consumer products were doing more harm than good. That meant it became, in turn, a time when the nation's chemical industry honed a set of tactics to defend their bottom lines: denying unfavorable evidence about the risks associated with their products, distracting public attention away from such evidence, and producing their own countervailing evidence. This presentation uses the case study of the history of the pesticide DDT to explore how corporate tactics evolved over the course of half a century to defend not just specific chemicals or classes of chemicals but production of consumer goods generally that exposed humans to a broad range of chemical components. Eventually, these strategies evolved to incorporate deliberate dissemination of disinformation, laying the groundwork for the widespread erosion of public trust in science we see today. Even more importantly, these strategies shaped the production and public reception of scientific knowledge in ways that actively undermined regulatory advances from the 1970s onward, as scientific understanding of the nature of risk posed by chemical exposures expanded to encompass concepts like endocrine disruption and other new mechanisms of carcinogenicity.

W 1035 Environmental Exposures and Pulmonary Fibrosis: What Do We Know and Where Do We Go?

E. Corteselli. University of North Carolina at Chapel Hill, Chapel Hill, NC.

Exposure to ambient air pollutants has long been known to contribute to the pathogenesis of respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD), and cancer. However, there has been historically less focus on the role of environmental exposures in the context of interstitial lung diseases (ILD). ILD encompasses different subtypes, often classified based on the causative agent, and include treatment-induced (chemotherapy, radiation), occupational exposure-related

(dusts, fibers, and fumes), and autoimmune. If no causative agent can be identified, the disease is classified as "idiopathic pulmonary fibrosis (IPF)," which is currently the most common type of ILD. IPF is a devastating disease, with a median survival of three-five years after diagnosis, few effective therapeutic options, and potential for exacerbations with a high mortality rate. There is a growing body of evidence that demonstrates the contributions of environmental exposures to IPF. This includes epidemiological studies that have found increased risk of disease incidence, progression, and exacerbations associated with levels of ambient air pollutants, particularly ozone, particulate matter, and nitrogen oxides. This association is not surprising considering many of the initiating molecular events thought to be involved in IPF are also known to be caused by exposure to air pollutants, including oxidative stress, airway epithelial cell injury, and telomere shortening. Mechanistic studies in animals and *in vitro* models have interrogated the link between exposure to ambient air pollutants and fibrotic phenotypes, finding evidence of lung remodeling, airway epithelial cell dysfunction, and fibroblast activation. Further, the interplay between environmental exposures and genetic predisposition has been recently appreciated, and it has been suggested that exposure to air pollutants in genetically susceptible individuals drives the incidence and progression of IPF. Thus, environmental exposures are of concern to the IPF community, and a greater understanding of the types of exposures and mechanisms by which they influence disease pathogenesis is needed. This Workshop focuses on presenting the current state of knowledge regarding the role of environmental exposures in fibrotic lung disease. Experts will provide insight into the epidemiological evidence linking air pollutants to IPF and the potential mechanisms by which this may occur and will discuss genetic factors that may predispose individuals to development or exacerbation of IPF following environmental exposures. The goals of this session are to highlight research in this emerging area, identify knowledge gaps, and encourage discussion and collaboration among epidemiologists, basic scientists, and clinicians to engage in a multidisciplinary approach to better understand this public health issue.

W 1036 Unmasking Environmental Factors in Interstitial Lung Disease

C. Sack. University of Washington Seattle, Seattle, WA. Sponsor: E. Corteselli

Dr. Cora Sack will review the evidence demonstrating that exposure to air pollution may be a modifiable environmental risk factor for the incidence, progression, and mortality of interstitial lung disease (ILD). Her talk will focus on the short- and long-term impacts of air pollution exposure in ILD and explore potential biologic mechanisms, including epigenetic changes and gene x environmental interactions.

W 1037 Air Pollution Fuels Progressive Pulmonary Fibrosis

J. Larson-Casey. University of Alabama at Birmingham, Birmingham, AL.

Epidemiological evidence indicates that exposure to particulate matter is linked to the development of idiopathic pulmonary fibrosis (IPF) and increases the incidence of acute exacerbations of IPF (AE-IPF). In addition to accelerating the rate of lung function decline, exposure to fine particulate matter (PM2.5) is a risk factor for increased mortality in IPF subjects. Exposure to PM2.5 in mice with established pulmonary fibrosis led to monocyte recruitment and fibrotic progression. In mice with established fibrosis, bronchoalveolar lavage cells showed monocyte/macrophage heterogeneity after exposure to PM2.5. These cells had a significant inflammatory and anti-inflammatory signature. The mixed heterogeneity of cells contributed to the pro-inflammatory and anti-inflammatory response. Although monocyte-derived macrophages were recruited to the lung in bleomycin-injured mice treated with PM2.5, recruitment of monocytes expressing Ly6Chi to the lung resulted in progression of fibrosis, reduced lung aeration, and reduced lung compliance. Ly6Chi monocytes isolated from fibrotic mice exposed to PM2.5 showed enhanced expression of proinflammatory markers compared to fibrotic mice exposed to vehicle. Therapeutically targeting Ly6Chi monocytes inhibited fibrotic progression in mice after PM2.5 exposure, while the adoptive transfer of Ly6Chi monocytes exacerbated established fibrosis. These observations suggest that enhanced recruitment of Ly6Chi monocytes with a proinflammatory phenotype mediates acute exacerbations of pulmonary fibrosis, and targeting these cells may provide a novel therapeutic target to protect against AE-IPF.

W 1038 Ozone, Diabetes, Obesity, and Pulmonary Fibrosis: Is There a Link?

R. Tighe. Duke University, Durham, NC.

Ozone (O₃), a significant component of air pollution, is associated with increased respiratory-related hospital and emergency room visits and elevated overall morbidity and mortality rates. Despite efforts to reduce O₃ exposures, ambient levels continue to escalate globally, posing a substantial public health hazard. Addressing this requires focus on mechanism(s) of O₃-induced host-responses to target with precision in susceptible individuals. While susceptibility has traditionally been attributed to host genetics, recent evidence suggests that co-existing metabolic conditions, such as diabetes and obesity, also play a critical role in influencing sensi-

tivity to air pollution. Given their increasing prevalence, defining the mechanisms by which pre-existing metabolic conditions enhance O₃ sensitivity will focus urgent public health interventions. Epidemiology and rodent studies demonstrate that O₃ exposure exacerbates the incidence and severity of metabolic conditions such as diabetes and obesity. However, it remains less clear whether pre-existing metabolic disorders amplify the severity of O₃-induced inflammation. This presentation will highlight recent evidence suggesting that ozone exposure in diabetic/obese rodent strains drives lung injury and fibrosis and define a mechanism via the impact of adipokine signaling.

W 1039 Pattern Recognition Receptors in Environmental Exposure-Mediated Pathogenesis and Exacerbations of Fibrotic Lung Disease

S. Hussain. West Virginia University, Morgantown, WV.

Environmental exposures are known triggers for exacerbations of fibrotic lung disease. However, their role in disease development and the molecular mechanisms by which they induce exacerbations remain poorly understood. Understanding these mechanisms and pathways can potentially identify novel therapeutic targets. Pattern recognition receptors are an essential component of the innate immune system and play a significant role in the pathogenesis of pulmonary disorders. Over the past decade, we have studied the role of toll-like receptors and Nod-like receptors in the context of environmental exposures and chronic pulmonary disorders, including fibrotic lung disease. In this presentation, novel data demonstrating the role of toll-like receptor 5 (TLR5) and Nod-like receptor X-1 (NLRX1) in the development and exacerbation of pulmonary fibrosis will be presented. We will provide functional, biochemical, and histological characterization of the role of these receptors using organoids and whole-body/cell-type-specific knockout animals, as well as in a human cohort. Finally, evidence of the therapeutic benefit of targeting these receptors in the fibrotic disease will be presented. In summary, in addition to providing the mechanistic role of these receptors in the pathogenesis of fibrotic lung disease, it will provide information on the novel therapeutic targets for fibrotic lung disease in the context of environmental exposure-induced exacerbations.

W 1040 Gene-by-Environment Interactions with Ozone-Induced Fibrosis in the Mouse Collaborative Cross Strain CC002/Unc

G. Smith. University of North Carolina at Chapel Hill, Chapel Hill, NC.

Ozone (O₃) exposure exacerbates and worsens outcomes for people with fibrotic interstitial lung disease (fILD) and evidence from rodent and non-human primate studies show that repeated O₃ exposure causes fibrotic changes in the lung, consistent with fILD. However, there is insufficient epidemiological data to establish a link between O₃ and new onset fILD. Most cases of fILD are thought to be caused by the interplay of genetic and environmental factors (GxE). A thorough understanding of underlying GxE will allow for identification of O₃-exposed individuals at most risk for developing fILD. Compared to methods for studying GxE in humans, GxE studies using rodent models are both more tractable and powerful. However, such studies are limited. In this talk we will describe an investigation of GxE using CC002/Unc (CC002), a strain of the mouse Collaborative Cross (CC) predisposed to O₃-induced pulmonary fibrosis. We found that following a relatively low concentration (0.8 ppm O₃) and short exposure paradigm (4h per day for 9 days), CC002 mice develop an aberrant and exaggerated pulmonary eosinophilic inflammation and centriacinar fibrotic lesions. We identified genetic loci underlying CC002's unique inflammatory and fibrotic responses by phenotyping a population generated from backcrosses of CC002 and a low responding CC strain, CC005/TauUnc and conducting quantitative trait loci (QTL) mapping of BAL eosinophil frequency and fibrosis related gene (Col1a1 and Tgfb1) expression quantification across ten batches of backcross mice (n = 304). For O₃-induced eosinophil frequency, we identified five QTL (Oief 1-5), including a QTL of large effect (Oief 4) on chromosome 11 accounting for 18% of phenotypic variation and encompassing 345 protein coding genes. Additionally, the talk will further highlight the process of prioritizing six candidate genes at Oief 4 based on a tabulation of coding and regulatory variants in CC002, prior annotations in the Mouse Genome Informatics database, and known roles in innate immunity, inflammation, and wound healing/fibrosis. In total, our data demonstrates the use of the O₃-sensitive CC002 strain as a new model for studying the adverse respiratory health effects of O₃ such as fILD. Further genetic and functional analysis of the candidate genes we identified using CC002/Unc will provide new insights on the mechanisms of O₃-induced fILD.

L. Anger. Genentech Inc., South San Francisco, CA.

The future of preclinical research is being reshaped by the convergence of innovation, technology, and scientific rigor. This evolution in preclinical research parallels the adoption of digital measures like wearable sensors and artificial intelligence (AI)-powered monitoring in clinical settings, providing a foundation for building a translational bridge and connecting preclinical studies with clinical outcomes. Broader adoption in preclinical toxicology and safety assessment requires building confidence through validation, addressing data security, and navigating regulatory acceptance. This session explores how AI and digital measures are poised to transform *in vivo* studies, enhancing data quality and reproducibility and enabling real-time, noninvasive assessments. The session will showcase examples of real-world applications of these novel technologies in tolerability/safety studies, efficacy studies, and behavioral neurotoxicology studies. Rapid advances in AI and noninvasive sensor technologies offer unprecedented opportunities to monitor health and disease in animal studies. These technologies, including AI-supported home cage monitoring systems, provide a more precise, cost-effective, and objective alternative to traditional observations. This enables more thorough assessments of therapeutic tolerability and earlier detection of adverse events and supports advancement of the 3Rs (replacement, reduction, and refinement) by improving detection of health and welfare events. Importantly, digital measures can also enhance the translational relevance of preclinical studies by providing more human-relevant, continuous, and objective data. Ultimately, by capturing richer insights from each study, this may reduce the need for repeat or additional studies, helping to lower overall animal use over time. Commercially available systems or in-house developed platforms integrate video capture and other sensors in the home cage to generate detailed measurements of behavior and physiology. These systems offer solutions to issues of reproducibility and subjective measurements in preclinical *in vivo* experimentation by enabling continuous monitoring and reducing natural human bias and, at times, the stress and variance introduced by human influence. The application of machine learning to the extensive data collected can predict potential liabilities of compounds and accurately reflect the onset and severity of symptoms in wild-type or disease models. At the same time, these digital caging technologies will advance computational toxicology research to the next level by providing detailed, real-time data on animal behavior and physiology, enabling the development of more predictive models of toxicity and improving the overall accuracy of risk assessments. This session will provide insights into important contributors to a roadmap for integrating AI-powered digital tools into preclinical research. Experts will share a validation framework for establishing analytical and clinical confidence in digital measures and outline strategies for their integration into preclinical and clinical research. There is a critical need for active efforts toward alignment with regulatory authorities to support the integration and eventual acceptance of digital measures in regulatory filings. Open collaboration and early communication between preclinical and clinical communities, as well as with regulators, are essential to ensure these tools are developed and validated in a way that meets regulatory expectations. Speakers will provide learning objectives, discuss validation strategies, data security, translational decision-making, and regulatory engagement, highlighting how collaborative efforts across preclinical and clinical research and across sectors can establish confidence in digital measures and ensure their suitability for broad adoption. The session will conclude with a panel discussion expanding on these essential topics, including strategies for building confidence and navigating regulatory acceptance.

W 1042 Digital Opportunities in Toxicology and Safety Assessment

B. Berridge. B2 Pathology Solutions LLC, Cary, NC.

Rapid advances in Artificial Intelligence (AI) and non-invasive sensor technologies are offering unprecedented opportunity to monitor health and disease in animal studies. These technologies are increasingly used in academic settings (particularly in neurobehavioral research) but are also finding their way into the more standardized and regulated practice of drug safety and environmental hazard assessment. Precedent adoption of digital health measures in the form of "wearables" in the clinical setting provides the foundation for building a more translational and predictive bridge from preclinical studies to clinical outcomes in patients. A growing portfolio of digital measures that may serve as biomarkers of toxicity can increase the sensitivity of our studies for identifying patient-relevant liabilities, provide insights into the onset and progression of those liabilities, and be translated to the clinics to mitigate those risks. This presentation will provide an introduction and overview of AI-supported, digital sensor-based, invasive and non-invasive technologies that are being applied to animal studies in and outside the home cage environment. It will represent those technologies in the context of usual approaches to drug safety assessment, identify specific application opportunities, recognize challenges to their adoption (e.g. data management, data security, costs, etc.), and share vignettes of the efforts of a multi-disciplinary, multi-sector collaboration (the Digital In Vivo Alliance - DIVA) aimed at building capabilities and an ecosystem to support the application of digital measures and biomarkers. Additionally, it will explore approaches to building confidence in these novel measures in a regulated setting applying a framework for analytical and clinical validation. Also, it will discuss the importance of and approaches to engagement with regulatory stakeholders.

W 1043 Improved Toxicity Screening for New Therapeutics Through Use of Machine Learning and Home Caging Systems

C. Lutz. The Jackson Laboratory, Bar Harbor, ME. Sponsor: L. Anger

The Jackson Laboratory is a non-profit research institute with a large program in preclinical development through the Rare Disease Translational Center. Detecting adverse events in therapeutics during the preclinical stages of drug development is a crucial step in minimizing risks and advancing new products to clinical trials. Typically, the initial tolerability assessment of small molecules and biologics takes place in mouse models during efficacy evaluations. Promising therapeutics are then progressed to more expensive manufacturing and toxicology phases of IND enabling studies, which involve the use of rats, pigs, or non-human primates. While acute toxicity can be identified through urine and biomarkers, as well as pathological examination of organs, subtle signs of intolerance are often overlooked during traditional cage side observations. These technician based observations typically monitor changes in behavior, gait, posture, and response to handling. In this case study, we will demonstrate that utilizing automated home cage monitoring systems equipped with cameras and machine learning tools provides a more precise, cost-effective, and objective alternative to conventional cage side observations. Our monitoring systems and developing software have been integrated into studies involving mice, revealing dose-dependent alterations in behavior and physiology in response to treatment challenges. Our home cage machine learning systems allow for more thorough assessments of therapeutic tolerability at an earlier stage in the drug development process, saving time and dollars. The continuous 24-hour monitoring in the animals' home cage environment eliminates stressors like technician handling, sleep disruptions, and subjective technician-driven assessments, addressing the need for enhanced rigor and reproducibility in preclinical studies. Improving the translational relevance of toxicology assessments can reduce the reliance on larger species and the overall use of animals in preclinical studies, addressing both ethical and cost savings. Importantly, our home cage monitoring systems enhance the detection of adverse events before they progress to clinical trials, ultimately improving the safety profile for patients.

W 1044 Smart Cages, Smarter Insights: Advancing Preclinical Pharmacology and Toxicology with AI-Enabled Digital Biomarkers

M. Potter. Sanofi, Cambridge, MA. Sponsor: L. Anger

Sanofi has made a bold commitment to become the first pharmaceutical company powered by AI at scale. This initiative is a key part of Sanofi's long-term modernization and digital leadership strategy. A comprehensive and translational digital biomarker strategy for preclinical studies, supporting our vast pipeline across therapeutic areas, is a key component of this objective. In the age of precision medicine and AI-driven discovery, the humble rodent home cage has the opportunity to be transformed into a powerful data-generating platform unlocking behavioral insights not feasible through traditional observation techniques. This talk will explore how continuous, 24/7 non-invasive home cage monitoring (HCM), powered by computer vision and AI/ML, is reshaping preclinical research by enabling the discovery of digital biomarkers - quantifiable behavioral signatures that reflect pharmacological and toxicological responses in real time. By capturing subtle changes in activity, sleep, social interaction, and more, these systems offer unprecedented insight into disease progression, drug responses, toxicological indicators as well as animal welfare. Tracking spontaneous behavior 24/7 in a naturalistic setting, HCM improves reproducibility, reduces stress-induced variability, and supports longitudinal monitoring across treatment paradigms. This approach not only refines animal welfare but also strengthens the translational bridge between preclinical and clinical studies. At Sanofi, we are taking a diversified approach to explore several promising HCM technologies, as well as opportunities at our external partners. This presentation will discuss our global strategy and progress on adoption of this technology, covering case studies demonstrating how HCM enhances behavioral phenotyping across neurodegenerative, immunological and muscle disease models, offering unbiased, non-invasive endpoints for efficacy evaluation. Additionally, we will highlight its growing role in toxicology, where subtle behavioral deviations can serve as early indicators of adverse effects, often preceding traditional clinical signs in both mice and rats. The results generated to date indicate that this technology will add significant value to our Immunoscience pipeline and enhance our ability to translate preclinical findings into successful and safe clinical outcomes.

W 1045 Use of Automated Home Cage Technologies in Behavioral Neurotoxicology Studies

J. Cushman. NIEHS, Research Triangle Park, NC. Sponsor: L. Anger

Assessing the neurobehavioral impact of environmental exposures is an essential part of screening putative neurotoxins and investigating the mechanisms of toxicity. Neurobehavioral testing plays an essential role in this, however, it is time consuming, labor intensive and very sensitive to variables that are often difficult to control for, such as the handling technique and skill of the experimenter. Automated home cage technologies have the potential to solve these challenges by allowing investigators to fully automate behavioral phenotyping, which allows for greater throughput, repro-

ducibility, and reliability. Integrating machine vision also allows for more sophisticated assessments of behavior that may reveal behavioral changes that might otherwise be missed. This lecture will discuss recent progress in implementing these technologies to examine the neurobehavioral impact of toxicant exposures in both mice and flies (*Drosophila*), including the organophosphate pesticides chlorpyrifos and terbufos. The learning objectives are to provide a general overview of progress in this field, including both commercial and custom-built systems, as well as a preliminary evaluation of their efficacy and discussion of future directions.

W 1046 Strategies for Clinical Adoption of Patient-Centric Novel Digital Endpoints

I. Karahanoglu. Pfizer Inc., Cambridge, MA. Sponsor: L. Anger

Advancements in digital health technologies (DHTs), combined with advanced analytical tools, offer significant opportunities for drug discovery and development in pre-clinical and clinical studies, and for adoption by regulatory authorities. Capturing measurement of activity and physiological data in naturalistic settings with sensors helps facilitate enhanced safety and efficacy monitoring, streamline various phases of clinical development, improving reliability and replicability. For example, continuous vital sign monitoring can provide valuable insights into toxicity in pre-clinical studies, thereby contributing to the drug safety profile. Additionally, it can help in managing and mitigating the risks of life-threatening conditions, such as cytokine release syndrome, following treatment in clinical trials. The use of advanced AI/ML techniques to derive the novel digital endpoints (NDEs), such as gait and physical activity, from complex digital data rapidly paves NDEs' way into clinical use to establish digital phenotypes relevant to diseases. Traditional clinical outcome assessments are episodic, thus cannot capture day-to-day variation, and might suffer from observer bias. The DHTs can transform clinical trials, enhance diversity through remote monitoring, and improve patient care by providing non-invasive, quantitative measures of quality of life. While the use of DHTs has increased tremendously over the past decade across the drug development life cycle and in multiple indications, only a few novel digital endpoints (NDEs) were accepted by regulatory bodies. The 95th percentile stride velocity for Duchenne's Muscular Dystrophy and moderate-to-vigorous physical activity for Fibrotic Interstitial Lung Disease were endorsed by EMA and FDA, respectively, as primary endpoints. Demonstrating NDEs' reliability and analytical validity with device-agnostic AI/ML algorithms are fundamental steps to increase adoption and bolster the body of evidence supporting their use. It is also important to demonstrate the clinical validity and meaningfulness of NDEs within a target population, to be able to reliably measure treatment effects and determine a success criteria. This talk will include examples of analytical and clinical validation strategies that support the clinical adoption and regulatory acceptance of novel digital endpoints in support of drug development.

IS 1047 Wildland Urban Interface Fire Beyond the Smoke: Exploring the Broader Environmental and Health Impacts

Y. Choo. Chemical Insights Research Institute, Marietta, GA.

As climate change accelerates and human development continues to expand into fire-prone wildland areas, wildfires—particularly those occurring in the wildland-urban interface (WUI)—are becoming more frequent, more destructive, and more chemically complex. These fires now impact not only forests and grasslands, but also densely populated communities, critical infrastructure, and thousands of homes constructed with synthetic and engineered materials. The combustion of these synthetic building materials, vehicles, and household goods during WUI fires releases a unique and poorly characterized mixture of toxic pollutants, including volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons (PAHs), heavy metals, microplastics, etc. While the visible plumes of smoke are often the most immediately noticeable and discussed aspect of WUI fires, the full scope of their effects on communities, the environment, and public health extends far beyond the smoke itself. Despite the growing number of affected communities and the scale of environmental devastation, our understanding of the acute and chronic health consequences of WUI fire exposures remains fragmented. Traditional wildfire research has focused primarily on ambient air quality and biomass-derived emissions; the WUI environment introduces new layers of complexity involving indoor exposures, structural damage, ash and residue toxicity, and emerging contaminants. There is an urgent need for a cohesive, interdisciplinary framework that bridges environmental science, toxicology, epidemiology, and disaster response planning to address this evolving public health threat. This Symposium aims to bring together researchers at the forefront of WUI fire research to present novel findings from recent major wildfire events, innovative exposure models, and cutting-edge environmental analyses. Using real-world case studies, controlled laboratory exposures, and advanced analytical techniques, the session will examine how WUI fires produce unique toxicants, how these toxicants enter environments and human bodies, and how they may lead to adverse health outcomes—particularly in vulnerable populations, such as returning residents, children, and emergency responders.

S 1048 Human and Ecological Risk Assessment: Has the Time Come for Greater Integration of Approaches?

L. Brown. ToxStrategies LLC, Ann Arbor, MI.

Human and environmental health are intrinsically connected; yet, chemical risk assessment has historically proceeded along distinct and separate tracks when evaluating each. This often results in parallel evaluations with minimal coordination between assessors. However, with recent scientific advances, particularly the rapid development and validation of nonanimal, mechanistically based methods, new avenues are opening for integrating human and environmental safety decision-making. At the same time, regulatory initiatives such as the proposed "one substance, one assessment" approach, along with growing pressure to incorporate sustainability principles (e.g., the Safe and Sustainable by Design framework), are challenging (eco)toxicologists to move beyond traditional silos and adopt more holistic, unified approaches to chemical safety evaluations. With these driving forces, it is time to understand what innovations can enable more holistic, efficient, and protective assessments that transcend species boundaries. This session will present current activities and frameworks developed for integrating data across human and environmental health. It will focus on how data derived from new approach methodologies (NAMs) can inform both human and environmental safety. The session will begin with an overview of the international scientific initiative PrecisionTox, which researches species, tools, and models to uncover molecular toxicity pathways shared across the animal kingdom. Initial findings from this project will also be shared. Next, a proposed framework, created by an industry consortium that incorporates both next-generation risk assessment principles and integrated human and environmental health, will be presented, along with a state of the research in this area. Lastly, a case study will demonstrate how one may conduct an integrated human and ecological risk assessment from a cosmetics industry perspective. By bringing together expertise from multiple sectors, this session will pose the question: What would it take to truly unify human and environmental risk assessment? Are we ready to take that step?

S 1049 Searching for the Evolutionary Origins of Chemical Toxicity

J. Shaw. PRECISIONTOX, Bloomington, IN. Sponsor: L. Brown

Regulatory toxicology faces the fundamental challenge of setting chemical exposure safety limits for many species, including humans, based on experimental data obtained from only a few standard test species. This pragmatic approach to testing "representative" species of the animal kingdom cannot achieve the desired protection of human health and the environment without an evolutionary and ecological understanding of why toxicity observed in one species can be extrapolated to other distantly related organisms. PrecisionTox, a European H2020-funded project, provides evidence on the feasibility of understanding the health impact of chemicals on all animals, including ecological keystone species and humans, based on the evolutionary conservation of pathways to toxicity. Additionally, PrecisionTox builds an empirical case for a concerted effort to protect people and the environment from the harmful effects of chemical pollution by leveraging evolutionary theory for an integrated risk assessment approach. The talk will cover PrecisionTox's work creating and assembling transcriptomic and metabolomic data into putative biomolecular key toxicity events which are shared among five biomedical model species (*Danio rerio* embryos, *Xenopus laevis* embryos, *Daphnia magna*, *Caenorhabditis elegans*, *Drosophila melanogaster*) and human cells. Specifically the talk will describe how in PrecisionTox, molecular initiating events for adverse outcome pathways and chemical modes of action are identified using a Multimodal Co-Response Graph Analysis (MOCHA) and how the toxicological results across species are phylogenetically assigned to their common ancestors. The speaker will also provide an overview of the end goal for the project, which will be a data explorer that will allow users to view the results from testing 200 substances to i) view the multi-omics data for each tested substance in each organism by dose and time point, ii) compare results across species to identify functionally homologous genes and metabolites, iii) explore trends for any gene, metabolite or their network interactions across all chemicals, doses, and time points. The speaker will demonstrate how the analysis of a relatively small dataset can provide initial insights into the efficacy of the concept of "toxicity by descent" for cross-species extrapolation of chemical modes of action.

S 1050 Development of an Integrated Human and Environmental Health Safety Assessment Framework: Initial Findings and Progress from a Consortium Effort

L. Brown. ToxStrategies LLC, Ann Arbor, MI.

Given the recent push to more holistic and integrated safety assessments, a team has been formed by the International Collaboration on Cosmetic Safety with the goal of developing a non-animal based decision framework to assess both human and environmental safety in an integrated manner. Given the inherent multi-disciplinary nature of proposing a NAM-based framework which includes both human and ecological health, a diverse working group of cosmetics and personal care companies with varying areas of expertise collaborated to develop a process by

which a safety assessor can consider when data from one field of discipline (e.g., human health toxicology) may inform another (e.g., ecotoxicology) or vice versa. This presentation will cover both the approach used to develop the draft framework and present the framework as it stands in March of 2026. A landscape review of existing frameworks for integrated or NAM-based safety assessments was performed to identify existing frameworks for human and/or environmental health safety assessments, areas of overlap between the two, and data gaps that could be addressed in the development of the integrated framework presented herein. In addition, the talk will cover case studies that are being used to “stress test” the framework to identify both the robustness of available tools and the gaps in our ability to ensure safety of relevant ingredients without the use of animal tests. Further, the talk will discuss available findings as it pertains to NAMs that are available to address relevant biological targets including ones of common interest between human health and environment, along with tools that can be used to evaluate to feasibility of cross-species extrapolation based on knowledge of a compound mechanism of action. These key considerations to integrate human and environmental health include addressing relevance of outcomes in one species (e.g., humans) to other species of interest (e.g., fish, invertebrates), using NAMs to understand metabolic pathways, protein/chemical interactions, and gene similarity. The talk will conclude with planned next steps of the working group in the process of finalizing the framework.

S 1051 Case Study-Led Evaluation of an Integrated Human Health-Environmental Next-Generation Risk Assessment Framework

B. Campos. Unilever, Sharnbrook, United Kingdom. Sponsor: L. Brown

Implementation of NGRA concepts in chemical safety assessment is now increasingly feasible through combining non-animal New Approach Methodologies (NAMs) and high resolution exposure datasets to establish bioactivity exposure ratios. The NGRA paradigm shift offers increased opportunities to integrate human health and environmental safety assessment to improve their efficiency and effectiveness. In this presentation, we will share a case study implementing a novel integrated tiered framework to assess human health and environmental safety, capturing opportunities to integrate across the two fields when performing exposure and hazard characterization to reach a safety decision. By using Benzophenone-3 as an example, the talk will describe various aspects of implementing the framework. This will include demonstrating how in the initial tier of evaluation, *in silico* prediction tools, and available data can be leveraged to predict and establish physico-chemical properties, fate and hazard characterization and mechanisms of toxicity covering both human and environmental species. The presenter will also describe how in tiers 1 and 2, the data generation steps, a battery of non-animal NAMs, inclusive of both human and fish cell-line models, covering a broad biological and taxonomical space can be used to provide protective points of departure for the species of interest. These will include High Throughput Transcriptomics (HTTr) and Phenotypic profilers (HTPP) in human and fish cell lines, *in vitro* pharmacological profiling panel (IPP), TOXCAST/ TOX21 assays, RTGill Acute data (OECD 249) and *in vivo* data in algae and invertebrates. To demonstrate how a safety decision may be made with the generated data, the speaker will outline how the PoDs derived from the battery of NAMs are compared to external exposures using robust Human and Fish PBK models. The speaker will also discuss how genomic conservation of biological targets and/or relevant toxicological processes across species can be evaluated using tools such as SeqAPASS and G2P-SCAN. While the objective of the case study was to test and optimize a common battery of NAMs covering both human and environmental species and how all the information can be organized together to support a robust decision, the speaker will also address the inherent differences in when considering human and ecological risk assessments.

R 1052 Integration of Kinetics and Mechanisms in Safety Assessment

J. Fredericks. Gowan, Yuma, AZ.

As new testing approaches are developed to generate more relevant and reliable toxicological data, a more complete understanding of the intricate relationship between kinetics and mechanisms is needed within the broader toxicology community. The literature clearly shows that mechanisms of direct toxicity are dose-dependent, while some adverse effects occur in a mechanistically indistinct or nonspecific manner secondary to high-dose administration. Clarifying the distinction between direct and nonspecific toxicity has the potential to advance the field by providing a deeper understanding of how dose influences toxicological outcomes. Nonspecific toxicity arises when a chemical dose exceeds the biological and metabolic processing capacity of an organism, leading to effects such as cytotoxicity or physical-chemical disruption of cell membranes causing effects that would not occur without such overload. Several developments and advancements in toxicology present an opportunity to refine how these two forms of toxicity are distinguished. Direct mechanisms of toxicity may involve molecular interactions, such as gene expression modulation, receptor binding, or enzyme activity changes. In contrast, toxicity resulting from overload reflects a loss of physiological equilibrium rather than a targeted molecular event. Two complementary strategies can help differentiate these effects. Mechanistic toxicology studies can identify specific molecular and organ-level targets and characterize their dose-response relationships. Kinetic studies can determine the internal concentrations at which biological systems become saturated, identifying a kinetic

maximum dose (KMD) beyond which nonspecific effects may dominate. Integrating these approaches can provide insight into the dose ranges at which chemicals act through targeted biological mechanisms versus those at which generalized biological stress responses are triggered. This session will feature three concise presentations to introduce the topic and provide key context, followed by a panel discussion designed to promote active audience engagement through questions, comments, and debate. Central discussion points include whether current methodologies can reliably and efficiently distinguish direct from nonspecific toxicity and how these distinctions might shape future research and interpretation of toxicological data. The session will incorporate diverse perspectives from industry, academic researchers, and method developers.

S 1053 AI in Toxicology: Navigating Promise, Pitfalls, and Practical Implementation

S. Auerbach. NIEHS/DTT, Research Triangle Park, NC.

Artificial intelligence (AI) is poised to transform toxicology, offering unprecedented opportunities for accelerating chemical safety assessment, enhancing predictive accuracy, reducing reliance on animal testing, and increasing workflow efficiency. From high-throughput screening analysis to predictive modeling of complex endpoints, AI tools promise significant advancements. However, realizing this potential requires a clear-eyed assessment of profound challenges. This Symposium critically examines the current landscape, moving beyond the hype to address the fundamental limitations inherent in applying AI to complex biological systems. The session will explore the successes of AI in areas like early stage prioritization and data analysis, while dissecting key hurdles, including the pervasive issues of data scarcity, quality, and applicability domain constraints; the lack of predictive granularity essential for human health translation; the persistent “black box” problem hindering interpretability and trust; and the infeasibility of purely *ab initio* prediction for emergent toxicological outcomes. Furthermore, the session will confront the practical implications of these limitations, delineating strategies for leveraging AI as a powerful decision-support tool to maximize efficiency while carefully managing the significant liabilities associated with its misapplication in definitive safety assessments. This Symposium convenes experts to foster realistic dialogue on best practices, validation requirements, and the path toward responsible, impactful integration of AI in toxicology research and regulation.

S 1054 Using Deep Learning to Extract Toxicologically Relevant Information from Cell Images

J. Ewald. EMBL-EBI, Hinxton, United Kingdom.

High-content imaging assays like Cell Painting, as well as histopathology whole slide images, provide rich visual data for understanding cellular responses to chemical exposures. Traditionally, these cellular images have been manually examined by eye or quantified using engineered features that measure specific things like the shape, texture, and intensity of cells and subcellular compartments. In more recent years, various deep learning approaches have been used to learn meaningful, quantitative representations of images. When applied to images of cells and tissues, these learned profiles can improve prediction of biological and toxicological endpoints, suggesting that the deep learning approaches are capturing subtle, complex phenotypes that may be missed by the human-defined features. However, these methods also come with trade-offs—including reduced interpretability, reliance on GPU resources, data leakage, and performance issues when models are applied to data that differ from the training set. The presenter will discuss strategies to mitigate these limitations and explore how deep learning can be integrated into toxicology workflows in a practical way, highlighting both the promise and pitfalls of applying modern computer vision techniques to biological image data for safety assessment.

S 1055 The Role of AI in Advancing Drug Safety

N. Greene. Recursion, Salt Lake City, UT.

Artificial intelligence (AI) has the potential to accelerate drug development at every stage of the process including drug safety. By leveraging machine learning, we could reduce the reliance on animal testing, lower development costs, and discover safer drug candidates. This presentation will examine how AI and machine learning methodologies are being applied in the identification and assessment of toxicity early in the drug discovery pipeline. It will explore applications in virtual screening, high-throughput screening data analysis, and the development of more predictive *in vitro* and *in vivo* models. This presentation will also discuss the challenges and limitations of AI and the future applications of this technology in advancing drug discovery toxicology.

S 1056 Bridging Mechanistic AOP Elucidation Using Knowledge Graph Mining and Machine Learning for Chemical Safety Assessment

A. Tropsha. University of North Carolina at Chapel Hill, Chapel Hill, NC.

Biomedical knowledge graphs (KG) have emerged over the last decade as universal means of re-representing and mining biomedical and clinical knowledge integrated from dozens of databases. For instance, ROBOKOP Knowledge Graph developed in our team, currently integrates data from 40 databases and comprises 10M chemical and biological entities and 140M relationships between them. Knowledge graphs have been used for multiple knowledge mining tasks including drug repurposing, searching for clinical outcome pathways, and of course, elucidation of Adverse Outcomes Pathways of chemical toxicity. We will consider methods of rule mining as well as graph embedding to discover novel linkages between chemicals of concern or molecular initiating events and adverse outcomes. We will also discuss approaches to automated discovery of adverse outcome pathways via KG mining that discuss how integration of QSAR models developed for different elements (key events) in the AOP can improve the overall accuracy of *in chemico* to *in vitro* to *in vivo* extrapolation (ICIVIVE). As part of the presentation, we will discuss the value of data curation and model validation in assuring external predictive accuracy of machine learning models.

S 1057 Transforming Drug Safety Reviews: Integrating AI Models into the US FDA DILI Assessment Process

W. Tong. US FDA/NCTR, Jefferson, AR.

The FDA Modernization Act 2.0 promotes alternatives to animal testing in drug development. The release of the FDA Roadmap to Reducing Animal Testing in Preclinical Safety Studies further reinforces the FDA's commitment to advancing the 3Rs principles in drug reviews through New Approach Methodologies (NAMs). To support the FDA's efforts, we established SafetAI Consult to assist the FDA's Drug-Induced Liver Injury (DILI) Team, a specialized group responsible for assessing DILI-related risks during drug safety reviews. SafetAI Consult utilizes a broad range of in-house developed AI models trained on large-scale, validated datasets, including chemical structures, FDA labeling documents, and synthetic data, to predict DILI risk in new drug candidates. The platform's objectives align with three main goals in supporting the FDA DILI Team: (1) To analyze historical drug approval data (e.g., chemical structures and prior adverse event profiles), we provide regulatory reviewers with relevant data by helping identify DILI risk patterns and guiding the review of new drug applications based on previously approved drugs; (2) To assess the likelihood of human DILI concerns early in the review process with the SafetAI models that provide predictive insights, enabling the DILI Team to identify potential safety concerns and alert reviewers to request additional clinical or preclinical data before approval; (3) To guiding labeling recommendations by using integrated insights from both AI model results and other related information, the DILI Team can suggest data-driven adjustments to drug labeling language, ensuring that the drug's safety profile is accurately reflected, and potential risks are communicated clearly to healthcare professionals. The fit-for-purpose AI models undergo a rigorous validation process, comparing them with animal models and other NAMs to assess effectiveness in real-world drug safety assessments. This presentation will discuss how SafetAI Consult's AI models are specifically integrated into the FDA review process, and share lessons learned from their application in supporting the DILI Team's work. Through these efforts, SafetAI Consult plays a critical role in enhancing the FDA's regulatory review process, helping to identify potential risks earlier in the drug development lifecycle while reducing reliance on animal testing through NAMs.

S 1058 Knowing What You Know and Don't Know: Progress Toward Interpretable and Uncertainty-Aware Machine Learning for Chemical Points of Departure

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Lack of points of departure (PODs) representing the dose at which toxicologically-relevant effects begin to manifest are a major bottleneck in the evaluation and management of chemical human health risks and impacts. Widespread use of machine learning (ML)-based *in silico* predictions of PODs has been hindered in part by the desire for greater biological interpretability, more consistent applicability across chemicals, and improved quantitative characterization of uncertainty. We demonstrate recent progress towards addressing these challenges through novel ML approaches. To address interpretability, we implemented a "two-stage" ML framework whereby physical, chemical, and biological properties predicted from "first stage" ML models are used as features in a "second stage" ML to predict PODs. To address applicability and uncertainty, we have demonstrated how "uncertainty-aware" ML models can quantify both data- and model-related uncertainty and provide uncertainty estimates that are closely aligned with observed prediction errors, assigning more "unfamiliar" chemicals higher prediction uncertainty.

S 1059 Extracellular Vesicles as Mediators of Environmental Stress in Carcinogenesis and Other Human Diseases

W. Bisson. Integrative Toxicology and Cancer Prevention, Research Triangle Park, NC.

Extracellular vesicles (EVs), found in blood or other biological human fluids, play key roles in intercellular communication in both normal cell physiology and in disease processes, including inflammation, neurodegenerative and carcinogenic signaling. Nomenclature for EVs varies according to the cell, tissue of origin, size, and subcellular origin. EV cargo contains (and can transfer to host cells) cell components such as RNA, DNA, receptors, proteins, and lipids that are involved in intercellular signaling and cellular processes. Together, EVs and extracellular signaling make up a significant focus of current investigation for mechanistic basic research, discovery of new biomarkers, and clinical applications (e.g., early detection and diagnosis, safer drugs). Biomarkers detectable in body fluids (i.e., liquid biomarkers) offer the unique advantage of being identified by minimally invasive methods compared with markers found in samples prepared from tissues. There is rapid growing interest in (1) studying the roles for EVs in altering cell-cell communication in response to environmental stress; (2) identifying EVs function in environmentally related diseases; (3) discovering EV biomarkers involved in human health and disease; and (4) characterizing EV signaling in environmentally related health conditions. This Symposium is composed of scientists from academia, government, and the private sector, with high expertise in the field of EVs—from methods development, including EVs isolation and separation, and mechanistic basic research to clinical applications and translational toxicology. The first presenter will discuss the carcinogenicity of 1,4-dioxane, a probable human carcinogen, that is largely dependent on Nrf2 activation, which drives the biogenesis of EVs with epithelial-to-mesenchymal transition (EMT)-promoting functions. The next speaker will focus on endothelial EVs as biological messengers and indicators of lung carcinogenesis upon exposure to polycyclic aromatic hydrocarbons (PAH)s. In addition, the speaker will report findings suggesting that an integrative approach combining 'omics and imaging techniques with machine learning could enable the detection and isolation of a specific subpopulation of EVs of interest. The third speaker will then demonstrate how EVs using *in vitro* 3D cell models can serve both as a route for propagating toxic phenotypes and as a source of biomarkers for precision prevention of carcinogenesis and other diseases or the development of safer therapeutics. The fourth speaker will discuss how circulating ex-miRNAs contain tissue-specific signals from EVs and non-EV components and the composition varies by tissue type. As robust biomarkers of tissue health and diseases, these signals enable the tissue-specific assessment of pathophysiological responses to environmental stress, representing a significant advancement toward precision prevention. Finally, the last speaker will explore the clinical application of blood brain-derived EVs (BEVs) as a powerful tool for discovery of biomarkers of disease risk and monitoring neurodegenerative pathologies. To conclude the Symposium, there will be a panel discussion with Q&A.

S 1060 1,4-Dioxane Induces Carcinogenesis Through Nrf2-Dependent Extracellular Vesicle Release

F. Chen. Stony Brook Cancer Center, Stony Brook, NY.

The carcinogenic potential of the environmental pollutant 1,4-dioxane (1,4-D) in humans is not yet fully understood or recognized. In this study, we provide evidence that 1,4-D acts as a carcinogen in human epithelial cells. Using the human bronchial epithelial cell line BEAS-2B, with or without CRISPR-Cas9-mediated Nrf2 knockout, we demonstrate that continuous exposure to environmentally relevant concentrations of 1.25-20 ppm 1,4-D over two months induces malignant transformation in an Nrf2-dependent manner. Transformed cells exhibit enhanced anchorage-independent growth in soft agar, increased migration and invasion, and tumorigenic potential in a xenograft mouse model. Integrated RNA sequencing (RNA-seq) and proteomics analyses reveal that 1,4-D robustly activates Nrf2 signaling, driving the biogenesis and cargo loading of extracellular vesicles (EVs) with syndecan 4 (SDC4) and other proteins, including COL12A1, CAPG and NNMT, associated with epithelial-mesenchymal transition (EMT) and metastasis. Nrf2 knockout reduces SDC4 expression and its incorporation into EVs, leading to diminished EV uptake in recipient cells. Unlike EVs from 1,4-D-transformed WT cells, which enhance the proliferation, migration and invasion of recipient cells, EVs from 1,4-D-transformed Nrf2 KO cells show a reduced ability to promote these EMT properties. Furthermore, we provide evidence that the Nrf2 target gene SDC4, which is induced by 1,4-D and enriched in EVs, plays a critical role in EV uptake by recipient cells, thereby facilitating the propagation of EMT. Overall, our findings suggest that 1,4-D is a human carcinogen, with its carcinogenicity largely dependent on Nrf2 activation, which drives the biogenesis of EVs with EMT-promoting functions.

S 1061 Endothelial Extracellular Vesicles: Biological Messengers and Indicators of Lung Carcinogenesis upon Exposure to Polycyclic Aromatic Hydrocarbons

E. Le Ferrec. Institut de Recherche en Santé, Rennes, France. Sponsor: W. Bisson

Polycyclic Aromatic Hydrocarbons (PAHs) are pollutants to which humans are commonly exposed through breathing or diet. Increasing amount of evidence suggests that air pollutants can modify the production of EVs, induce EV-mediated communication, and facilitate a tumor-promoting microenvironment during the development or progression of non-small cell lung cancer (NSCLC). Furthermore, tobacco-smoke, air pollution (PM2.5) and PAHs have been shown to trigger EV release from different cell types in the lung tissue (e.g., macrophages, bronchial epithelial and endothelial cells). Specifically, EVs derived from endothelial cells in the stroma are also often associated with inflammation, immune activation, and angiogenesis. We have demonstrated that individual PAHs such as benzo[a]pyrene (B(a)P) or mixtures of PAHs are able to increase EV levels in plasma and urine *in vivo*, as well as EV production *in vitro* by endothelial cells, human peripheral blood mononuclear cells or hepatocytes. Among the EVs overproduced by endothelial cells in response to PAHs, we identified vesicles containing intact mitochondria and glycolytic enzymes. These EVs can thus generate extracellular ATP, which is associated with cancer development and resistance, notably through the Warburg effect that we have previously linked to PAHs toxicity. Endothelial EVs can thus be considered as: indicators of cancer risk or cancer pathological states; contributors to disease propagation or exacerbation; biomarkers of exposure or toxic effects; and even as vehicles and mediators of toxicity. To investigate EVs specificities and assess their alterations in pathological contexts such as carcinogenesis upon exposure to environmental contaminants, it is essential to identify and sort EVs (based on size, surface markers, or cargo content) both qualitatively and quantitatively. Recently, the combination of Raman microscopy and tip-enhanced Raman spectroscopy with omics and machine learning allowed us to identify phenotypic alterations (particularly lipid modifications) in EVs produced by endothelial cells exposed to a low concentration of B(a)P. This ability to detect a specific signature proved to be a means of identifying EVs derived from B(a)P-exposed cells. These findings suggest that an integrative approach could enable the detection and isolation of a specific subpopulation of EVs of interest.

S 1062 Extracellular Vesicles in Disease Etiology and Precision Biomarker Discovery

E. Tokar. NIEHS/DTT, Research Triangle Park, NC.

Cells can communicate with one another within the microenvironment and systemically by secreting extracellular vesicles (EVs). These EVs are released both during normal growth and development and in response to toxicity, playing key roles in intercellular communication and signaling mechanisms. Accumulating evidence shows that EV signaling can play critical roles in the development and progression of a wide spectrum of diseases, such as cancer. However, the role of EV signaling in environmental toxicant-induced disease etiology remains poorly understood. We have used several *in vitro* 2D and 3D cell models to investigate the role of EVs in toxicant-induced developmental cardiotoxicity and neurotoxicity and in carcinogenesis. Using a 2D non-contiguous cell co-culture model, we demonstrated how arsenic, a common environmental human carcinogen, affects EVs to impact the tumor microenvironment. Exposure to arsenic increased both the secretion of EVs by the cells and the packaging of myriad cancer-associated cargo molecules (i.e. miRNAs, KRAS, inflammatory cytokines, anti-apoptotic genes) that play key roles in the recruitment of stem cells to a cancer stem cell phenotype. EVs were also isolated from 3D cerebral or cardiac organoids and their cargo (RNA) was characterized using ddPCR and NanoString. Characterization of the cargo in these organoid-derived EVs identified miRNAs known to target several pathways involved in neural or cardiac development and neurodegenerative diseases. Together, the studies and data presented here will highlight the use of various 2D/3D model systems for studying carcinogenesis as well as brain- and cardiac-related diseases and show their utility as tools for the discovery of EV-associated cargo involved in disease and toxicity. The presentation will demonstrate how EVs can serve both as a route for propagating toxic phenotypes and as a source of precision biomarkers for various diseases. Elucidating how EV cargo and signaling properties change during dynamic cellular events and disease etiology and progression may aid precision prevention of toxicity and development of safer therapeutics.

S 1063 Circulating microRNAs as Tissue-Specific Biomarkers of Human Health and Disease

W. Li. Columbia University Mailman School of Public Health, New York, NY. Sponsor: W. Bisson

Early pathological changes in many organ systems are difficult to detect, creating challenges in disease prevention, diagnosis, and treatment. MicroRNAs are highly tissue-specific and constantly released into circulation, either encapsulated in extracellular vesicles (EVs) or bound to proteins, presenting new opportunities to capture early pathophysiological changes in diverse tissue types. We leveraged total circulating extracellular microRNAs (ex-miRNAs) and EV-encapsulated miRNAs (EV-miRNAs) and applied deconvolution to derive miRNA-based tissue specificity scores (ex-TS

and EV-TS scores, respectively) for 17 tissues (e.g. heart, lung, pancreas). We validated these scores against a variety of clinical markers and phenotypes, disease trajectory, and exogenous exposures in population-based cohorts. We then examined whether the proportion of ex-miRNA that were encapsulated in EVs modified the observed effects. Both ex-TS and EV-TS scores were robustly associated with indicators of health across cohorts, with each score uniquely and specifically linked to health outcomes related to the tissue of origin. Interestingly, we observed opposite directions of associations for artery and kidney ex-TS scores versus EV-TS scores, suggesting EV-miRNAs may represent distinct signals as compared to total ex-miRNAs. This is reinforced by composition analysis, which showed that brain and liver-derived ex-TS were mainly enriched in the EVs, whereas artery and kidney-derived ex-TS were correlated with depleted EV signals. Using prospective data, the ex-TS scores also functioned as predictive biomarkers that preceded significant health events including acute myocardial infarction, cerebrovascular accidents, cognitive impairment, and the onset of airflow limitation. Finally, we showed that ex-TS scores can capture acute toxicity and recovery of the liver following acetaminophen overdose, and long-term health effects of smoking and smoking cessation on the lung, with high tissue specificity. Circulating ex-miRNAs contain tissue-specific signals from EVs and non-EV components, and the composition varies by tissue type. As robust biomarkers of tissue health and diseases, they enable the tissue-specific assessment of pathophysiological responses, representing a significant advancement toward precision medicine.

S 1064 Blood Brain-Derived Extracellular Vesicles as a Biomarker Platform for Identification of Neurodegenerative Disease Pathologies

E. Eitan, and O. Volpert. NeuroDex Inc., Natick, MA. Sponsor: W. Bisson

Most neurodegenerative diseases involve mixed pathologies; for instance, 80% of dementia patients show multiple pathological features, while fewer than 5% present with pure Alzheimer's pathology (amyloid beta and tau only). Shifting disease definitions from symptom-based to pathology-based would enable precision medicine (intervention and prevention) and improve drug development. Blood brain-derived extracellular vesicles (BEVs) offer a minimally invasive platform for accessing molecular brain information. Using BEV profiling, we identified Lewy body pathology in dementia patients with 100% specificity and 80% sensitivity (AUC=0.87) across four cohorts (N=140), including postmortem-confirmed cases. BEV-associated alpha-synuclein was also significantly elevated in Parkinson's disease and multiple system atrophy (MSA), with BEV-amyloid-beta in MSA significantly correlating with shorter survival time, highlighting the effect of mixed pathologies across diseases. In ALS, BEV-TDP43 was significantly increased, with 85% sensitivity and 82% specificity (AUC=0.82, N=95), and elevated levels were also observed in dementia (N=310). BEVs also enable measurement of functional pathways: brain lysosomal activity can be assessed via Cathepsin D, while insulin resistance and other metabolic functions are captured through AKT pathway proteins. In a GLP-1 agonist (liraglutide) trial (N=15), BEV-AKT protein levels showed significant modulation. Together, these findings highlight BEVs as a powerful tool for discovery of biomarkers of environmentally related disease risk and monitoring neurodegenerative pathologies. Our automated, scalable platform is now integrated into multiple clinical trials.

S 1065 From Correlation to Causation: Development and Application of Novel Approaches to Implicate Epigenetic Mechanisms in Toxicological Responses

P. Allard. University of California Los Angeles, Los Angeles, CA.

Toxicoeugenetics seeks to understand how toxicant exposures interact with the epigenome to influence disease risk. However, while most environmental epigenetic studies have successfully uncovered associations between a multitude of environmental factors—such as diet, toxicants, or stress—and epigenetic changes, proving that these modifications are directly implicated in the etiology of outcomes is challenging. This difficulty is compounded by confounding variables, individual genetic variability, and the dynamic nature of the epigenome, which can respond to multiple environmental cues simultaneously and over time. Consequently, the field often struggles to move beyond correlation to definitive causality via mechanistic insights. This session will bring together an exciting slate of speakers who have significantly contributed to advancing the field of epigenetics into mechanistic epigenetics through epigenetic editing, drug targeting, and other approaches. The first speaker will describe strategies to show how piRNAs act as mediators of early lead (Pb) exposure in the disruption of placental function and their alteration of gene regulation in a sex- and tissue-specific way. The second talk will be technology focused and will describe the development of a new technological platform, Tagmented, Indexed and Pooled ChIP-Seq, built to overcome current limitations in mechanistic research caused by low-throughput and sample size. The third talk will show how in-depth profiling and comparative analyses across toxicants and tissues can be used for mechanism identification. The speaker will discuss results from the NIEHS TaRGET II Consortium showing that early life exposure to various environmental toxicants causes lasting epigenomic reprogramming and disrupts adult liver gene expression. Despite different modes of action, toxicants like BPA, TBT, TCDD, and PM2.5 produced similar aging-related transcriptomic changes, particularly in genes tied to liver function and disease. These findings suggest early exposures can reshape epigenomic aging and increase long-term disease risk. The fourth talk

will show how epigenetic drugs can be used for environmental epigenetic reversal. Specifically, the speaker will show that treatment with a weak epigenetic modifier reverses BPA-induced transgenerational NAFLD in the medaka and corrected associated molecular changes, suggesting potential for mitigating environmentally induced metabolic disorders in future generations. Finally, the fifth talk will discuss seminal studies on epigenetic editing to induce discrete changes in DNA methylation and monitor their behavior during cellular differentiation and across generations. Together, the session will be of high interest to SOT members for demonstrating how classic and novel strategies can be leveraged to directly implicate epigenetic mechanisms in the etiology of environmentally induced outcomes.

S 1066 Reversal of Environmentally Induced Transgenerational Metabolic and Reproductive Impairments Through Reprogramming of DNA Epimutations

R. Bhandari. University of Missouri, Columbia, MO.

Epigenetic inheritance of environmentally-induced phenotypes has been demonstrated in many model organisms in a laboratory setting, suggesting that individuals in current generations could be harboring ancestral environmental exposure-induced molecular memories. Thus, it is essential to develop strategies to correct transgenerational abnormalities before they are transferred to offspring via germline transmission. As a proof of concept, using medaka fish as an aquatic model organism of human and ecosystem health, we investigated whether the environmentally induced transgenerational non-alcoholic fatty liver disease (NAFLD) can be reversed by epigenetic modifier treatments. Ancestral bisphenol A (BPA) exposure (10 µg/L) caused transgenerational NAFLD leading to nonalcoholic steatohepatitis (NASH) in offspring at F2 generation, which persisted for five generations. The severity of the disease was sexually dimorphic: with females affected more than males. Past F2 generation, the transgenerational NAFLD started after puberty, developed in adulthood, and progressed toward NASH and fibrosis as the fish aged. We performed transcriptomic, metabolomic, and methylome analyses to understand the mechanisms involved. DNA methylation profile of the F1 paternal sperm predicted the DNA methylation and transcriptional landscape of the F2 offspring liver revealing distinct pathways associated with NAFLD. A larger percentage of transgenerational differentially methylated promoters (DMPs) present in the NAFLD liver at the F2 generation were removed in the F4 generation during germline transfer. Still, the phenotype persisted but with comparatively reduced severity. Treatment of the F4 fish with a weak epigenetic modifier resulted in: 1) A reversal of NAFLD phenotype to normal, 2) Correction of the majority of BPA-induced DMRs, irrespective of their methylation status, and 3) restoration of transcriptional network comparable to that in untreated controls. The present results suggest that low-concentration nutritional epigenetic modifiers may be useful for correcting EDC-induced transgenerational liver injury. Results open avenues for further research towards understanding the role of environmentally established epigenetic landscape in metabolic health and disease and the mitigation of this multifactorial metabolic disease in humans.

S 1067 Early Life Environmental Exposures Reprogram Epigenomic Aging to Alter Gene Expression Trajectories

C. Walker. Baylor College of Medicine, Houston, TX. Sponsor: P. Allard

To understand how early-life environmental exposures shape health and disease risk across the lifecourse, the NIEHS TaRGET II Consortium exposed mice to diverse toxicants from pre-conception through weaning, and followed individual animals into adulthood, generating over 800 epigenomic and transcriptomic profiles. These profiles revealed that early-life exposures induced persistent epigenomic reprogramming and significantly disrupted the adult transcriptome. Notably, despite their diverse mechanisms of action, the exposure signatures of the xenoestrogen BPA, obesogen TBT, dioxin TCDD, and air pollutant PM2.5, all converged on genes normally differentially expressed during liver aging. Epigenetic histone modifications at enhancers—and, to a lesser extent, promoters—emerged as key targets for this reprogramming. Despite differing mechanisms of action, these four toxicants imparted similar “fingerprints” on the adult liver, characterized by direction- and cell type-specific polarization of the transcriptome. Hepatocyte genes that typically increase with age, particularly those in metabolic pathways, were downregulated, while conversely, non-parenchymal cell genes that typically decrease with age, such as those involved in extracellular matrix production, were upregulated. A similar anti-correlation with programmed aging was also found in the transcriptome of patients with liver disease and hepatocellular carcinoma (HCC), and was effective at distinguishing healthy from diseased human livers. These findings demonstrate that the plasticity of epigenomic aging is vulnerable to early-life environmental exposures, which can reprogram the epigenome with lasting impacts on the transcriptome, and disease risk, later in life.

S 1068 DNA Methylation Editing to Study the Transgenerational Inheritance of Epigenetic Memory at CpG Island Promoters in Mammals

Y. Takahashi. Kumamoto University, Kumamoto, Japan. Sponsor: P. Allard

Whether transgenerational epigenetic inheritance occurs in mammals, to what extent epigenetic information can be passed to offspring, and whether it is associated with disease heritability have been attractive scientific questions. We demonstrated that DNA methylation of promoter-associated CpG islands (CGIs) can be transmitted from parents to their offspring in mice. We generated DNA methylation-edited mouse embryonic stem cells (ESCs) in which CGIs of two metabolism related genes, the Ankyrin repeat domain 26 (Ankrd26) and the low-density lipoprotein receptor (Ldlr), were specifically methylated and silenced using our DNA methylation editing strategy. DNA methylation-edited mice generated by microinjection of the methylated ESCs exhibited abnormal metabolic phenotypes. Both the acquired methylation of the targeted CGI and the phenotypic traits were maintained and transmitted across multiple generations. The heritable CGI methylation was subjected to reprogramming in parental PGCs and subsequently reestablished in the next generation at post-implantation stages. This indicates that it was not the DNA methylation itself, but rather an epigenetic memory that was transmitted—one that can lead to the reestablishment of CGI methylation. In this presentation, I would like to discuss the underlying mechanisms and how such epigenetic editing strategies may be applied to the study of environmental alterations of the epigenome.

S 1069 Deciphering Epigenetic Mechanisms of Lead Exposure During Mouse Development

B. Perera. University of California Berkeley, Berkeley, CA.

Environmental lead (Pb) exposure during critical developmental periods can have long-lasting health effects through epigenetic mechanisms. Despite extensive research on Pb toxicity, the epigenetic pathways through which Pb exposure influences long-term health remain poorly understood. The current work investigates (1) the effects of gestational Pb exposure on the placental weight and expression profiles of imprinted genes, and (2) sex- and tissue-specific effects of perinatal Pb exposure on PIWI-interacting RNA (piRNA) pathways in adult mice at 5 months. Gestational exposure to Pb significantly increased placental weight regardless of fetal sex, with subsequent RNA-seq revealing substantial Pb-induced changes in placental gene expression, including those related to genomic imprinting. Perinatal Pb exposure identified differentially expressed piRNAs specific to the male mouse cortex. Despite cessation of Pb exposure at weaning, the piRNA downstream target Taok3 exhibited decreased DNA methylation and increased gene expression in adult mouse brains. Collectively, these findings suggest that Pb exposure disrupts placental function and fetal development, likely through imprinting dysregulation. Perinatal Pb exposure also has long-lasting effects on longitudinal piRNA expression in a tissue- and sex-specific manner, potentially via DNA methylation-directed mechanisms. This work highlights piRNA as a potential disease biomarker and as a promising candidate for interventions through epigenome-editing mechanisms.

S 1070 Overcoming Sample Limitation in Mechanistic Epigenetics: Introducing TIP-ChIP, a High-Throughput Alternative to ChIP-seq

S. Traynor. Active Motif, Carlsbad, CA. Sponsor: P. Allard

Difficulty in accessing sufficient experimental material is a common bottleneck in the assessment of epigenetic changes and often informs the number of conditions that can be simultaneously tested and which epigenetic mark will be examined. In this method-oriented talk, we will present Tagmented, Indexed and Pooled ChIP-Seq, or TIP-ChIP, to generate high throughput, multi-sample results from low input samples. TIP-ChIP is a novel chromatin profiling method designed to overcome the limitations of traditional ChIP-seq. Capable of processing up to 96 samples simultaneously in a single reaction, TIP-ChIP significantly reduces batch effects and sample-to-sample variation while requiring substantially lower input material. The streamlined workflow minimizes hands-on time and increases overall efficiency. A robust barcoding strategy enables accurate sample deconvolution without compromising resolution. TIP-ChIP has been validated across multiple cell types, including human primary cells, and shows strong concordance with conventional ChIP-seq data. This high-throughput method provides a powerful and scalable solution for mapping histone modifications in large-scale epigenomic studies.

S 1071 Rebuilding Immunity in the Lab: Complex *In Vitro* Models for Smarter Safety Science

M. Collinge. Pfizer Inc., Groton, CT.

The immune system is one of the most complex and dynamic networks in the human body—and one of the most challenging to evaluate in toxicological risk assessment. Traditional approaches to immunotoxicity testing have relied heavily on animal models, which often fail to capture the nuance of human-specific immune responses, especially in cases of immune sensitization or dysregulation or immunosuppression. As scientific and regulatory communities pivot toward more human-relevant, mechanism-based safety assessment, new approach methodologies (NAMs) are becoming essential tools in the evolving immunotoxicology toolbox. This session explores the cutting-edge application of NAMs, including complex *in vitro* models such as microphysiological systems (MPS), immune-competent organoids/ spheroids, and 3D co-culture systems, in the context of immuno-safety assessment. These models enable the recapitulation of key immune functions—such as antigen presentation, cytokine signaling, immune cell trafficking, and barrier integrity—in a controlled and human-relevant environment. When integrated with high-throughput screening, 'omics technologies, and computational modeling, these approaches offer powerful new ways to predict, detect, and understand immune system perturbations caused by pharmaceuticals, chemicals, biologics, and environmental agents. Speakers will present recent breakthroughs in the development and validation of NAMs for key immune assays, with a focus on early stage de-risking and compound selection through assays intended to reduce animal studies and support a regulatory package. The session will address critical questions around translational relevance, regulatory uptake, and model standardization. The session will also include discussion of regulatory frameworks that are beginning to incorporate or accept NAM-based data. This session aims to bridge scientific innovation with practical application, offering toxicologists, immunologists, and risk assessors a forward-looking view of how NAMs are redefining the landscape of immuno-safety across academia, industry, and government. Attendees will leave with a deeper understanding of both the scientific potential and the implementation challenges of complex NAMs, as well as opportunities for future collaboration across sectors.

S 1072 New Approach Methodologies in Immunotoxicology: Current Landscape and Future Directions

E. Corsini. Università degli Studi di Milano, Milan, Italy.

The ability of chemicals to alter immune function—causing immunosuppression or inappropriate activation—is a significant public health concern. Immune disruption has been linked to asthma, autoimmune diseases, cancer, and increased infection risk, highlighting the need for reliable, human-relevant immunotoxicity assessments. Historically, such evaluations have relied on animal models using tiered testing strategies to assess various immune components. While these models have advanced understanding, they present ethical concerns, high costs, and limited predictive value for human outcomes. Driven by the goal of reducing animal use, recent efforts focus on New Approach Methodologies (NAMs), including *in vitro* assays, *in silico* models, and systems biology tools. These approaches aim to enhance mechanistic insight and human relevance. However, the complexity of the immune system means no single method can cover the full spectrum of potential adverse effects. Nonetheless, progress is evident. Alternative assays—such as cytokine profiling, T-cell proliferation, and dendritic cell function tests—are gaining validation and regulatory interest. Similarly, *in vitro* and *in silico* sensitization models have advanced, with some incorporated into OECD guidelines. This lecture will highlight recent scientific and regulatory advances in alternative immunotoxicity testing, address persistent challenges such as assay standardization and applicability domains, and explore future directions including Adverse Outcome Pathways and systems immunology. Together, these innovations support a more predictive, ethical, and human-centered approach to immunotoxicity evaluation.

S 1073 The Future Is Adaptive: Modeling Immune Diversity and Function in Microphysiological Systems

R. Pompano. University of Virginia, Charlottesville, VA. Sponsor: M. Collinge

Although the past decade of advances in animal studies and human multi-omics platforms yielded dramatic advances in understanding of immune processes, effective vaccines and immunotherapies remain challenging to develop. One reason is the lack of accurate models that recapitulate immune responses in human disease. Microphysiological systems (MPS) offer the potential to fill this gap, but integration of adaptive immune function into MPS is still in early stages. In addition, factors such as sex and other aspects of human variation are rarely considered in MPS models, but significantly impact immune responses to infection and vaccination. This lecture will briefly review current MPS models of lymphoid function and describe our recent work on an MPS that predicts antibody production from naïve human lymphocytes. The model starts with purified naïve T cells and B cells from human blood, co-cultured in a 3D matrix in a microfluidic chip that allows the option to pattern cells into T cell and B cell regions. Naïve CD4 T cells were differentiated into T follicular helper cells

and allowed to interact with B cells. This model yielded plasmablast and plasma cell differentiation, spontaneous formation of lymphoid clusters, and IgM production in response to model (super)antigens, making it applicable to donors with a broad range of HLA alleles. In ongoing work, we are advancing the system to predict the response to vaccine-relevant antigens, and incorporate elements of human variation such as age and sex.

S 1074 Reconstructing Human Lymph Node Function *In Vitro* Using a Microphysiological System

L. van den Broek. MIMETAS, Oegstgeest, Netherlands. Sponsor: M. Collinge

Lymph nodes play a crucial role in orchestrating immune responses in the human body. However, existing *in vitro* models fall short of replicating the full complexity of human lymph node architecture and function. Microfluidic technology presents a promising solution to bridge this gap by enabling stable chemical gradients, continuous perfusion, and membrane-free stratified co-cultures. These features provide physiologically relevant signals and support complex 3D tissue organization that closely mimics *in vivo* lymph node. To advance this field, NC3Rs and four pharmaceutical companies funded a CRACK IT Challenge aimed at developing a human *in vitro* T-cell dependent antibody response (TDAR) assay. In this talk, we present the human lymph node-on-a-chip platform developed in this challenge. This platform co-cultures human adaptive and innate immune cells (PBMC derived), along with supporting stromal cells (eg. endothelial cells and fibroblasts), in a 3D configuration. The immune cells in the 3D lymph node model were assessed by flow cytometry to look at the state of the immune cells and different subtypes and assessed by immunofluorescent staining to look at their interaction. Upon exposure to the SARS-CoV-2 spike protein, the *in vitro* human lymph node showed changes in immune cell development and humoral immune responses, including plasma cell differentiation. Additionally, B and T cell activation and proliferation were observed, along with dynamic reorganization of immune cells. Fluorospot was performed to look at specific SARS-CoV-2 IgG and total IgG production. In 50% of the experiments specific IgG for SARS-CoV-2 were detected upon exposure to the SARS-CoV-2 spike protein. This is demonstrating the potential of microfluidic platforms for modeling human-specific immune responses *in vitro*.

S 1075 From Simple to Complex: Leveraging a 2D *In Vitro* Assay for Primary Antibody Response to KLH

L. O'Donnell. Pfizer Inc., Groton, CT. Sponsor: M. Collinge

Lack of an *in vitro* assay capable of measuring a primary antibody response, equivalent to the *in vivo* T cell-dependent antibody response (TDAR), is an important gap in immunosafety assessments. While development of 3D and microphysiological systems (MPS) are advancing our understanding of many aspects of the immune system, development of successful complex models of a functional primary immune response remains a challenge. 2D approaches, with their relatively simple composition of defined immune cells co-cultured in suspension, are often considered less relevant since they lack the architectural and spatial complexity of immune organs. But the assays can be sufficient to generate relevant immune functional data and the higher throughput and versatility of 2D assays can be leveraged to establish the necessary components for higher complexity approaches, such as antigen-priming, co-culture cell type composition, provision of exogenous factors, etc. In this talk, we present the development of a 2D *in vitro* assay, using human PBMC and cells differentiated from human PBMC, capable of generating antibody responses to the neo-antigen KLH. Findings that support successful generation of a KLH-specific immune response including critical cell responses (such as activation and/or proliferation of dendritic cells, B cells and T cells), provision of exogenous factors and anti-KLH IgM production, will be presented. Efforts to transfer the 2D assay to more physiological 3D and MPS models and development of an alternative 2D assay using immune tissue (ie, tonsil) will be discussed.

S 1076 Regulatory Considerations for Incorporating NAMs-Based Immuno-safety Assessments into the Drug Development Process

R. Wange. Aclairo Pharmaceutical Development Group, Bethesda, MD.

The immuno-safety assessment of new drug has always been a somewhat challenging endeavor. This, in part, derives from differences in the immune system between species, as well as the tiered approach to the assessment of immuno-safety, which generally does not directly query the effects of a pharmaceutical on functional aspects of the immune system. Appropriately constructed and qualified NAMs hold the promise of being able to bridge the gap from both of these shortcomings. This presentation will consider some of the regulatory considerations and expectations regarding incorporation of NAM-based immuno-safety assessments into the drug development process.

W 1077 **Reevaluating Adversity and the Maximum Tolerated Dose in the Modern Regulatory Environment**

F. Sewell. NC3Rs, London, United Kingdom.

While alternative approaches are rapidly being developed, animal testing remains a regulatory requirement as part of safety assessment for many sectors. It is essential that where *in vivo* studies are required, they are performed to ensure the highest standards of animal welfare. Establishing appropriate scientifically justified high dose levels or maximum tolerated dose (MTD) in regulatory toxicology studies conducted in animals is essential to generate meaningful data for human risk assessment while minimizing unnecessary harm and distress for the animals. This is especially important when data are used in hazard-based regulatory environments, as data from underdosed studies are not considered informative for hazard identification purposes. Increased regulatory focus on this topic has led to concerns around the definition of the MTD and increased the potential to test at excessively high dose levels that risk flawed data and present welfare concerns. This session will discuss regulatory expectations in terms of dose level selection and establishing adversity, focusing on differences across geographies and within OECD test guidelines, and the need for more alignment and guidance in this area to ensure humane endpoints whilst maximizing the scientific validity of the data. The first presentation will provide the background to this topic, information on how consistently the MTD concept is applied across CROs and why these well-established principles on adversity are now being revisited. Other presentations will discuss considerations for dose level selection, including body weight loss versus reduced body weight gain, food consumption, and other clinical signs, calling for more tailored and objective guidance on humane endpoints for specific study types and/or life stages to better inform decisions. Finally, case studies from CROs and industry will be presented to illustrate the complexities of dose level selection and the consequences of inappropriate dose level selection and provide alternative approaches to refine dose selection using data-driven approaches with the predictive power of NAMS in hazard-based environments.

W 1078 **Introductory Background on Dose Selection of *In Vivo* Studies Within Hazard-Based Regulatory Environments and Why First Principles of Dose Adequacy Are Now Being Reevaluated**

E. Richmond. Exponent, Harrogate, United Kingdom.

Establishing adversity levels within *in vivo* toxicity studies for chemical regulatory purposes is fundamental both scientifically and legislatively. Despite being a common principle established for many decades, in recent years there has been increased European regulatory focus on suitability of dose levels to fulfil these requirements and reliably inform on hazard endpoints for human safety. European regulatory concerns arose from evidence of underdosed studies rendering data meaningless for classification purposes. However, establishing adversity has to be balanced against the flawed scientific data and ethical issues arising from dosing at excessive levels exceeding the Maximum Tolerated Dose (MTD). Whilst OECD guidance exists to define humane endpoints, this only reflects frank effects clearly exceeding the MTD, and there is no guidance to establish more refined evidence of adversity across a spectrum of change. Reflecting this lack of guidance and perceived increases in risk over regulatory acceptability, there are emerging patterns for *in vivo* testing at excessive doses in an effort to empirically prove the eventual high dose has been fully maximized. In order to reverse this concerning trend, and support better regulatory confidence in dose setting, there is now a pressing need to reevaluate adversity and the concept of the MTD in the modern testing paradigm and it is proposed that harmonized guidance be developed. To facilitate discussion, and provide background, this introductory workshop session will outline what information already exists to support dose selection and adversity within European guidance and legislation, and provide context to why this well-established principle on identifying adversity setting is now being revisited. This session will also present information on a data scoping exercise conducted across CROs to establish how consistent the concept of the MTD and humane endpoints are applied across laboratories.

W 1079 **Refining Humane Endpoints by Providing More Objective Guidance**

F. Sewell. NC3Rs, London, United Kingdom.

The use of appropriate humane endpoints to minimise pain and suffering is a key consideration when conducting *in vivo* studies, including for making decisions regarding dose selection, to determine the maximum tolerated dose (MTD) and/or the need for interventions. Whilst there is debate around what constitutes an MTD in terms of limits for body weight loss and/or reduced body weight gain, some guidance exists within the OECD guidance document on limits relating to humane endpoints (OECD Guidance Document 19, 2000). However, this is generic and subjective, and not specific to different study types, duration, species and/or life stages (e.g. juvenile or pregnant animals). This subjectivity can mean differences in opinions, and therefore decision-making, which can impact the in-life experience of the animals involved. There is a need to update current advice on humane endpoints to support

the use of tailored and objective humane endpoints that are appropriate for the species used, the procedure/study performed, and the organ/body system involved. This can be used to inform study designs and in-study decisions, and to better define early interventions to avoid and/reduce suffering in animals. This presentation will present the need to update current guidance on humane endpoints that considers factors such as the duration and severity of clinical signs that can help provide a more objective definition of humane endpoints.

W 1080 **Evaluation of Adversity and Selection of Dose Levels for Toxicology Studies Using Clinical Signs, Body Weight, and Food Consumption**

M. Beekhuijzen. Charles River Laboratories, Den Bosch, Netherlands. Sponsor: F. Sewell

In the last couple of years there has been a lot of debate on the selection of dose levels and evaluation of adversity between industry and ECHA. Working at a contract research organization (CRO) we are in the midst of this discussion, with scientific arguments against regulatory arguments. A resolution for this problem is urgently needed. With this workshop we would like to start moving towards consensus and produce a guidance document that aligns the approach for dose level selection and adversity evaluation. In this presentation, several case studies will be presented with examples of clinical signs and effects on body weight and food consumption. These will be put against published literature and regulatory guidance (for pharmaceuticals, industrial chemicals and pesticides), followed by a discussion with the audience. Examples from both general toxicology as developmental and reproductive toxicology studies will be presented as well as rat and rabbit data.

W 1081 **Evidence-Based Dose Level Selection for Toxicology Studies Using Prior Knowledge and New Approach Methodologies**

D. Cowie. Syngenta Crop Protection, Bracknell, United Kingdom. Sponsor: F. Sewell

Toxicology studies are crucial for assessing the safety of novel compounds, informing regulatory decisions, and protecting human and environmental health. A critical aspect of study design is dose selection, which must balance hazard identification with ethical considerations and resource efficiency. Traditionally, identifying the Maximum Tolerated Dose (MTD) involved initially testing high doses that were subsequently reduced if found excessive, often resulting in unnecessary animal testing and potentially misleading toxicological profiles. This presentation introduces a systematic, evidence-based framework for optimizing dose selection that integrates historical knowledge with modern technologies. We demonstrate how comprehensive analysis of existing toxicological databases—including clinical pathology parameters and organ toxicity profiles—can establish scientifically justified starting points for toxicology studies. These data-driven approaches can be further refined by the implementation of physiologically based pharmacokinetic/pharmacodynamic (PBPK-PD) modeling to extrapolate *in vitro* findings to *in vivo* scenarios across species. In combination, these tools enable the extrapolation of *in vitro* and *in vivo* data to predict potential effects across different dose levels and species, thereby refining dose selection and reducing reliance on extensive animal testing. By integrating prior knowledge with the predictive power of NAMS, this presentation will demonstrate how to design more scientifically sound, ethically responsible, and resource-efficient toxicology studies.

W 1082 **Petroleum Substance Testing Strategy and Results: Trying to Demonstrate Use of the “Highest Possible Dose Without Severe Suffering or Death” and Still Obtain Meaningful Results**

N. Synhaeve. Concawe, Brussels, Belgium. Sponsor: F. Sewell

Petroleum substances are complex hydrocarbon substances with unknown and variable composition (i.e. UVCBs), posing significant challenges for toxicity assessment. To evaluate their safety under the REACH legislation, *in vivo* toxicological tests are mandated, particularly for reproductive and developmental toxicity endpoints. Historically these substances have been assessed by dermal and inhalation routes of exposure, as the main routes of (limited) human exposure. Over the years further knowledge has been built on the overall properties of the various petroleum streams and the constituents that cause toxicity (e.g. 3+ ring poly-aromatic hydrocarbons). For UVCBs it is difficult to demonstrate systemic exposure, and per regulatory request new testing via the oral route of exposure was initiated. We conducted a series of OECD TG 422 screening studies for 14 gas oil substances with the aim of selecting suitable candidates for higher tier testing, provide range finding for further work and, to demonstrate biological similarity for read across. We will discuss how and why we amended our study design to accommodate the new guidance from ECHA on dose-level selection for the conduct of reproductive toxicity studies. Following the OECD TG 422 studies, we have conducted a number of higher tier tests which have included the limit dose of 1000 mg/kg/day. In some of these studies we have observed (predictable) secondary effects due to reduced bodyweight gain and

food consumption as well as excessive liver hypertrophy. We will also discuss the difficulties encountered due to dosing levels in differentiating adaptive and non-adverse findings from potentially toxic observations.

W 1083 Talking the Tox: Effective Risk Communication of Toxicological Hazards for Public Consumption

B. Cummings. Wayne State University, Detroit, MI.

This Workshop uses an interactive approach with case studies to help attendees develop critical strategies for effectively communicating the risks that environmental toxicants, pollutants, medical devices, and therapeutics pose to humans. Attendees will gain knowledge of initial steps in risk communication, development of communication teams, and implementation of communication plans that inform the public of the risk of toxicants during health emergencies, environmental disasters, and drug development. Case studies presented will aid attendee's understanding of key steps in the initial design and implementation of risk communication using scientific rationale. Attendees will be first be exposed to an overview of risk communication and will be introduced to stakeholders in risk communications and to the ethical frameworks that serve as guiding principles for scientists to engage and communicate with the community-at-large. The following presentations will focus on risk communication from the pharmaceutical, government, and emergency response perspectives and will utilize case studies to emphasize concepts introduced in the initial presentation. An interactive format will be utilized allowing attendees to identify key concepts using their mobile devices and review these concepts audience-wide after each study is presented. The Workshop will end with a roundtable allowing panel members to share sound practices and barriers to effective risk communication. Attendees will be provided test cases as part of the engagement activity and will be encouraged to present their approach to communication of risk to the panel to aid their understanding of key strategies of effective risk communication.

W 1084 Critical Strategies in Effective Risk Communication of Adverse Outcome Events That Threaten Public and Environmental Health

W. Al-Delaimy. University of California San Diego, CA. Sponsor: *D. Germolec*

Science involves several processes from design to implementation, and communication of potential risks or hazards to human health requires proper and ethical community engagement. This is more critical in environmental health research where results can lead to adverse impacts on the livelihood and well-being of impacted communities. Furthermore, in environmental health there are many stakeholders with vested interest that do not support open scientific evidence if it harms their industry or livelihood of communities exposed to such risks. In this session, real-life scenarios will be shared with the audience and address the process of engaging, as well as reporting results of studies. The ethical frameworks will be the guiding principles for scientists to engage and communicate to study participants and the community at large.

W 1085 Risk Communication in Pharmaceutical Development: Educating the Government and the Public on Drug Safety and Development

L. Lehman-McKeeman. Bristol Myers Squibb, Princeton, NJ.

This presentation will inform attendees about approaches used by pharmaceutical companies to communicate the application of hazard identification and risk assessment to drug discovery and development. Relevant stakeholders include non-toxicology colleagues, government regulatory agencies, medical practitioners and the public. Examples will be used to highlight the combination of a broad spectrum of research tools and approaches that can be used to determine human safety. Discussion of communicating findings on mechanistic research to define the relevance of animal data for human risk assessment for mouse lung tumors observed with the antiviral drug entecavir (hepatitis B) will be highlighted. The critical need to discuss exposure-related adverse effects at margins that exceed therapeutic exposures will be discussed with examples including anti-coagulant therapies and kinase inhibitors. These examples will highlight effective communication strategies for exaggerated pharmacology or dose-dependent off-target effects. Strategies for communicating these approaches in a cogent, integrated and simple manner will be discussed and will align the case study with key concepts from the first presentation.

W 1086 How Do Governmental Agencies Communicate the Risk of Environmental Chemicals to the Public?

L. Birnbaum^{1,2}. ¹Duke University, Durham, NC; and ²NIEHS (Retired), Research Triangle Park, NC.

This presentation will inform attendees about approaches used by government research and regulatory agencies to communicate the risk of regulated and emerging environmental toxicants to human and animal health. PFAS are known as "forever" chemicals because they are environmentally persistent, mobile, and often toxic. They

represent a huge class of synthetic chemicals which have multiple uses but have also been associated with a plethora of adverse effects in multiple animal species as well as people. This speaker will highlight the communication of hazards and risks of exposure to "forever" chemicals, PFAS, and align the strategies with key concepts presented by the first speaker.

W 1087 Communication of Risk on the Frontlines: Lessons from High Profile Chemical Emergency Responses

P. Nony. Center for Toxicology and Environmental Health, Little Rock, AR.

This presentation will inform attendees about approaches used to assess and communicate risk during some of the highest profile chemical emergency responses of the past 20 years, including the 2010 Gulf Oil Spill. It will highlight crisis communication during emergency situations and strategies used when dealing directly with key stakeholders, such as emergency responders and private citizens. It will also present strategies from a toxicologist with over 20 years of expertise in emergency response to spills impacting the public and align these strategies with key concepts in risk and crisis communications.

W 1088 Toxicological Risk Assessment of Medical Devices Used for Neonatal and Pediatric Populations

S. Rawat. Becton Dickinson, Irvine, CA.

Medical devices can be used to treat patients across the lifespan, from infancy to old age. However, there are particular challenges associated with the development of pediatric devices, both in terms of clinical applicability and the potential toxicological risk posed by patient exposure to compounds released from the devices. Clinically, innovators must modify adult-sized products to address the smaller size of the patient and their rapid changes in growth. From a toxicological perspective, the need exists to derive tolerable intake (TI) values that are protective of adverse effects that may occur in this potentially more sensitive subpopulation of patients. While instrumental updates have recently been made to the ISO 10993-17 standard to address the potential risk posed by exposure of pediatric patients to compounds released from device materials, there are still unique challenges that exist when conducting toxicological risk assessments for neonatal and pediatric populations. The goal of this workshop is to explore the clinical challenges associated with developing pediatric devices; provide an overview of considerations that need to be accounted for when performing toxicological risk assessments for neonatal and pediatric populations; and provide case studies that illustrate approaches that can be used to address the challenges.

W 1089 Regulatory Overview and Discussion of the Challenges and Benefits Associated with Developing Pediatric Devices

A. Komiya. Bluestocking Health LLC, San Diego, CA. Sponsor: *S. Rawat*

Few medical devices are designed and marketed specifically for children. Instead, adult devices are often repurposed and used off-label in pediatrics. The innovation gap between pediatric and adult devices is complex and multifactorial. This talk will provide an overview of the medical device landscape, describe barriers (and benefits) to pediatric device development, and include strategies that are available to help overcome these obstacles. Key topics include the complexities of designing devices that accommodate the anatomical and physiological differences in pediatric patients, the importance of well-designed biocompatibility studies, and strategies for addressing the limited availability of clinical data in this population. Participants will also learn about the latest FDA guidelines and initiatives aimed at fostering innovation while maintaining rigorous safety standards.

W 1090 Overview of Challenges Related to the Toxicological Risk Assessment of Infants and Children from Exposure to Medical Devices

S. Parker. SParker Toxicology Consulting LLC, Davie, FL.

The presentation will describe the differences in neonate and general population systemic toxicity associated with medical devices. Further, the challenges with evaluating the toxicological risk of extractable and leachable compounds to neonates and children will be outlined. This will include a discussion of ISO 10993-17 risk assessment principles and a description of how each step of the risk assessment paradigm needs to be adapted for neonates and children. Specifically, the challenges will be discussed in deriving TI values for infants and children; establishing actual clinical use scenarios; and considering foreseeable misuse as a new focus of ISO 10993-1.

W 1091 Availability and Use of Developmental Toxicity Data for Evaluating the Risk of Adverse Events to Infants and Children

K. Ehman. Eurofins Lancaster Laboratories, Saint Paul, MN.

Ideally, data derived from studies in which neonates are exposed to a potentially hazardous material are preferable when calculating TIs for medical devices intended for use by neonates. However, to capture different developmental milestones in a human, multiple animal studies may need to be considered. This presentation will provide an overview of commonly available developmental and reproductive study designs as well as the reported endpoints that define effect levels. The objective is to provide toxicologists with an understanding of the strengths as well as the limitations to be able to effectively select a point of departure (POD) and appropriate uncertainty factors (UF) for the population being evaluated.

W 1092 Is an Additional UF Needed for the Derivation of TI Values Intended to Be Protective of Infants and Children?

R. Brown. Risk Science Consortium, Arnold, MD.

ISO standards and technical specifications have been developed to provide guidance on methods to derive Health Based Exposure Limits (HBELs) for compounds released from medical devices. These HBELs include Tolerable Intake (TI) values and Threshold of Toxicological Concern (TTC) values. The method for deriving TI values is described in the ISO 10993-17:2023 standard. Specifically, the TI and TE values for compounds released from devices intended for use in children should include consideration for the potential immaturity of the principal routes of elimination of the material and the potential for higher accumulation. Ideally, data derived from studies in which neonates are exposed to the hazardous material are preferable when calculating TIs for medical devices intended for use by neonates. However, data from toxicity studies conducted in juvenile animals are not readily available, therefore, ISO 10993-17:2023 outlines the application of an uncertainty factor (UF) of 3 for derivation of TI using data from studies conducted in adult animals. This talk will explore whether the existing UF to account for interindividual variability is sufficient to account for the potential increased sensitivity of neonates and children to extractable and leachable compounds.

W 1093 Case Studies Evaluating Neonatal Susceptibility to Chemical Exposures from Medical Devices and Implications for Toxicological Risk

J. Cohen. Gradient, Boston, MA.

Medical devices intended for neonatal populations are typically evaluated for potential systemic toxicity and carcinogenicity risks via chemical characterization and subsequent toxicological risk assessment of the extractable chemicals profile. In accordance with ISO 10993-17:2023, chemical specific tolerable intakes require an additional uncertainty factor of 3 to account for immaturity of the metabolic capacity and renal function for this specific patient population (infants <6 months of age). The immaturity of neonatal toxicokinetics (metabolism, distribution and elimination) can potentially yield blood levels that are higher for longer durations than those typically observed in older populations. Furthermore, neonatal susceptibility is a concern for several commonly observed medical device extractables. For example, neonatal exposures to benzyl alcohol are associated with metabolic acidosis, respiratory distress and neurological toxicity. Isopropyl alcohol is associated various toxic effects including central nervous system depression in neonates. Potential susceptibility to di-(2-ethylhexyl) phthalate (DEHP), a reproductive toxicant in animals, is of particular concern in light of its frequent use in medical tubing and blood storage bags found in neonatal intensive care units. In this presentation, case studies detailing specific mechanisms of neonatal susceptibility, and the implications for potential toxicological risk associated with exposures associated with chemical released from medical devices will be discussed.

W 1094 Considerations in the Toxicological Risk Assessment of Gas Pathway Devices for Neonates and Children

R. Nguyen. Philips, Perrysburg, OH.

Medical devices with breathing gas pathways intended for neonates and infants present unique challenges in biological and toxicological risk assessments. These challenges are due to the route of exposure via the respiratory tract with different forms of potential hazards such as Volatile Organic Substances (VOS), particulate matter, and leachables found in the condensate, and warrant special consideration as they are coupled with the susceptibilities of low body weight patient populations. This presentation will provide an overview of the anatomical and physiological differences between the respiratory system and metabolism of adult and infant populations, and how these differences influence toxicological risk assessment for gas pathway devices. It will also review current approaches for evaluating VOSs and

aldehydes (ISO 18562-3), leachables in condensate (ISO 18562-4), and derivation of tolerable intakes (TIs) and tolerable exposures (TEs). Approaches for calculation of exposure and inhalation doses for neonates and infant population per ISO 18562-1:2024 and ISO 10993-17:2023 will also be discussed.

R 1095 Chemical Exposures and At-Risk Populations in California

A. Reddam. OEHHA, Oakland, CA.

California is a large state with a heterogeneous population and wide-ranging topography. Populations in California have multiple and varied environmental exposures that can impact health. These can include exposures from oil and gas refineries, air pollution, pesticides, and extreme weather events such as wildfires. We need a better understanding of California's unique chemical exposure patterns, with the goal of identifying potential sources and reducing exposures by implementing policy changes. Furthermore, this information can help inform how populations are exposed to similar environmental exposures in other regions of the US. This session brings together scientists from academia, government, and nongovernmental organizations to discuss the different ways in which Californian's exposures to chemicals are being assessed, with a focus on the role of biomarkers as an exposure tool. Following a brief introduction to the topics covered in this session, the first speaker will discuss the development of analytical methods used to assess both chemical exposures and metabolic profiles indicative of birth defects in amniotic fluid. Next, we'll hear from speakers on the implementation and findings from biomonitoring studies carried out in different regions and communities within California. The second speaker will discuss metals exposures in urban communities that live near oil drilling sites in Los Angeles using toenails, a noninvasive biological matrix. The third speaker will talk about biomarkers of air pollution exposure and biomarkers of response in a population in the Central Valley of California. Finally, the fourth speaker will discuss community experiences and real-world impacts of biomonitoring data in California, with a focus on a community of farmworkers in the Central Valley and firefighters who were first responders to wildfires across the state. The session will end with a panel discussion. This session will provide a well-rounded discussion on novel methods and approaches used in biomonitoring and the value and impact of the data generated from these studies.

R 1096 Prediction of Birth Defect Cases in Amniotic Fluid Using Machine Learning on Non-targeted HRMS Features: Integrating Metabolomics and Environmental Contaminants in the Exposome

X. Ji. CalEPA Department of Toxic Substances Control, Berkeley, CA. Sponsor: A. Reddam

Birth defects remain a major global public health concern, with growing evidence implicating environmental exposures alongside genetic factors. The full range of exogenous chemicals linked to adverse fetal outcomes remains unclear. Amniotic fluid, reflecting both maternal and fetal exposures, offers a valuable matrix for profiling metabolic and chemical signatures relevant to fetal development. We applied LC-HRMS-based non-targeted analysis (NTA) to amniotic fluid from pregnancies affected by birth defects to: (1) assess metabolic differences between case and control samples and predict status via machine learning; (2) annotate discriminative features using database searches and *in silico* fragmentation; (3) identify altered metabolic pathways; and (4) investigate exogenous chemicals and their associations with disrupted profiles. Although specific contributors could not be confirmed, several significantly different features (Mann-Whitney U test, $p < 0.01$) showed decreased signal in cases. These represented diverse metabolite classes, with 76% remaining unidentified. Pathway analysis revealed pronounced changes in long- and medium-chain fatty acid metabolism, phospholipid metabolism, and tryptophan metabolism. Network analysis highlighted metabolites in primary bile acid biosynthesis and fatty acid metabolism intersecting with purine metabolism, amino acid exchange, and the acetyl-CoA pool from nitrogen metabolism. We identified exogenous chemicals positively correlated with altered endogenous metabolites, suggesting potential interference with lipid and mitochondrial enzymatic function during pregnancy. Notable examples include butyl levulinate (solvent, fuel additive, fragrance ingredient), oleic acid methyl ester (industrial, agricultural, cosmetic, biodiesel uses), and lauryl sulfate (anionic surfactant). Our findings demonstrate the potential of NTA-integrated exposomics to inform early diagnostics and clarify links between environmental exposures, pregnancy complications, and fetal development disorders.

R 1097 Metal Exposures in Residents Living Near Urban Oil Drilling in Los Angeles, California

J. Johnston. University of California Irvine, Irvine, CA. Sponsor: A. Reddam

Los Angeles (LA) California, is situated atop one of the largest urban oil fields in the United States. Land development, population growth, and oil exploration occurred concurrently, leaving a patchwork of thousands of active oil wells operating in very close proximity to homes, schools, and parks. There is limited informa-

tion about potential biomarkers of exposures. As part of community-based participatory research, we assessed exposure to arsenic (As), cadmium (Cd), mercury (Hg), manganese (Mn), nickel (Ni), lead (Pb), and antimony (Sb) in toenail clippings collected from two cohorts in South LA. We used non-negative matrix factorization (NMF), a dimension reduction technique, to identify potential sources of toxic metal exposures, and to understand how these metals cluster. In the LAS VOCES cohort, consisting of adults living within 1 km of an active oil drilling site on the Las Cienegas oil field (n=239), we identified three factors, including a Ni and Mn factor, which is hypothesized to be related to oil drilling. Ni is the most abundant trace metal in oil and Mn has been linked to oil drilling; both Mn and Ni have been found together in particulate matter near drilling sites in the LA area. Expanding this work to the BELLA cohort, consisting of Latina women living throughout South LA neighborhoods (n=369) we found this same factor. Moreover, we observed a statistically significant increase in the Ni-Mn factor score with increased oil and gas production within 1 km of the participants home in the prior 12 months. This research builds evidence of clustered Ni and Mn exposures near active oil and gas production in LA. Utilizing NMF, we identified a source factor high in Ni and Mn in residents living near oil and gas production in urban LA.

RI 1098 Urinary Biomarkers of Air Pollutants in Two Communities in the San Joaquin Valley

A. Reddam. OEHA, Oakland, CA.

The San Joaquin Valley (SVJ) in California (CA) has some of the nation's worst air quality, often failing to meet federal standards. Exposure to air pollutants is associated with serious health effects, with children being particularly vulnerable. The objective of the Biomonitoring Component of the SVJ Pollution and Health Environmental Research Study (BiomSPHERE) was to measure biomarkers related to air pollution exposure in Stockton and Fresno, CA. Urine samples were collected from 64 parent-child pairs in 2023. Metabolites of four polycyclic aromatic hydrocarbons (PAHs), six volatile organic compounds (VOCs), and nicotine were measured in urine by LC-MS/MS. Additionally, four biomarkers of response (8-isoprostane, 8-OHdG, PGE2, and CC16) were measured by ELISA. A survey was administered to collect information on demographics and potential exposure sources of PAHs and VOCs. The average age of adults and children was 42 and 9 years, respectively. Most participants were Hispanic/Latino (69%). Metabolites of fluorene (2-hydroxyfluorene), naphthalene (2-hydroxynaphthalene (2-naphthol)), phenanthrene (1-hydroxyphenanthrene), and pyrene (1-hydroxypyrene) were detected in more than 85% of samples. Metabolites of acrolein (3-hydroxypropyl mercapturic acid), acrylonitrile (2-cyanoethyl mercapturic acid), crotonaldehyde (3-hydroxy-1-methyl-propyl mercapturic acid), and propylene oxide (2-hydroxypropyl mercapturic acid) were detected in more than 90% of the samples. Levels of PAH and VOC metabolites in urine were similar or lower than NHANES, except for 2-naphthol. Concentrations of 2-naphthol were around 3 times higher than NHANES and significantly positively correlated between adults and children suggesting a common exposure source. Additionally, 2-naphthol levels had a high interclass correlation coefficient, suggesting consistent exposure to naphthalene. 2-naphthol levels were significantly higher in Hispanic/Latino participants and in participants who used cleaning products, air fresheners, and scented products. Each of the biomarkers of effect were detected in all participants. Average levels of 8-isoprostane, 8-OHdG, and CC16 were higher in adults compared to children, and PGE2 was higher in children. Further work is needed to identify exposure sources for naphthalene and associations between the biomarkers of exposure and response.

RI 1099 Report-Back of Environmental Exposure Data and Impacts of Biomonitoring Studies in California

S. Patton. Commonweal Biomonitoring Resource Center, Bolinas, CA. Sponsor: A. Reddam

This presentation will examine two case studies of community-based biomonitoring research projects in California. The first case study will focus on firefighter exposures to PFASs and other chemicals and the second case study will explore pesticide exposures in a farmworker population in the Central Valley. This presentation will report on the different steps of community-based participatory research, from requests for biomonitoring, to sample collection in the field, to dissemination of results. We will highlight how the "exposure experience"—the embodied, personal experience and understanding of chronic exposure to environmental pollutants—is shaped by community context and the report-back process itself. We analyze themes of expectations and learning, trust, and action and how these may differ depending on various facets of the community. This presentation unravels the complex relationship between lay experience and lay understanding of science and discusses the importance of community-based participatory research for policy development and/or social change in California and beyond.

S 1100 It's Time to OVARY-Act: New Insights into the Effects of EDCs on Ovarian Function

B. Rickard. North Carolina State University, Raleigh, NC.

Endocrine-Disrupting chemicals (EDCs) have the potential to disrupt normal ovarian functioning by interfering with key processes such as hormone regulation and ovulation. Impairment of these processes is detrimental, as, locally, it can lead to menstrual irregularities, infertility, and polycystic ovarian syndrome, while, more broadly, hormone imbalances can contribute to osteoporosis and cardiovascular disease. In some cases, EDC exposures can also contribute to the onset and metastatic progression of ovarian cancer, the deadliest gynecologic malignancy largely due to the limited effectiveness of existing therapies. This Symposium brings together scientists of varying expertise and career stage to discuss the latest research on EDCs, ovarian health, and their underlying mechanisms. The session will begin with a brief introduction of normal ovarian processes and organ-specific targets of EDCs. The first speaker will then provide an overview of the effects of EDCs on ovarian health from a pathologist's perspective. Next, the second speaker will highlight the effects of environmentally relevant phthalate mixtures on ovulation in multiple primary ovarian cell culture models. The third speaker will follow by providing new evidence of mechanistic pathways driving phthalate-induced adverse effects on several ovarian cell types and fertility outcomes using a single-cell approach. Shifting gears to another class of EDCs, the fourth speaker will present compelling findings illustrating the adverse effects of gestational exposure to the herbicide atrazine on ovarian follicle health in a porcine model. Finally, shifting the focus to ovarian cancer, the fifth speaker will share novel findings suggesting that chronic (35-day), human-relevant perfluoroalkyl substance (PFAS) exposures drive chemotherapy resistance through enhancing mitochondrial functionality. Altogether, this session will highlight the importance of considering the impact of EDC exposures on the ovary over multiple life stages (i.e., *in utero*, early life, and later cancer outcomes) and will shed light on the novel mechanisms driving such effects. A 10-minute discussion led by the Chair and Co-Chair will conclude the session, providing time for additional questions from audience members and a chance to discuss future research topics of importance, such as emerging EDCs or potential therapeutic interventions for effects discussed during this session.

S 1101 Introduction: New Insights into the Effects of EDCs on Ovarian Function

L. Sapienza-Lundie. University of North Carolina at Chapel Hill, Chapel Hill, NC. Sponsor: B. Rickard

This session will cover EDCs and their effects on normal ovarian function and overall women's health. Humans are exposed to EDCs through multiple routes on a daily basis, such as through use of personal care products and even in drinking water supplies. While awareness of EDCs, like phthalates or PFAS, and their adverse health impacts has increased in recent years, further research is needed to fully identify how these environmental chemicals alter ovarian cell types and functions, such as menstrual cyclicity, hormone levels, and fertility. Throughout this session, we will explore how exposure to EDCs shapes ovarian function and women's health, mechanisms driving alterations, and ways to change the trajectory of women's health for the future.

S 1102 Endocrine-Disrupting Chemicals and the Ovary: A Pathologist's Perspective

B. Dray. Charles River Laboratories, Ashland, OH. Sponsor: B. Rickard

Endocrine disrupting chemicals (EDC) are ubiquitous in the environment and there are a multitude of ways in which they can impact ovarian function. Females are born with a finite number of oocytes and therefore EDCs that effect oocyte/follicular health can have a permanent impact on fertility and hormonal regulation. Furthermore, some EDCs have been shown to cause multigenerational effects. The effects of EDCs on the ovary vary depending on the particular chemical and exposure level, but often include altered follicular growth, increased follicular atresia, abnormal luteal regression, poor oocyte quality, alteration to ovarian steroidogenesis, and/or inhibition of ovulation. These effects can differ depending on when the exposure occurs in relationship to reproductive maturity. The ability to avoid exposure to EDCs is nearly impossible, so identifying and characterizing the impact of these chemicals is of critical importance. Veterinary pathologists play a key role in evaluating the endocrine disrupting potential of these chemical compounds. Evaluation of the female reproductive tract can be complicated by the cyclical changes that occur in hormone levels and in the morphology of the organs, along with notable differences in reproductive anatomy and physiology between species. Depending on the length of the study and the life stages examined, differences in reproductive maturity and senescence can be important considerations. Thorough knowledge of the different estrus cycle (or menstrual cycle) stages and common background findings in each species is essential when evaluating the ovary for potential test substance-induced changes. Although organ weights and histopathology are the most obvious endpoints evaluated by pathologists on regulatory studies, there are several other

endpoints that are important to consider when evaluating potential EDC impact on the female reproductive tract which may include hormone levels (FSH, LH, E2), age at vaginal opening and first estrus, estrus cyclicity over time as measured by vaginal lavage, ovarian follicle and corpora lutea counts, implantation sites, and fertility indices. Integration of all available end points is vital in determining which chemicals exhibit endocrine disruption and potential far-reaching effects on reproduction and health.

S 1103 Exposure to an Environmentally Relevant Phthalate Mixture Inhibits Ovulatory Prostaglandin Function in Human Ovarian Granulosa Cell, Cumulus Cell, and Endothelial Cell Models

K. Land. University of Kentucky, Lexington, KY.

Exposure to phthalates, ubiquitous EDCs, is unavoidable, and is now recognized as contributing to the prevalence of infertility in women. Measurable levels of phthalates are found in human ovaries, and since previous studies established that phthalates disrupt ovarian function, exposures may also impair steroidogenesis and ovulation. While many studies have focused on individual phthalate exposures in animal models, little is known regarding the mechanistic effects of phthalate mixtures on ovulation in women. This is alarming since ovulatory defects are the leading cause of infertility. Ovulation is characterized by immense production of prostaglandins (PGs). PGs are vital mediators of ovulation due to their involvement in cumulus-oocyte complex (COC) expansion (stabilized matrix for egg transport to the site of fertilization) and angiogenesis (proliferation and migration of endothelial cells to the ovulating follicle). PGs, specifically PGE2 and PGF2a, are synthesized by granulosa and cumulus cells within the ovulating follicle. PGs bind to their receptors, PTGER1-4, on cumulus and endothelial cells to facilitate COC expansion and angiogenesis, respectively. Primary human granulosa cells, cumulus cells, and ovarian endothelial cells (hOMECs) were collected from women undergoing IVF to test the hypothesis that exposure to an environmentally relevant phthalate mixture (MPTmix) inhibits PG production and function. MPTmix was derived from urinary phthalate metabolite measurements in pregnant women. In human granulosa cells, ovulatory increase in PGE2 and PGF2a levels was inhibited by MPTmix exposure compared to controls. In human cumulus cells, MPTmix exposure decreased PGE2 levels compared to controls. In hOMECs, MPTmix exposure decreased PTGER1 mRNA levels and altered PTGER4 mRNA levels (dose-dependent) compared to controls. These data suggest that MPTmix exposure decreased PG production in granulosa and cumulus cells, which can disrupt expansion of the COC necessary for ovulation. These data further suggest that MPTmix exposure altered the levels of PG receptors on endothelial cells, which can disrupt angiogenesis that is vital for ovulation. Ultimately, exposure to an environmentally relevant phthalate mixture disrupted the essential ovulatory PG pathway in 3 human ovarian models; thus, exposure to phthalates may impair women's fertility.

S 1104 Single Cell Insights into Chemical Toxicity in Human Ovaries

P. Damdimopoulou. Karolinska Institutet, Solna, Sweden. Sponsor: B. Rickard

Human-made chemicals have become an unavoidable part of modern life. The estimated 350,000 chemicals on the market have contributed to an increased standard of living; however, historically poor regulations of their use now jeopardize human health. For example, although the EU has the strictest chemical legislation in the world, 70% of chemicals on the European market have not been assessed for safety. These chemicals end up as contaminants in the environment throughout their life cycle: manufacture, transport, use, and waste handling. As a result, individuals are continuously subjected to complex mixtures of poorly characterized chemicals, including persistent organic pollutants and EDCs. These exposures have been linked to various adverse health outcomes, including infertility. Our analyses of northern European women have revealed complex mixtures of persistent pollutants, parabens, and phthalates in ovarian follicular fluid. These exposures correlated with altered ovarian sensitivity to gonadotropin stimulation during medically assisted reproductive technology. Notably, phthalates were linked to disrupted cholesterol and steroid biosynthesis as well as increased inflammation in somatic cells of the follicles. To provide causal evidence for these observed epidemiological links, we conducted controlled exposure studies using human ovarian tissue culture as a model. As human ovaries are composed of multiple cell types, we analyzed the impacts at a single-cell resolution. We discovered that although ovarian stromal cells exhibited the highest number of significantly differentially expressed genes in response to phthalate treatment, the fold changes were modest. In contrast, rare cell types in the ovary, such as glial cells, immune cells, and endothelial cells, responded to phthalate treatment with high fold changes, suggesting elevated susceptibility. The targeted cellular processes were similar across cell types, relating to metabolism, mitochondria, cell cytoskeleton, and cell-to-cell signaling. Altogether, this study provides novel insights into the molecular pathways through which phthalates may disrupt human ovarian function and highlights sensitive cell types. Several somatic cell populations were found to be more responsive than follicles. This emphasizes the need to assess ovarian toxicity holistically, considering all components, not just follicles.

S 1105 Maternal and Female Offspring Ovarian Follicle Number and Chemical Metabolism Protein Abundance Are Altered by *In Utero* and Perinatal Atrazine Exposure

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Atrazine (ATZ) exposure during perinatal development is linked to reproductive dysfunction, which is proposed to be attributed to endocrine disruption. Also, gestational ATZ exposure can lead to adverse outcomes, including low birth weight, delayed fetal growth, and delayed onset of puberty. To investigate the effects of ATZ exposure on the maternal and female offspring ovary in a porcine model, timed pregnant nulliparous sows were provided *ad libitum* access to water that contained an environmentally relevant concentration of ATZ (20 µg/L) or vehicle control [0.002% (v/v) ethanol] to achieve a final ATZ dose of ~0.0014 mg/kg BW. Piglets were exposed to control or ATZ *in utero* from gestation day 28 through birth and lactation (~127 days total). The number of ovarian follicles and abundance of chemical biotransformation and steroidogenic proteins in postnatal day 10 offspring and maternal ovaries were assessed. In offspring, ATZ exposure did not alter the abundance of primordial, primary, and secondary follicles but tended to decrease the abundance of atretic follicles. In sows, ATZ exposure did not affect primary, secondary, antral, or atretic follicle number, however, the number of preantral follicles decreased due to ATZ exposure. Phase I and III chemical biotransformation proteins were also evaluated and found to be altered in both piglets and sows, and there were also impacts on steroidogenic proteins. Taken together, ATZ exposure during gestation and lactation may impact female fertility due to altered ovarian follicle number and ovarian abundance of chemical biotransformation and steroidogenic proteins.

S 1106 Chronic Perfluoroalkyl Substances Exposure Induces Chemoresistance in Ovarian Cancer Cells via Promoting Mitochondrial Alterations

B. Rickard. North Carolina State University, Raleigh, NC.

Ovarian cancer is the deadliest gynecologic malignancy due to high rates of recurrent disease characterized by resistance to platinum-based chemotherapeutics. Thus, identifying drivers of chemoresistance is critical to improving survival outcomes. Environmental exposures have long been studied in the context of oncogenesis, but few studies have examined the link between environmental exposures and therapy response. Since environmental exposures occur daily and toxicants bioaccumulate over time, it is plausible that the exposure to and buildup of toxicants over a lifetime may interfere with response to treatment. Our lab focuses on exposures to perfluoroalkyl substances (PFAS), which are global drinking water contaminants that have been linked to adverse reproductive outcomes. To evaluate the effects of human-relevant PFAS exposures on ovarian cancer cells, we developed a PFAS chronically-exposed ovarian cancer cell line (35 days) and measured response to carboplatin (first-line therapy) and doxorubicin (second-line therapy for platinum-resistant disease). Compared to shorter durations of PFAS exposure (2-6 days), where cell survival increased by up to 50% following chemotherapy treatment, survival fractions increased by an average of 200% in PFAS chronically-exposed ovarian cancer cells treated with chemotherapy. These findings suggest that PFAS induce chemoresistance in a duration-dependent manner and motivated us to understand mechanisms driving this malignant adaptation. Studies have shown that mitochondrial health is pivotal for determining whether an ovarian cancer cell will respond to chemotherapy. As a result, we evaluated mitochondrial structural and functional parameters in PFAS chronically-exposed ovarian cancer cells and found that mitochondrial content and mitochondrial DNA copy number significantly increased compared to controls. Additionally, PFAS-exposed cells demonstrated increased maximum respiratory capacity and extracellular acidification rates, indicative of remodeled metabolism. These findings are critical, as increased mitochondrial content and metabolic reprogramming have been implicated in chemoresistant ovarian cancer. Altogether, this research provides novel insight into the mechanisms underlying PFAS-induced effects on chemotherapy efficacy and may be used to inform personalized treatment regimens based on PFAS exposure levels.

S 1107 Milk Matters: Assessing Breast Milk Through an Environmental Health Perspective

J. Zelikoff. NYU Grossman School of Medicine, New York, NY.

Breast milk is an infant's primary source of water, vitamins, minerals, and fats but also a potential source of environmental toxicants. Maternal exposures can directly translate to infant exposures in milk, particularly in the case of lipophilic compounds. This Symposium will gather experts in breast milk toxicology research from throughout the country to discuss exposure assessment and biological effects of contaminants commonly found in breast milk, such as the per- and polyfluoroalkyl substances (e.g., PFAS, PFOA), microplastics, and brominated fire retardants (BFRs). A brief overview will introduce the topic and speakers and provide a five-minute summary of the overall content of the program. After the introduction, the first presentation will focus on microplastic contamination in breast milk, with a discussion of the physiochemical characteristics and distribution of microplastics in human milk. The second presen-

tation will discuss methods for quantifying BFRs and PFAS in human breast milk and the current levels seen in North American populations, while the third talk will focus on the relationship between PBDE-47 exposure and extracellular vesicle miRNA in human breast milk samples. The fourth presentation will provide insight regarding a possible intervention strategy wherein clay sorbents could be used to remove PFAS from human milk prior to feeding. All speakers will participate at the end of the Symposium in a panel discussion to address preselected and audience-based questions regarding common toxicants in breast milk and their potential short- and long-term health effects, risk-benefits of breast feeding, and potential interventions to protect infant health.

S 1108 Quantitative Analysis of Microplastics in Breast Milk Using Integrated Microscopy and Spectroscopic Approaches

L. Barela, and M. Garcia. University of New Mexico School of Medicine, Albuquerque, NM.

The pervasive presence of micro- and nanoplastics (MNPs) in the environment has raised growing concerns about their potential effects on human health, particularly during vulnerable life stages such as infancy. MNPs are the result of widespread plastic production and degradation, infiltrating ecosystems, food chains, and the environment. Recent studies have identified MNPs in various human biological samples, including blood, lungs, and placenta, suggesting that no organ system is entirely protected from exposure. While previous research has detected MNPs in human breast milk using techniques such as infrared imaging and Raman micro-spectroscopy, these approaches are limited by particle size detection thresholds and potential misidentification of polymer types. To date, no studies have employed a combination of chemical digestion and Pyrolysis Gas Chromatography/Mass Spectrometry (Py-GC/MS) for identifying and quantifying a broad range of polymer types in breast milk analysis. This study addresses that gap by analyzing breast milk samples, including both donor milk and infant formula, collected in 2022 through a collaboration with the NYU Grossman School of Medicine. By optimizing detection sensitivity for 12 distinct polymers, this work provides a comprehensive assessment of microplastic presence in breast milk. The results raise critical questions about potential links between MNP accumulation and maternal lactation challenges, with preliminary associations observed between MNP presence and conditions such as breast engorgement, mastitis, and low milk supply. These findings have broader implications for maternal and infant health, as well as for ongoing research into how plastic-derived pollutants could influence human physiology. Ultimately, this study not only expands the current understanding of MNP distribution in human tissues but also underscores the urgent need for environmental and public health policies aimed at reducing plastic exposure during critical developmental stages.

S 1109 Legacy and Emerging Contaminants in Breast Milk from United States Mothers

A. Salamova. Emory University Rollins School of Public Health, Atlanta, GA. Sponsor: J. Zelickoff

Recent research demonstrates that nursing infants are exposed to a mixture of legacy chemicals that have been phased out from production and use, along with chemicals that replaced them. This presentation will focus on the occurrence, patterns and trends of legacy and replacement chemicals in breast milk from North America, focusing on per- and polyfluoroalkyl substances (PFAS) and brominated flame retardants (BFRs). Breast milk was analyzed for 39 PFAS, including 9 short-chain and 30 long-chain compounds, and 37 polybrominated flame retardants (PBDEs), 18 bromophenols, and 11 other BFRs. Sixteen PFAS compounds were detected in 4-100% of the samples, with several short-chain PFAS detected in the majority of the samples. A total of 26 BFRs were detected, including 9 PBDEs, 8 bromophenols, and 8 other BFRs. Analysis of available breast milk PFAS data from around the world (between 1996-2019) revealed that while the levels of phased-out PFAS have been declining, the detection frequencies of current-use short-chain PFAS have been increasing with a doubling time of 4.1 years. Analysis of time trends in PBDE concentrations in North American breast milk indicated a significant decline since 2002, with a halving time of 12.2 years; comparison with previous samples from the northwest U.S region showed a 70% decline in median levels. Overall, these results demonstrate that while the levels of the phased-out chemicals in milk from US mothers are dropping, the levels of replacement chemicals are increasing, posing continuing health risks for nursing infants.

S 1110 Adverse Effects of Maternal PBDE Exposure on Milk EV-miRNA Profile and Infant Neurodevelopment

R. Khan. NYU Grossman School of Medicine, New York, NY.

Though PBDEs were discontinued in the US in 2009, as stable, persistent contaminants they still exist both in the environment and as body burdens. PBDE exposure has been linked to preterm birth and adverse neurological consequences in exposed infants. Preterm birth has also been independently linked to neurological

consequences. Studies have shown that PBDEs can alter miRNAs within extracellular vesicles (EVs) and that EVs can be stably absorbed by the infant gastrointestinal epithelium. This study examines the relationship between breast milk PBDE levels and EV-miRNAs that support infant neuronal development. Milk PBDE levels were quantified by GC-MS from preterm, full-term, and donor human milk. The average PBDE level of donor milk samples, which are aggregate samples of full-term milk, were used to establish a high PBDE threshold. Results indicate that ~30% of preterm breast milk samples contain high levels of PBDEs. Within this population, there is also an inverse correlation between PBDE level and neuroprotective EV-miRNAs, as quantified by droplet digital PCR. These data suggest a multiple hit scenario for preterm infant neurodevelopment resulting from direct toxicant exposure, preterm status, and decreases in protective EV-miRNA.

S 1111 Novel Sorption Strategies to Reduce Per- and Polyfluoroalkyl Substances in Milk

K. Dinh. Texas A&M University, College Station, TX.

Given the growing concern regarding PFAS contamination in human breast milk, there is a need for mitigation strategies that do not impact milk nutrient composition. Currently, technologies to reduce PFAS in milk, including human breast milk and cow's milk, are absent. This presentation details the application of calcium montmorillonite clays as an innovative solution to remove PFAS from milk. Our approach entails comprehensive kinetic studies to evaluate binding efficiency and milk nutrients post-filtration. Findings demonstrate the initial proof-of-concept that clay-based sorption effectively reduces PFOA and PFOS, two legacy PFAS, in milk without compromising its nutritional composition. Findings highlight clay-based sorption as a viable strategy for mitigating infant PFAS exposure through milk.

S 1112 New Insights into Manganese Neurotoxicity: From Molecular to Clinical Studies

R. Tjalkens. Colorado State University, Fort Collins, CO.

The neurotoxicity of manganese (Mn) has been well-described for over a century, but key molecular events linking cellular and systemic dysfunction across the CNS have remained elusive. Manganese neurotoxicity, or manganism, is characterized by a spectrum of neuropsychological and neuromotor deficits that map heavily to the basal ganglia of the midbrain, although centers for cognitive function and memory are also involved, such as the cortex and hippocampus, among other regions. This Symposium will bring together a diverse group of experts to highlight recent findings in this field to shed new light into the underlying cellular mechanisms of manganese neurotoxicity, including changes in metabolism and gene regulation that impact innate immune function and behavior. In addition, the Symposium will feature lectures on new population and clinical studies that provide insight into how these molecular alterations influence neurological disease status at a population level, as well as how neuroimaging studies shed light on molecular mechanisms relevant to clinical outcomes. The first speaker will provide an overview of the Symposium and then describe the consequence of mutations in the critical Mn transporters SLC30A10 and SLC39A14 and how SLC30A10 functions as the only specific Mn efflux transporter that controls cellular and organismal Mn levels via efflux activity. This talk will examine how HIF signaling modulates expression of this transporter by inducing metabolic reprogramming that generates neurotoxic metabolites of the kynurenine pathway. The second speaker will discuss emerging evidence that Mn-induced dysregulation of transcription factor RE1-silencing transcription factor/neuron-restrictive silencer factor (REST/NRSF) is linked to neurotoxicity, including TH reduction and oxidative stress in neuronal cultures. Using a combination of over-expression studies and cell-specific knockout studies, the speaker will discuss how REST directly modulates the cytotoxic effects of Mn in dopaminergic neurons. The third speaker will discuss how Mn stimulates mitochondrial-dependent activation of the cGAS-STING pathway in astrocytes, a key activator of type-I interferon responses in immune cells as well as in glia. This talk will examine how exposure to Mn in the developing brain leads to activation of innate immunity in glia through cGAS-STING that promotes immune memory through altered genome stability and DNA methylation. The fourth speaker will discuss how Mn exposure at a population level in children affects rates of attention deficit hyperactivity disorder (ADHD). These findings will be compared to parallel animal models of ADHD, with a focus on novel pharmacological interventions that modulate long-term changes in attention, learning, and sensorimotor functions by modulating both neuroinflammation and neurochemistry. The final speaker will discuss new clinical sequelae of Mn exposure in a population of welders, focusing on cutting-edge neuroimaging modalities to understand changes in dopaminergic neuronal function, as well as neuroinflammation, that result from exposure to Mn. These studies will describe the use of PET and MRI to identify key molecular changes in Mn-exposed Parkinsonian patients that correlate closely with the Unified Parkinson's Disease Rating Scale, Motor Subsection Part 3 (UPDRS3) and with a cognitive battery. Collectively, the presentations in this Symposium will highlight new research that aims to link changes in key molecular events in CNS following exposure to Mn that underlie the development of chronic neuropsychological and

neuromotor disease. Speakers in the Symposium bring together expertise in clinical neurology and epidemiology, public health and environmental exposures, and mechanistic neurotoxicology.

S 1113 HIF Signaling at Crossroads of Manganese Homeostasis and Neurotoxicity

S. Mukhopadhyay. University of Texas at Austin, Austin, TX.

While Manganese neurotoxicity is recognized since the 1800s, it is only over the last 12 years that a defined understanding of mammalian Mn homeostasis is emerging due to the discovery of hereditary forms of Mn neurotoxicity that occur as a consequence of mutations in the critical Mn transporters SLC30A10 and SLC39A14. Our previous work characterized SLC30A10 as the only specific Mn efflux transporter that controls cellular and organismal Mn levels via efflux activity. More recently, we have discovered a novel interaction between the hypoxia inducible factor (HIF) signaling cascade, SLC30A10 expression, and Mn homeostasis. Elevated Mn activates HIF signaling by directly blocking HIF degradation. HIF activation has two consequences – first, it directly upregulates SLC30A10 expression to lower cellular and organismal Mn levels as a protective response; and simultaneously, HIF activation induces metabolic reprogramming that generates neurotoxic metabolites of the kynurenine pathway, which induce neurotoxicity. This presentation will discuss novel data on the mechanisms and consequences of Mn-induced HIF activation in the homeostatic control and neurotoxicity of Mn, and present possibilities for leveraging these basic science findings for therapeutic development.

S 1114 Role of REST/NRSF in Manganese-Induced Neurotoxicity

E. Lee. Florida A&M University, Tallahassee, FL.

Recent emerging evidence reveals that dysregulation of transcription factor RE1-silencing transcription factor/neuron-restrictive silencer factor (REST/NRSF) is linked to several neurological disorders. We found that REST was critically involved in manganese (Mn)-induced neurotoxicity. Mn decreased REST expression concomitant with its toxicities in neuronal cultures, while REST overexpression attenuated Mn-induced toxicities, including TH reduction and oxidative stress, in neuronal cultures. To understand the role of REST in Mn toxicity *in vivo*, we generated dopaminergic neuron-specific REST knockout (REST-cKO) mice, since Mn preferentially accumulates in the basal ganglia of the brain and causes dopaminergic toxicity. Mn-induced neurotoxicity and behavioral deficits in wild-type mice were exacerbated in REST cKO mice, along with a further decrease in dopamine levels and oxidative stress in nigrostriatal regions. In addition, REST restoration in the substantia nigra of Mn-exposed REST-cKO mice with neuronal REST AAV vectors mitigated Mn-induced neurotoxicity, dopamine reduction, and behavioral deficits. Tamoxifen, a selective estrogen receptor modulator, induced protection against Mn toxicity, at least in part by REST upregulation via activation of Wnt signaling. These novel findings suggest that dopaminergic REST in the nigrostriatal pathway is critically involved in Mn-induced neurotoxicity and could be a potential target to develop therapeutics for Mn toxicity.

S 1115 Manganese Stimulates Release of Damage-Associated Molecular Patterns in Glial Cells to Activate Innate Immunity in the Developing Brain

R. Tjalkens. Colorado State University, Fort Collins, CO.

Developmental exposure to manganese (Mn) results in immune priming of glial cells that amplifies neuronal injury to other neurotoxic exposures later in life. Using a 'two-hit' model approach, we have previously reported that mice orally exposed to Mn from weaning through puberty were more sensitive to Mn when re-exposed as adults. Likewise, early-life exposure to Mn has a similar immune priming effect on reactive gliosis and neuronal damage following secondary exposure to other neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone, as well as neurotropic viruses. This suggests that Mn-induced immune priming of glial cells broadly impacts the resulting inflammatory response to other neurotoxic exposures, but the basis for this heightened immune sensitivity in the brain is poorly understood. In probing the molecular basis for these effects, cell-specific knockout of critical inflammatory pathways in astrocytes, such as NF- κ B/I- κ B kinase β (I κ B2), is profoundly neuroprotective, but the signaling factors responsible for activation of this critical inflammatory pathway remain poorly defined. We therefore examined the effect of Mn on mitochondrial-dependent activation of the cGAS-STING pathway (cyclic GMP-AMP synthase), a key activator of type-I interferon responses in immune cells as well as in glia. Mn directly increases the sensitivity of cGAS for dsDNA and increases binding affinity for STING. In addition, Mn increases levels of mitochondrial calcium in response to ATP stimulation, resulting in release of ds mitochondrial DNA (mtDNA) that amplifies activation of STING and increases expression of both INF β and TNF. Both increased STING activation and ROS from mitochondrial injury alter genome stability and DNA methylation patterns that may underlie the immune priming effects of Mn in glia. Astrocyte-specific knockout of STING greatly attenuates

these changes. Understanding how Mn alters innate immunity in glial cells within the neuro-immune axis to coordinate inflammatory responses of the brain to environmental exposures may therefore help to delineate the etiology of early prodromal neurological disease.

S 1116 Congruence of Human and Animal Model Findings Shows that Developmental Manganese Exposure Causes Lasting Neurodevelopmental Deficits Accompanied with Prefrontal Cortex Dysfunction

D. Smith. University of California Santa Cruz, Santa Cruz, CA.

Neurodevelopmental disorders, including attention deficit hyperactivity disorder (ADHD), are among the most prevalent disorders among children worldwide. Developmental manganese (Mn) exposure from contaminated drinking water, diet, and/or air pollution is associated with deficits in attention, inhibitory control, and psychomotor function in children and adolescents, though the causal relationship(s) between Mn exposure and these deficits, their underlying mechanisms, and efficacy of potential for therapeutic treatments are not known. We conducted epidemiological studies to determine the risk of cognitive, behavioral, and psychomotor deficits in children and adolescents exposed to ferromanganese alloy plant emissions and their legacy impact on environmental Mn levels. In parallel, we developed a rat model to determine whether developmental Mn exposure causes lasting deficits in attention, impulse control, and sensorimotor function, and their underlying mechanisms, and whether pharmacologic treatment with methylphenidate (Ritalin) and guanfacine ameliorates the Mn deficits. Results: Our epidemiological studies established a suite of biomarkers for environmental Mn exposure that are associated with cognitive, behavioral, and psychomotor deficits in children and adolescents. Our animal model studies show that developmental Mn exposure causes lasting impairments in attention, impulse control, and sensorimotor functions that are associated with a proinflammatory cellular environment, altered morphology of PFC-VTA and sensorimotor cortex brain areas, and an overall hypofunctioning catecholaminergic system in fronto-striatal regions. Disrupted mTOR and Wnt signaling is identified via transcriptomics and methylomics as a key regulatory pathway underlying the lasting catecholaminergic system deficits. Finally, methylphenidate and guanfacine are efficacious in ameliorating the behavioral impairments. Conclusions: 1) Elevated environmental Mn exposure in children and adolescents presents a significant public health threat by causing lasting neurodevelopmental, ADHD-like and psychomotor deficits; 2) These Mn deficits are associated with a hypofunctioning catecholaminergic system in the PFC; and 3) A clinically-relevant oral regimen of methylphenidate or guanfacine is fully efficacious in ameliorating these deficits.

S 1117 Multimodal Imaging of Manganese Neurotoxicity

B. Racette. Barrow Neurological Institute, Phoenix, AZ. Sponsor: R. Tjalkens

Manganese (Mn) is an environmental neurotoxicant that causes a clinical syndrome that overlaps with Parkinson's disease. The role of the nigrostriatal system in manganese toxicity remains controversial. The clinical phenotype of Mn-exposed workers overlaps substantially with PD. To investigate the pathophysiology Mn neurotoxicity, we performed multi-modal neuroimaging using positron emission tomography (PET) and brain MRI. We performed 385 PET scans in Mn-exposed welders and non-welder controls, using the radioligands FDOPA, NMB, and DTBZ. We also performed 288 multi-sequence brain MRIs, including standard T1 weighted MRI and Diffusion Basis Spectrum Imaging (DBSI), to quantify brain Mn concentrations and investigate the role of neuroinflammation in the pathogenesis of Mn neurotoxicity. Participants were examined by a neurologist using the Unified Parkinson's Disease Rating Scale, Motor Subsection Part 3 (UPDRS3) and with a cognitive battery. Compared to controls, mean caudate FDOPA uptake was 0.0014min⁻¹ lower in Mn-exposed welders, indicating pre-synaptic dopaminergic dysfunction. In cross-sectional analyses, dopamine type 2 receptor (D2R) nondisplaceable binding potential (BPND) in the substantia nigra (SN) had the greatest discriminant power among exposure groups. Age-adjusted SN D2R BPND was 0.073 greater in Mn-exposed welders compared to non-welder controls. After adjustment for age, SN D2R BPND was 0.0021 higher for each year of Mn-exposure. Each 0.10 increase in SN D2R BPND was associated with a 2.65 increase in UPDRS3 score. Longer term Mn exposures resulted in a dose-dependent reduction in SN D2R BPND. On brain MRI, basal ganglia region of interest analyses revealed higher average caudate DBSI-derived nonrestricted fraction and DBSI-derived radial diffusion values in Mn-exposed welders relative to non-welder controls. Caudate DBSI-derived nonrestricted fraction was also associated with greater cumulative Mn exposure and higher UPDRS3 scores. Multi-modal brain imaging demonstrates that Mn-exposed welders have dose-dependent dopaminergic dysfunction and inflammation in the basal ganglia, including the substantia nigra. With short term exposures, the dopaminergic system upregulates to maintain homeostasis, but this compensation fails with long term exposure consistent with a neurodegenerative pathophysiology.

W 1118 Assessment of Oncogenicity Risk Associated with Cell Therapies: From Preclinical Studies to the Clinic

J. Clingan. Sonoma Biotherapeutics, South San Francisco, CA.

Cell therapies encompass a broad range of therapeutics, including engineered autologous or allogeneic cells (e.g., CAR T cells), as well as iPSC-derived cells. Although cell therapies have great therapeutic potential and some have been approved for a number of indications, there are theoretical risks for malignancy upon administration of cell therapies that could result from either genetic engineering of transferred cells or due to the differentiation state of the cell product (in the case of iPSC-derived cells). In particular, this topic has come under attention recently due to identification of CAR-positive lymphomas in cancer patients treated with CD19- or BMCA-directed CAR T therapies and the resultant label changes applied to some cellular therapies highlighting a malignancy risk. It is especially important to understand the risk of product-related malignancies in the context of non-oncology indications (e.g., autoimmune disease) where a much larger and younger population may be treated with different risk/benefit considerations. A number of methods have been used to evaluate the tumorigenic potential of cell therapies preclinically and in post hoc root-cause analyses to predict the risk of malignant transformation in the product and determine causality in tumors arising following cell therapy treatment, respectively. This session will discuss the current understanding of malignancy risk arising from engineered T cell therapies, and the preclinical evaluation of oncogenicity risk of engineered T cell and iPSC-derived cell therapies. Discussion of preclinical analyses will encompass evaluation of risks associated with genetic integration (insertion site analysis), cytokine- and antigen-independent growth (cytokine-independent growth assay), and oncogenicity potential assessment for *in vitro* and *in vivo* engineered CAR T and iPSC-derived therapies. The session will end with a discussion aimed at understanding how to perform a root-cause analysis in case of a clinically identified malignancy following cell therapy. Following this session, participants will have a thorough understanding of observed malignancy risk associated with cell therapies and common methods used to evaluate oncogenic risk of cell therapies preclinically.

W 1119 The Illusion of Precision in Assigning Risk of Secondary Malignancies Post-CAR T-Cell Therapy

B. Levine. University of Pennsylvania, Philadelphia, PA. Sponsor: J. Clingan

Since the 1990's, we have conducted clinical trials of gene modified T cells. Chimeric antigen receptor (CAR) T cells targeting CD19 on B cells leukemias and lymphomas have induced durable complete responses in patients who are relapsed or refractory to all other available treatments. Determining the critical quality attributes, dose, potency, and anticipating pharmacokinetics of a living, dividing drug presents unique challenges. Another challenge is dissecting potential toxicities, causality and risk. The US FDA has been investigating cases of T cell malignancies following BCMA-directed or CD19-directed CAR T cell immunotherapies. The relative risks of conventional chemotherapy, stem cell transplantation, lymphodepletion, and gene insertion of CAR need to be considered in the context of refractory cancers for which patients might be eligible for CAR T cell therapy.

W 1120 Assessing Insertional Oncogenesis Risk in Gene and Cell Therapies

T. Lanz. Pfizer Inc., Groton, CT. Sponsor: J. Clingan

Gene therapies and many cell therapies involve transferring therapeutic nucleotide cargo into patient cells, which may pose a risk of insertional oncogenesis depending on the modality. Chimeric antigen receptor (CAR) T-cell therapy and the use of lentiviral or retroviral vectors inherently establish insertional mutagenesis, though not necessarily with oncogenic risk. Similarly, transforming patient or autologous cells with adeno-associated viral (AAV) vectors, transposons, or gene editing tools results in genomic insertions, albeit at a lower frequency. Various next-generation sequencing tools have been developed to analyze insertion site patterns. While the recommended analytical methods and interpretations depend on the modality, the ultimate goal of any insertion site analysis is to understand the risk of a particular therapy causing insertional oncogenesis. This talk will provide an overview of current methods, suggest best practices for method validation, and highlight key elements of secondary analysis to aid in risk assessment.

W 1121 Standardized Protocol for the Cytokine Independent Growth Assay for Gene Modified CAR T Therapy

S. Xu. Bristol Myers Squibb, Seattle, WA. Sponsor: J. Clingan

Genetic engineering in CAR-T therapy has raised concerns of secondary malignancies. As the CAR-T therapy moves to treat solid tumors and autoimmune diseases with extensive genomically edited cells, there is a need to have tools to understand the risk of genomic changes. The cytokine-independent growth (CIG) assay is recognized by health authorities as a critical component of non-clinical safety evaluations

for CAR-T cells, as a positive result in this assay would be a biomarker of transformation. Despite its extensive use, there is not a standardized protocol including the absence of an established positive control. We developed a modified CIG assay with a biological relevant positive control, defined the assay length, specific culture conditions and other standardized culturing parameters. This defined protocol is being assessed by multiple pharmaceutical companies within HESI consortium and has the capability to assess the transformational risk associated with gene-edited CAR-T products.

W 1122 Nonclinical Safety Assessment of *In Vivo* CAR T-Cell Therapy Products

A. Brandes. Umoja Biopharma, Seattle, WA. Sponsor: J. Clingan

Autologous CAR T cell therapies have transformed treatment paradigms for B-cell malignancies; however, many challenges remain that limit patient access to these therapies, including operational and logistical complexity and high manufacturing and distribution costs. Recently, CAR T cell therapy has demonstrated clinical efficacy in non-oncology indications such as autoimmunity, thus, demand for these therapies is anticipated to increase as these modalities expand into new indications. To address this, "off-the-shelf" therapies that directly engineer CAR T cells within the patient's own body are an area of active development. These *in vivo* CAR T cell therapies utilize targeted technologies such as lipid nanoparticles or viral vectors to deliver a CAR payload directly to patients' T cells to engineer CAR T cells *in vivo*. Here, we will discuss the primary safety risks associated with these types of therapies, with a focus on the nonclinical assessment of oncogenicity risk evaluation. These considerations differ by modality (i.e. integrating versus non-integrating gene delivery mechanisms) and take into account details specific to *in vivo* CAR T products, such as the specificity of targeting T cells and potential oncogenic risk following transduction of off-target cell types. A case study will cover IND-enabling nonclinical assessment of a surface-engineered lentiviral *in vivo* CAR T cell therapy product, which assessed oncogenicity risk by evaluating genetic integration (insertion site analysis and vector copy number determination), cytokine- and antigen-independent growth (cytokine-independent growth assay), and specificity of on- and off-target transduction using *in vitro* and *in vivo* models.

W 1123 Evaluating Safety Risks of iPSC-Derived Cell Therapies

G. Rao. Genentech Inc., South San Francisco, CA.

Induced pluripotent stem cells (iPSCs) offer transformative commercial potential for off-the-shelf cell therapies (CTs), and iPSC-derived CTs are increasingly being tested in clinical trials. However, the clinical use of iPSC-derived CTs requires comprehensive preclinical risk assessments to address unique safety concerns, including teratoma formation from residual undifferentiated iPSCs and tumorigenic potential due to genomic instability and mutation accumulation. Various methodologies are used to assess tumorigenicity during the development of iPSC-derived CTs, including *in vivo* teratoma assays as well as more sensitive *in vitro* assays such as the Highly Efficient Culture assay, digital PCR, and transcriptomic analyses to detect residual undifferentiated cells. For regenerative CTs, teratoma formation is also assessed in dedicated tumorigenicity studies in immunodeficient rodents. Genomic integrity is evaluated using techniques such as G-banding, digital PCR, and next-generation sequencing to identify structural variants, copy number variations, and point mutations that may arise during reprogramming, culture, differentiation, or expansion. In the absence of clear regulatory guidelines, a thorough weight-of-evidence approach must be employed, integrating multiple data sources to assess the safety risks of iPSC-derived cell therapies during preclinical development.

W 1124 Diet and Toxicology: Examining the Novel and Underappreciated Interaction

K. Gowdy. Ohio State University, Columbus, OH.

The interaction of diet and toxicology is an emerging area of investigation. However, it is still unclear how dietary intake, deficiencies, or supplementation alter toxicological responses in multiple organ systems. This Workshop will bring together multidisciplinary researchers, including nutritional and dietary experts, and toxicologists with expertise in different exposure routes and xenobiotic-induced pathologies to discuss the current state of science regarding nutrition and the biological responses during health and diseases and to identify critical research gaps, challenges, and opportunities. The primary goal of this Workshop is to advance nutrition research in the field of toxicology and ensure that future investigations are conducted to best model human dietary intake. Diet is an underappreciated contributor to toxicant effects, acting as a partner in a complex exposure. The first speaker will give an epidemiological overview of nutrition and how current dietary patterns are modulating biological responses to xenobiotic exposures. The second speaker will provide data highlighting interactive effects between diet and the toxicological responses to PFAS. In the third talk, effects of air pollution and diet will be presented. The fourth presenter will describe results from animal studies investigating synergistic effects

of diet, sex, and microplastics exposure. The final talk will focus on how to model diet and xenobiotic synergistic interactions, taking into account the dietary needs of the animal models used. The diversity of speaker expertise in mechanistic toxicology, human cohort studies, nutrition, and epidemiology will enhance comprehension of the complex interplay between xenobiotics and diet and provide guidance on how to apply this information to improve inputs for risk assessments to best protect public health. This will provide valuable insights for research planning, funding allocation, and surveillance practices, as well as policy development.

W 1125 Nutrition to Mitigate the Adverse Impact of Exposures

S. Sumner. University of North Carolina, Kannapolis, NC. Sponsor: K. Gowdy

While exposures to medications, specific diets, supplements, or natural products can have beneficial effects, some exposures (e.g., pollution, tobacco use, drugs, foods, environmental chemicals) can individually or collectively damage our DNA, change how our proteins function, cause dysbiosis of the microbes in our gut, and lead to disruptions of our normal metabolic processes. Untargeted metabolomics of the internal exposome provides a means to reveal how exposures perturb our normal metabolic state, and how these perturbations are associated with states of health and wellness. Using a nutritional pharmacology/toxicology workflow, the high-information metabolomics/exposome data can be evaluated to design intervention strategies: such as needs in exposure reduction, or the formulation of nutrient cocktails that could protect against adverse exposures. This presentation will cover applications in areas of drug addiction, inflammation, and pregnancy complications that lead to informing nutritional guidance. Intervention with nutrient cocktails presents an opportunity to modify the effects of environmental exposures for more beneficial health outcomes. Ultimately, the nutritional pharmacology/toxicology approach will have a significant impact on public health by reducing the burden of disease caused by environmental exposures, and by enhancing the beneficial effects of specific diets or prescription drugs.

W 1126 PFAS and Diet Interactions: The Good, the Bad, and Maybe the Ugly

J. Schlezinger. Boston University, Boston, MA.

People are exposed to per- and polyfluoroalkyl substances (PFAS) in drinking water, food, air, dust, and by direct use of consumer products. Perturbation of lipid and cholesterol homeostasis are important adverse effects of PFAS in people. Yet, the vast majority of studies on these endpoints are conducted in animal models fed a rodent diet (57% carbohydrate, 23% protein, 21% fat as %kcal with low cholesterol, 12-15% fiber by weight). Dietary fat and cholesterol significantly modify the physiological handling and synthesis of these macromolecules. And dietary fiber significantly impacts lipogenesis-, lipid oxidation-, and cholesterol metabolism-related genes. We conduct our experiments with PFAS in female and male mice fed a diet based on the "What we eat in America" analysis in NHANES (47% carbohydrate, 16% protein, 37% fat as %kcal with 0.13% cholesterol, 5.5% fiber by weight). We find perfluorooctanoic acid significantly increases serum cholesterol in mice on this diet, but that effects on lipids in the liver depends upon time on the diet (6 vs 14 weeks). Furthermore, the effect of fiber supplementation in the diet depends upon the characteristics of the fiber (e.g., insoluble versus soluble, viscous vs non-viscous), both in terms of metabolic characteristics of the mice fed each diet and their response to a mixture of PFAS, as well as the sex of the mouse. Our results highlight the need for more careful consideration of diet as a variable that modifies the toxicological response to PFAS and the use of more human-relevant diets.

W 1127 Diet and Eating Pattern as Determinants of Air Pollution Susceptibility

P. Haberzettl. University of Louisville, Louisville, KY.

Inhalation of polluted air has emerged as an independent risk factor for developing cardiometabolic disease. It is also known that choosing either an unhealthy diet or bad sleep habits that consequently change eating pattern have adverse consequences on cardiometabolic health. The impact of these lifestyle choices on the susceptibility to ambient fine particulate matter (PM_{2.5}) exposure, however, is still unclear. To investigate this, we exposed mice maintained on either a high fat diet (60% kcal from fat; diet-induced obesity, DIO) or a disturbed light cycle (18/6h, light/dark; light-induced circadian dysynchrony, LICD) to concentrated ambient PM_{2.5} (CAP, 30 days, 6h/d). Control mice (12/12h, light/dark; 12% kcal from fat) inhaled either CAP or HEPA-filtered air (30days, 6h/d). Changes in adiposity, systemic glucose handling, tissue specific insulin sensitivity and inflammation as well as systemic and pulmonary redox changes were measured. While CAP exposure did not change any metabolic parameters, changing the light cycle impacted activity and feeding pattern. In male mice, exposure to CAP exacerbated systemic glucose intolerance, insulin resistance and inflammation in both DIO and LICD without increasing adiposity. Interestingly, CAP exposure did not impact cardiometabolic health in female mice. Testing for a possible protective mechanism, we found that CAP expo-

sure induced systemic and pulmonary oxidative stress in male, but not female mice that was accompanied by a higher expression of antioxidant enzymes in the female lungs. Our results indicate that choosing an unhealthy diet or sleeping/eating pattern could increase the susceptibility to PM_{2.5}.

W 1128 Sex, Diet, and Micro/Nanoplastics: Enhancing Translational Fidelity in Toxicological Models

S. Phatak. University of New Mexico, Albuquerque, NM.

Micro- and nanoplastics (MNPs) are increasingly detected in human tissues and food systems, raising concerns about their long-term health impacts. Many plastic particles and additives are lipophilic, suggesting that dietary components, particularly fat and fiber, may influence MNP absorption, bioavailability, and physiological effects. These interactions are further complicated by sex-specific differences in dietary intake and metabolism. However, a critical gap remains in environmental toxicology: understanding how sex-specific dietary responses influence MNP toxicokinetics and outcomes. Sex differences play fundamental roles in shaping developmental biology, immune function, metabolism, and disease susceptibility. These differences, driven by hormonal signaling, immune responses, and energy metabolism, are likely to influence how MNPs are processed and impact physiological systems. In our study, we examined the long-term effects of chronic dietary MNP exposure (0.5–5 µm polystyrene) in mice from adolescence to adulthood. To ensure translational relevance, we used defined purified diets to eliminate confounding non-nutrient variables found in standard chow. Despite equivalent MNP intake across groups, we observed sexually dimorphic physiological responses. Single-cell transcriptomics revealed divergent immunometabolic profiles by sex, underscoring the interactive effects of diet and sex on MNP biodistribution and toxicity. Our methodological approach addresses limitations of conventional exposure models: gastric gavage induces acute stress and bolus dosing, while drinking water models suffer from inconsistent intake and particle sedimentation. By incorporating MNPs directly into a defined diet, we achieved chronic, low-dose exposure under physiologically relevant conditions, better simulating real-world dietary exposure. This study highlights the importance of integrating sex as a biological variable and controlling dietary context in environmental toxicology. These findings have broad implications for both experimental design and public health strategies aimed at mitigating plastic-associated risks. Moreover, our data suggest that targeted dietary manipulation may offer a practical approach to modulate MNP uptake and reduce their physiological impact.

W 1129 Modeling Interactions Between Diet and Xenobiotic Exposure for Translation to Human Health

B. Mickelson. Inotiv, Madison, WI. Sponsor: K. Gowdy

The interaction of diet and how this influences biological responses to xenobiotics is an emerging area in toxicological research. This is often examined in animal models with dietary supplement and/or development of diets that reflect human consumption of macro and micro nutrients. However, there are several experimental factors to consider when designing experiments to examine diet in animal models. This talk will cover the following topics to increase rigor and reproducibility in this emerging area of toxicology including 1) what is the appropriate 'control diet' for an experiment; 2) what are the dietary needs are for the model and how will manipulating them will change the health of the rodent; and 3) how to test different dietary patterns/ingredients in rodents to model human dietary intake given known species differences. This multidisciplinary talk will provide researchers with information and considerations for designing dietary experiments so they can make the best possible diet decisions for toxicological models.

W 1130 Science Simplified Through Graphics

A. Bernstein. Rutgers, The State University of New Jersey, Piscataway, NJ.

In today's fast-paced digital world, the ability to communicate toxicological science visually is more important than ever. This interactive Workshop will equip participants with the tools and strategies to transform complex toxicology data into engaging, accurate, and accessible visual content. Through hands-on training, attendees will learn the fundamentals of effective graphic design, how to create impactful infographics, and best practices for developing graphical abstracts tailored for publication, social media, and public engagement. Whether you are explaining chemical risk assessments, mechanisms of toxicity, or environmental exposure data, visual storytelling can enhance comprehension and broaden the reach of your science. Participants will explore key design principles—including color, layout, hierarchy, and typography—and receive guidance on selecting the right format and graphics for different audiences and platforms. Examples from publications, social media, and community outreach materials will be used to highlight effective practices and common pitfalls. By the end of the session, participants will have drafted their own visual piece and gained practical insights into tools like Canva, BioRender, NIH BioArt, and PowerPoint. This Workshop is ideal for toxicologists at all career stages

who want to improve their science communication through compelling visuals that inform, educate, and inspire. No prior design experience is necessary. Bring a laptop to fully immerse in this hands-on training.

W 1131 Essentials First: Define What You Are Communicating

A. Bernstein. Rutgers, The State University of New Jersey, Piscataway, NJ.

This opening activity sets the stage for the workshop by helping participants clarify the core message they want to communicate and define their target audience. After a brief introduction, each participant will identify one key aspect of their work they wish to convey visually and specify the intended audience. Participants will then have five minutes to work independently before regrouping to share and discuss their ideas with peers at the table. The session will conclude with a recall exercise designed to demonstrate the limited extent to which scientists can effectively convey content through visuals in a short period of time, reinforcing the necessity of concise and intentional communication.

W 1132 Align Your Message with Audience Interests

A. Tukker. Purdue University, West Lafayette, IN.

Effective communication is a two-way exchange that happens with an audience, not to an audience. In this session, participants will explore how to set audience-specific communication goals that align both with the scientist's intended message and the audience's interests or needs. Following a brief introduction by the moderator, participants will further refine their target audience and define clear goals for what they hope to communicate. The session will also highlight compelling examples that demonstrate how content can be strategically tailored to resonate with different groups.

W 1133 Visual Storytelling Through Tailored and Compelling Graphics

L. Aleksunes¹, and A. Jennings². ¹Rutgers, The State University of New Jersey, Piscataway, NJ; and ²Consultant, Bloomfield, NJ.

Building on audience-specific goals, this session guides participants in refining their content into clear, focused messages suitable for visual communication. A foundational overview of graphic design principles will be provided, including color theory, font choice, text alignment, effective use of imagery, and accessibility considerations. Participants will also learn how different communication platforms—such as social media, academic posters, and public-facing brochures—have unique features that influence graphic design decisions. This session will equip attendees with the tools to create visually compelling and platform-appropriate materials that enhance audience engagement. Andi Jennings is an artist and graphic designer with over two decades of experience in branding, marketing, and digital and social storytelling. Andi believes that data, content, and design work in harmony, and her projects translate complex data into compelling visual stories.

W 1134 Building Your Graphic from Concept to Creation

A. Torres-Berrios¹, and L. Aleksunes². ¹Michigan State University, East Lansing, MI; and ²Rutgers, The State University of New Jersey, Piscataway, NJ.

The final portion of the workshop will be fully hands-on and iterative. Participants will have dedicated time to begin designing their graphic using a platform of their choice (e.g., Canva, BioRender, PowerPoint, etc.), applying the principles and goals developed throughout the workshop. Small groups will then share their drafts and offer constructive feedback to one another. The remaining time will be used for participants to revise and refine their designs based on this peer input. A selection of graphics will be submitted in real-time to the moderators and showcased to the full group for broader feedback and discussion.

W 1135 The Quality Awakens: New Frontiers in the Appraisal of In Vitro Mechanistic Study Quality/Reliability

A. Wang. NIEHS, Research Triangle Park, NC.

Mechanistic data from epidemiological, animal *in vivo*, and various *in vitro* studies have traditionally been a part of the information used in hazard and risk assessments. To reach a scientifically sound decision, assessors prioritize high-quality data over poorer-quality data, especially when conflicting results are reported. Compared to well-developed study quality appraisal approaches for epidemiological and animal *in vivo* studies, appraisal for *in vitro* studies has a shorter history and is less often deployed in risk assessment. Several groups in the US and Europe are developing

tools and approaches to advance specifically the *in vitro* study quality and reliability appraisal. This session is designed for scientists conducting *in vitro* studies and/or using *in vitro* data results, including risk assessors and systematic review practitioners, to be aware of the concept, approach, and tools of *in vitro* study quality appraisal and keep abreast of recent innovations and directions of future development. Five speakers will present several examples of *in vitro* study appraisal in ways that fit for their respective assessment purposes, innovations in the tool and approaches of *in vitro* study quality and reliability evaluation, reporting templates and considerations to increase the regulatory value of your *in vitro* data, and a novel framework to choose a suitable *in vitro* study appraisal tool.

W 1136 Bridging Biology and Risk: How US EPA Program Offices Apply In Vitro Data to Modernize Chemical Evaluations

I. Druwe. US EPA, Research Triangle Park, NC.

The US Environmental Protection Agency (US EPA) has increasingly incorporated *in vitro* studies into chemical risk evaluations and or assessments to enhance mechanistic understanding, support biological plausibility and hazard identification, and inform-dose response characterization. Across EPA program offices- including the Office of Chemical and Pollution Prevention's (OCSPP) Office of Pollution Prevention and Toxics (OPPT) and Office of Pesticide Programs (OPP), the evaluation and application of *in vitro* data reflect evolving scientific methodologies and statutory frameworks. *In vitro* assay data, in particular that derived from high throughput screening assays (e.g. ToxCast) and other new alternative methodologies (NAMs), are being used to prioritize chemicals, refine points of departure, and evaluate modes of action. Integration practices of *in vitro* and mechanistic data vary across EPA program offices due to differences in regulatory mandates and risk assessment paradigms. But there is a growing emphasis on data quality, biological relevance, and translational validity to *in vivo* endpoints. Case studies from recent risk evaluations demonstrate how *in vitro* data can be used alongside traditional toxicological evidence. This presentation will highlight current practices, scientific advancements, and critical challenges- such as assay validation, interpretation of potency metrics, and incorporation of *in vitro* assays into dose-response analyses- that EPA risk assessors face in the incorporation of *in vitro* studies for human health risk assessments. *Disclaimer: The views expressed are those of the authors and do not necessarily represent the views or policies of the US EPA.*

W 1137 The SciRAP Tools: Facilitating Integration of Mechanistic In Vitro Data in Chemical Assessments and Supporting the Use of NAMs

A. Beronius. Karolinska Institutet, Stockholm, Sweden. Sponsor: A. Wang

The Science in Risk Assessment and Policy (SciRAP) platform (www.scirap.org) is an openly accessible online resource offering tools to evaluate the reliability and relevance of non-standard (eco)toxicity, including *in vitro* studies, and epidemiological data. These tools support evidence appraisal and Weight of Evidence assessments across various contexts, including chemical hazard and risk assessments, mode of action analysis, and the development of Adverse Outcome Pathways (AOPs). A core objective of SciRAP is to promote the use of academic research data in regulatory chemical assessments. The platform also aims to facilitate the integration of data from New Approach Methodologies (NAMs)—many of which are not yet validated or standardized—into chemical hazard and risk evaluations. SciRAP's tools, including the CRED criteria (Criteria for Reporting and Evaluating ecotoxicity Data), are increasingly adopted by researchers and regulators and are cited in EU regulatory guidance documents for chemical assessments. This presentation focuses on the SciRAP *in vitro* tool, which offers general criteria for the transparent and structured evaluation of *in vitro* data. These criteria are designed to be adaptable and fit-for-purpose across different regulatory contexts. Ongoing work includes the development of customized versions of the tool to optimize evaluation of data from specific assay types, such as receptor transactivation assays. Work is also ongoing to develop a family of SciRAP AI agents that automates the SciRAP evaluations and enables evaluation of large numbers of datasets at the same time. We will introduce the SciRAP *in vitro* tool, highlight current developments, and present examples of its application in various settings. Particular emphasis will be placed on how tools like SciRAP can support the regulatory use of NAMs. Additionally, we will discuss SciRAP's role in the education and training of future toxicologists and risk assessors.

W 1138 Enhancing the Regulatory Value of In Vitro Mechanistic Data Through Internationally Harmonized Reporting Templates

I. Langezaal. European Commission Joint Research Center, Lombardy, Italy. Sponsor: A. Wang

Internationally approved templates for the reporting of mechanistic information facilitate the exchange of data between organisations and their re-use for various purposes. Two such templates relevant for the reporting of *in vitro* results for chemical hazard assessment have been adopted by the Organisation for Economic Cooperation and Development (OECD). The first one, the OECD Harmonised

Template (OHT) 201, is for the reporting of mechanistic information obtained from *in vitro* or *in chemico* methods. For all adopted OECD *in vitro* test guidelines, OHT 201 features pre-compiled pick lists for easy selection of standardised terms. OHT 201 has been embedded into IUCLID 6, a major software tool for submitting data on chemical substances to (regulatory) authorities (e.g., REACH) and for recording, storing and exchanging chemical data in general. Reporting elements flagged in SciRAP (see Talk 2) and the elements of the OECD guidance on Good *In Vitro* Method Practice (GIVIMP) were included in OHT 201. Also, a bridge was created between real-life OHT 201 chemical data and the chemical-agnostic Key Event descriptions in the AOP-Wiki: Both can be tagged with the same "process, object, action" triplet of ontology terms, which can ultimately lead to a much more obvious linkage between mechanistic knowledge (AOP-Wiki) and experimental observation (OHT 201). Recently developed OHT-R template is for the reporting of *in vitro* data published in the scientific literature, as recommended by the OECD guidance document on the generation, reporting and use of research data for regulatory assessments. While based on OHT 201, OHT-R has limited use of free-text fields, to ensure consistent reporting of detailed information. The OHT-R will help researchers, funders, editors, publishers, and reviewers to understand the level of detail required to maximise the regulatory value of reported data. When implemented in software tools (e.g., IUCLID) and following recommended data reporting standards, sharing and re-using of research data for a wide range of (regulatory) purposes will become straightforward. The more OHT-R can be completed with information, the more useful the data will be for regulatory decision making. With additional guidance, a regulator should be able to assess the reliability of the data for inclusion in weight of evidence approaches in regulatory decision making.

W 1139 Realistic Workflow to Critically Assess Mechanistic and *In Vitro* Study Quality with the OHAT Approach in Considering NAMs Data in Chemical Risk Assessment

A. Rooney. NIEHS, Research Triangle Park, NC.

The OHAT approach is an established framework for assessing human, animal, and mechanistic evidence on potential health effects from environmental chemicals. OHAT and other systematic review methods increase the rigor, reproducibility, and transparency in the risk assessment process from the literature search, through selecting, critically assessing, and then synthesizing evidence. Critical appraisal of individual studies in terms of internal validity or risk of bias is a key step to assure that the conclusions are based on the most reliable data and that uncertainties in the evidence base are reflected. Increasingly we are faced with datasets that have a greater number and proportion of *in vitro* and mechanistic studies. This presentation will use an evaluation of immunotoxicity associated with exposure to perfluorooctanoic acid (PFOA) to illustrate how mechanistic and NAMs data should be considered throughout the systematic review process starting with problem formulation. A tiered approach for assessing mechanistic data with the OHAT tool for assessing risk of bias will be presented such that resources are focused on studies that are likely to inform the conclusions. The manual workflow used in the 2016 National Toxicology Program (NTP) assessment of PFOA immunotoxicity will be contrasted with recent workflow advances that incorporate automation with human-in-the-loop user verification. The advantages and challenges of incorporating artificial intelligence (AI) in study quality assessment will also be presented in terms of traditional machine learning (ML) and natural language processing (NLP) workflow enhancements as well as the more flexible and more powerful large language models (LLM) based methods.

W 1140 How to Select a Fit-for-Purpose Critical Appraisal Tool: Using the FEAT Principles

P. Whaley. Lancaster University, Leuchars, United Kingdom.

Critical appraisal is a key component of systematic review, chemical risk assessment, AOP development, and many other toxicology research contexts. We just heard a few out of dozens of published tools for appraising study quality in different regulatory and scientific contexts. The problem is, these many tools are designed for different tasks, with varying levels of rigour in their development and quality constructs as their target. The tools also differ in their validity for a given task, transparency levels in their use, and suitability of results for summarising study quality for a particular use. This creates a minefield for their prospective users. Of dozens of tools that could be used for a given purpose, how does one determine which tool is the best fit? If no tool is perfect, how does one decide what modifications need to be made to the nearest-fitting tool for the needed job that is needed? The purpose of this presentation is to solve these problems for the listener, by providing them with a simple and memorable framework for assessing study appraisal tools. The framework is FEAT, which stands for Focus (the appraisal concepts targeted by an instrument), Extensiveness (the comprehensiveness with which the instrument covers those concepts), Application (the validity with which the results of using the instrument can be applied in the research context), and Transparency (the explainability of judgments made when using the instrument). This talk will explain to participants how to apply FEAT to structure their thinking about critical appraisal tools, helping them transparently screen and modify potential tools to select the most fit for purpose

tool. Broad and easily memorisable, FEAT is an assessment framework designed to be "carried in the head, not in the pocket". FEAT helps researchers apply their critical appraisal skills and articulate the appraisal requirements of their research context, particularly if FEAT is applied in team discussions. Importantly, the lessons learned and principles presented in FEAT can be applied in any appraisal context, from peer-review to risk assessment to grant proposals. Already in use in environmental sciences, and part of editorial policy for at least two toxicology journals, FEAT stands to support toxicologists in a vital part of the scientific process.

IS 1141 Incorporating Toxicology Principles Essential to Establishing Plausibility of Environmental Epidemiology Studies

J. Doherty. Independent Toxicologist, Oakton, VA.

Environmental epidemiology studies (EES) frequently report potential chemical hazards that are incorporated into the risk characterization of chemicals with serious scientific, economic, and social consequences. Owing to these consequences, independent and well-qualified scientists should critically review EES to ensure the relevance of the reported adverse outcomes. The toxicology community has a public health responsibility to be intricately involved in the review of EES. A working principle for this session is that a report of an adverse outcome is also a report of a toxic response of clinical and public health relevance. Therefore, this session raises the issue that toxicologists should implement processes to ensure appropriate peer/expert review and engage with epidemiologists who will consider the needs of toxicologists in interpreting epidemiological data. This session aims to stimulate toxicologists to develop standard criteria for EES that can be applied to formulate a plausibility statement based on principles of toxicology. The plausibility statement would characterize whether or not the reported adverse outcome from EES is sufficient to support clinical relevance and, therefore, regulatory action. The toxicology community recognizes and appreciates that EES are inherently difficult to conduct. It is also recognized that because of their inherent complexity, EES cannot have the more rigid Good Laboratory Practices, Quality Assurance, and reporting standards associated with animal studies. Currently, most toxicologists are trained to judge animal studies, and few are trained to evaluate EES. This results in EES often being accepted essentially at their face value. In addition to the current situation, there is growing interest in reducing animal toxicity testing as the basis for safety evaluation with *in vitro* and computer modeling. Should these approaches eventually replace laboratory animal testing, there is reason to expect that the frequency of EES will increase further to challenge the safety of chemicals. Thus, standardizing criteria based on toxicology principles and means to develop a plausibility statement is one way to prepare toxicologists to address the significance of EES reports of adverse outcomes. This session will have representatives from the epidemiology and toxicology communities presenting their approaches to better mutual interaction. Suggestions for other means to involve toxicologists in the critical review of EES will be welcome.

IS 1142 Toxicology Meets Public Policy: Lessons Learned from NAMs Advocacy in the United States

G. Hilton. PETA Science Consortium International e.V., Stuttgart, Germany.

The traditional roles of scientists have predominantly revolved around research, product development, and teaching. Careers in advocacy and government affairs are relatively uncommon in the sciences. While some scientists have the opportunity to bridge sectors, merging research with advocacy, activism, and lobbying, few have experience in this domain. Changing policies affecting the scientific landscape have sparked a growing interest within the scientific community to understand their potential role in advocacy. Given the interest in advocacy, scientists are well-positioned to learn from key governmental developments like advocacy efforts supporting the US Food and Drug Administration (FDA) Innovative Science and Technology Approaches for New Drugs (ISTAND) program and other related policy actions. Decades of advancements in technologies have spurred scientists and policymakers to increasingly consider how predictive toxicological methods can be better integrated into laws, policies, and practices. For example, PFAS is used in many consumer products, but little is known about their exposure and related toxicity—this is a classic case of where predictive toxicological methods can be enforced by policymakers to ensure safe products are screened faster before being manufactured to come onto the market. With a growing need for scientists to communicate with lawmakers, this Informational Session aims to provide a foundational overview of how stakeholders can engage with lawmakers and decision-makers to advance effective science policies. Speakers will provide examples of how scientists are creating positive change for toxicology, with a focus on new approach methodologies (NAMs) advocacy in the US Congress and regulatory agencies.

RI 1143 Revisiting the DDT Dump Site off Southern California: Oceanographic Survey, Ecological Impacts, and Aquatic Toxicity

C. Moore. San Diego Zoo Wildlife Alliance, San Diego, CA.

Beginning in 2011, scientists from the University of California Santa Barbara uncovered several unknown dumpsites of the legacy contaminant DDT in the San Pedro Basin off the coast of Los Angeles. In 2021, an oceanographic survey led by Scripps Institution of Oceanography surveyed more than 35,000 acres of seafloor and found tens of thousands of waste barrels in varying states of decay lining the region. A 2022 SOT Annual Meeting Regional Interest Session invited these scientists, as well as biomonitoring and human health researchers, to provide their early research on the scope and impacts of this dumpsite. Since then, the California Sea Grant program and then-Senator Diane Feinstein's office secured support to sponsor research programs to investigate this persistent challenge. This session will feature up-to-date information about the dumpsite range and contents, monitoring in avian and marine wildlife, and toxicological assessment. First, the session will provide a historical review of this issue and provide an update about the dumpsite with spatial and chemical information from recent oceanographic surveys. The next presenter will then speak on biomonitoring projects involving wildlife and conservation groups around Southern California, quantifying DDT and its co-occurring metabolites and potential by-products (referred to as DDT+). The last speaker will discuss with an aquatic toxicology study comparing each DDT+ species, including mechanistic data yielding clusters of DDT+ species with similar modes of action. This session will conclude with a 25-minute moderated panel discussion and Q&A with the speakers to address audience questions and discuss future plans related to research and action.

RI 1144 Ongoing Ocean Pollution from Legacy Offshore Disposal of DDT Waste: What's Actually Down There?

D. Valentine. University of California Santa Barbara, Santa Barbara, CA. Sponsor: C. Moore

Ocean disposal of petrochemical waste was a common practice in the mid 20th century, particularly near industrial centers. The coastal ocean off Los Angeles, California is a prime example, with numerous waste streams disposed to deep water (900m) basins located close to the coast. This presentation will consider the offshore disposal of manufacturing wastes associated with synthesis of the pesticide DDT. Combining results of a sediment sampling campaign for chlorinated chemicals and metagenome-assembled genomes with analysis of historical records, this presentation will explore the spatial and temporal patterns of DDT waste disposal as well as transport and fate processes that distribute DDT wastes through the region. By defining a regional footprint of DDT waste contamination of more than 1000 square kilometers this work also explores target areas of potential ongoing exposure. Furthermore, this presentation will touch on the relationship of DDT waste with other observed contaminants and processes, and the current state of *in-situ* biodegradation.

RI 1145 Defining Biomagnified DDT+ Chemical Profiles and Investigating the Health Impacts on Sentinel Wildlife Species and Humans

N. Dodder. San Diego State University, San Diego, CA. Sponsor: C. Moore

During its production, DDT waste was discharged via a marine outfall to the Palos Verdes Shelf (PVS) near Los Angeles. Accordingly, the historical narrative and environmental monitoring focused on the PVS as the primary source of DDT to the regional environment. Although offshore dumping industrial waste was legal at the time, this alternate disposal route was largely dismissed until sedimentary DDT waste was (re)discovered further offshore in the San Pedro Basin in 2019. At approximately the same time, nontargeted analysis (NTA) based on comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC/TOF-MS) of regional marine mammal blubber identified not only the typically monitored DDT compounds (DDX) but also a suite of 45 DDT related compounds (DDT+). It was hypothesized the DDT+ profile reflects the persistent and transformed waste contaminants and is unique to the region. The average concentration of total DDX was 180 ug/g lipid mass, the highest known cetacean concentration in the world. Additionally, regional biological impacts were found to be associated with present-day DDT levels. A preliminary association of DDT+ with endocrine disruption was observed in cetaceans. A large-scale epidemiology study of California sea lions found an association between contaminant body burden (including DDX) and the occurrence of urogenital cancer. A reproductive decline due to eggshell thinning in endangered California condors was associated with coastal scavenging of marine mammal carcasses and subsequent exposure to DDX. DDX compounds were observed to activate estrogen receptors of the California condor, thus mechanistically linking exposure to condor reproduction impacts. At this point (ca. 2022), there was evidence of relatively high levels DDX in the southern California ecosystem, and a unique and chemically complex mixture of additional related compounds and structurally unidentified halogenated organic contaminants. There was evidence of biological impacts, but also a lack of contaminant monitoring data in historically

endangered or impacted species. Therefore, to systematically assess southern California wildlife and human exposure to DDT+ and address data gaps, the objective of our study was to measure current levels, temporal trends, the geographic distribution, and the association of DDT+ with biological outcomes.

RI 1146 A "Deeper Dive" into DDT+: Comparative Responses to DDT+ Chemical Species *In Vitro* and in Zebrafish

K. Sant. Michigan State University, East Lansing, MI.

DDT is a legacy pesticide with many existing environmental and biomonitoring programs, the recent research conducted in Southern California has demonstrated the persistence of many lesser-studied DDT metabolites and related products (DDT+) in marine environmental and coastal biota. For many of these DDT+ compounds, almost no toxicity information was yet available or was highly limited. During this presentation, we will build upon the research presented by Drs. Valentine and Dodder, but present our novel findings related to broader DDT+ toxicity. We will present *in vitro* research comparing cytotoxicity and estrogen receptor activity of DDT+ compounds, followed by aquatic toxicology research in zebrafish investigating impacts in developmental success, transcriptomics, and behavior. We will present data that suggest that the DDT byproducts tris(4-chlorophenyl)-methane and -methanol may be of particular interest for ecotoxicological and human health research due to conserved toxicity with DDT and similar bioaccumulation patterns. Likewise, estrogen receptor agonism is moderately conserved across humans and California condors, and there are clear differences in estrogen receptor activity between DDT+ species. Transcriptomics for exposed zebrafish embryos reveal distinct clusters of DDT+ compounds based on molecular response, and p,p'-DDT, -DDE, and -DDD have unique responses compared to the other DDT+ compounds assessed. Overall, we categorize these contaminants into groups of higher or lower priority for future investigation, mitigation efforts, and conservation.

S 1147 Current Nonclinical Approaches in Characterizing Local Tolerability with Long-Acting Injectable Therapies

S. Kulkarni. Gilead Sciences Inc., Foster City, CA.

Injection-site reactions (ISRs) are one of the most common adverse events, occurring in up to 40% of patients receiving injectable therapeutics. This Symposium is one of the first sessions at the SOT Annual Meeting to explore the current status of nonclinical characterization of ISRs with a focus on small molecule, depot-forming therapeutics or long-acting injectables (LAIs, long-acting anti-psychotics, antivirals, etc.) when administered via subcutaneous (SC) or intramuscular (IM) routes and their impact on drug development with industry, academic, CRO, and regulatory experts. ISRs are local inflammatory reactions originating around the injection site that manifest as discomfort, pain erythema, induration, and itching and, in some cases, more severely as ulceration or necrosis. While relatively mild ISRs could be acceptable, they are a cause for suboptimal patient experience and generally not desirable. On the other hand, higher-severity ISRs pose a significant challenge for patient compliance and commercial viability, hence requiring characterization and derisking early in drug development. The current practices in nonclinical species selection, study design and endpoint consideration for ISR evaluation will be presented. Histopathology assessment of ISRs and the impact of route and species differences on local tolerability determination will also be discussed. Regulatory guidelines on evaluating local tolerability are not specific and likely do not take into account the newer, long-acting type formulations for therapeutics intended for less frequent administration. A regulatory perspective on local tolerability and sponsor-generated ISR data in support of a clinical plan will be presented in this session. Furthermore, published literature indicates low confidence in the predictive capacity of severity of nonclinical ISRs to the clinic, and available *in vitro* systems fall short on capturing the temporal immune and inflammatory dynamics at the injection site. Given the implications, ISR evaluation in animal models and derisking for clinical progression remain challenging. LAI case studies will highlight the impact of therapeutic-related factors on ISRs in animal models and their progression through clinical development. Overall, this Symposium will provide an update on the current status, challenges, and mitigation in the evaluation and characterization of ISRs.

S 1148 Study Design Considerations and in Life Evaluation for Nonclinical ISR Evaluation

J. Wendel. Labcorp Early Development Laboratories Inc., Greenfield, IN.

Non-clinical safety assessment studies are used to determine tolerability and toxicity of pharmaceuticals prior to use in a clinical setting. Non-clinical safety assessment studies are also used to assess different forms, formulations, dose routes, or patient populations of currently clinically available pharmaceuticals. Based on the intended purpose of the non-clinical safety assessment study, the study may require a different design and/or specialized endpoints. This talk will focus on pharmaceutical compounds with a clinical route of injection such as subcutaneous or intramuscular and considerations that should be taken into account during the study design

process to meet the intended study objective. The risks and rewards of including local tolerance endpoints on a systemic toxicology study or completing a focused local tolerance study will be discussed. Opportunities for reduction in animal usage by assessing multiple dose routes in one animal or utilizing animals as their own control will also be discussed. Following the presentation of study design considerations, this presentation will focus on the endpoints needed to determine local tolerance and the differences in endpoints depending on the route of administration. When and if it is necessary to add specialized dermal observations and how to minimize variability in potentially subjective endpoints, will also be discussed.

S 1149 Pathology Considerations for Evaluation of Injection Site Reactions: Animal Models, Sampling, and Histopathologic Evaluation

K. Nelson. Inotiv, Portage, MI. Sponsor: S. Kulkarni

Injectable therapies utilize subcutaneous (SC) and intramuscular (IM) administration routes for delivery, with depot formation. Often, the injection site reaction (ISR) may be one of the key elements in evaluating the response. Careful collection and sampling of the injection site facilitates the accurate assessment of the ISR by the pathologist. The ISR is the site of test material delivery, the site of greatest test material concentration, the most common site for histopathological findings, and the determinant for local tolerability. Typical histopathological findings include a spectrum of inflammation, hemorrhage, pseudocyst formation, edema, necrosis, fibrosis, and delivery material presence. These findings may vary according to a range of factors, including the animal model, particle size, vehicle characteristics, trauma associated with administration, and nature of the therapeutic agent. Evaluation of the injection site should encompass thorough sampling of the region, consistent and descriptive terminology for both macroscopic and microscopic observations, and consideration of the ISR in the context to the overall health status of the animal, particularly when determining tolerability.

S 1150 Nonclinical Regulatory Perspectives on Injection Site Reactions

J. Hawes Oliphant. US FDA/NCTR, Jefferson, AR.

Regulatory expectations for evaluation of injection site reactions in nonclinical studies supporting therapeutic development programs for injectable products will be reviewed. Considerations and challenges associated with adverse ISR findings in nonclinical studies and translation to clinical relevance will be discussed, particularly in how they may potentially impact overall nonclinical risk assessments. Case study examples will be provided.

S 1151 The Development and Associated Challenges of Long-Acting Injectable Formulations

E. Pierson. Merck & Co., Rahway, NJ. Sponsor: S. Kulkarni

Long-acting injectable (LAI) formulations can provide several advantages over the more traditional oral formulation as drug product opportunities. LAI formulations can achieve sustained drug release for extended periods of time, which results in less frequent dosing requirements leading to higher patient adherence and more optimal therapeutic outcomes. This talk will provide an industry perspective on the development and associated challenges of long-acting injectable formulations, including considerations of the Active Pharmaceutical Ingredient, optimization of the formulation, biopharmaceutical properties and characterization of LAIs through *in vitro*, *in vivo* and *in silico* approaches.

S 1152 Nonclinical-to-Clinical Translation of Injection Site Reactions

D. Zane. Gilead Sciences Inc., Foster City, CA.

The translation of observed nonclinical injection site reactions (ISRs) to the clinic has been a challenge for all types of therapeutics. From all the available literature, there is poor interspecies concordance with respect to ISRs. In addition, nonclinical studies have been shown to overpredict or underpredict the potential for ISRs in humans. While some nonclinical studies have been useful in predicting that ISRs may occur with parenteral products, the magnitude of effect remains poorly correlated. Regardless of the material, inflammation is the most common response to a foreign material in the subcutis. This presentation will discuss an overview on the nonclinical strategy in the development of long acting injectables which will include considerations to general study design of ISR studies (also known as local tolerability studies) to support entry into the clinic, discussion of species selection, case example of nonclinical-to-clinical translation and lessons learned.

S 1153 It's Complicated: The Messy Relationship Between Environmental Exposures and Metabolic Health

J. Beier. University of Pittsburgh, Pittsburgh, PA.

Environmental pollutants significantly impact the development, progression, and outcomes of metabolic disorders with the liver serving as a primary target organ. Recent epidemiological and experimental evidence has firmly established associations between various environmental exposures and metabolic dysfunction-associated steatotic liver disease (MASLD), obesity, and related metabolic disorders. This Symposium will explore cutting-edge research on the complex relationships between environmental chemicals and metabolic health, with particular emphasis on hepatic outcomes and mechanisms. The liver plays a central role in xenobiotic metabolism and intermediary metabolism, making it uniquely vulnerable to toxic insult. Emerging research has identified several classes of environmental chemicals as metabolism-disrupting chemicals (MDCs) that can promote alterations in liver lipid metabolism, energy homeostasis, and insulin sensitivity. These include per- and polyfluoroalkyl substances (PFAS), persistent organic pollutants like polychlorinated biphenyls (PCBs), plasticizers, pesticides, and air pollutants. New epidemiological evidence demonstrates robust associations between these exposures and liver disease severity, metabolic dysregulation, and obesity across diverse populations. This Symposium will highlight recent advances in our understanding of exposure-related metabolic toxicity. Presenters will discuss innovative research applying multi-omics technologies, exposomics approaches, and novel biomarkers to identify chemical effects on liver pathology and metabolic health. Critical discussion will focus on sex-specific effects, developmental origins of exposure-related metabolic diseases, and gene-environment interactions that modify individual susceptibility. Emerging research on the hepatic effects of exposure mixtures that reflect real-world scenarios will be presented alongside mechanistic investigations elucidating molecular pathways of toxicity, including nuclear receptor signaling, oxidative stress, mitochondrial dysfunction, and inflammatory processes. The Symposium will conclude with translational perspectives on implementing environmental health science in clinical practice, exploring new approaches for exposure assessment, patient screening, and intervention strategies. By bringing together diverse expertise spanning environmental health sciences, hepatology, and metabolism research, this session will provide critical insights into the role of environmental exposures in metabolic health and disease, with implications for toxicological risk assessment, clinical practice, and public health policy.

S 1154 Proteomic Markers of Gut Dysfunction and Mortality in Alcohol-Associated Hepatitis

B. Schnabl. University of California San Diego, San Diego, CA. Sponsor: J. Beier

Patients with alcohol-associated hepatitis (AH) face a high risk of mortality, largely due to alcohol's damaging effects on the liver and its ability to disrupt gut health. Alcohol damages the intestinal barrier, facilitates microbial translocation to the liver and contributes to chronic liver inflammation and progression of liver disease. To better understand intestinal changes and identify potential mediators of gut barrier dysfunction, we conducted a study analyzing fecal proteins from individuals across three groups: healthy controls, patients with alcohol use disorder (AUD), and those with AH. Using Tandem Mass Tag (TMT) proteomics, we examined fecal samples from two large multicenter cohorts and identified distinct differences in the proteomic profiles of each group. These differences followed a clear pattern that aligned with disease progression. Notably, proteins associated with neutrophil granules became increasingly abundant as disease severity worsened. Among these proteins, myeloperoxidase (MPO), a key enzyme found in neutrophil granules, was strongly correlated with both disease severity and the likelihood of death within 60 days. Furthermore, fecal levels of cathepsin B, a secreted lysosomal protease, and activity progressively increased in patients with AUD and AH compared with controls and were associated with higher 90-day and 180-day mortality in patients with AH in the exploratory and validation cohort. Immunofluorescence analysis in patients with AUD revealed that cathepsin B is upregulated and predominantly expressed in macrophages in the intestine. Cathepsin B proteolytically cleaves occludin in enzymatic assays, and its inhibition prevented occludin degradation and barrier disruption in intestinal organoids and epithelial monolayers. These findings highlight intestinal neutrophils and cathepsin B as mediators of gut barrier dysfunction and therapeutic target in alcohol-associated liver disease.

S 1155 High-Throughput Chemical Exposomics: Understanding Body Burden of Environmental Chemicals

D. Jones. Emory University, Atlanta, GA.

Environmental disease often develops and progresses over decades. Unfortunately, cost and throughput have limited human toxicology studies to a relatively small number of the thousands of potentially harmful environmental chemicals to which humans are exposed. To address this problem, we developed a scalable workflow for gas chromatography-high resolution mass spectrometry analyses of human samples with capability to measure hundreds to thousands of chemicals in single analyses. We now have introduced software to rapidly identify and quantify envi-

ronmental chemicals relative to pooled reference materials with known concentrations of thousands of chemicals. The methods have been applied to serum samples from human cohorts to study environmental chemical contributions to breast cancer, prostate cancer, Alzheimer's disease, Parkinson's disease and idiopathic pulmonary fibrosis, as well as to thyroid cancer, non-alcoholic fatty liver disease, and hyperparathyroidism. Results show up to 600 environmental chemicals within individual samples, including POPs, PAHs, pesticides, commercial chemicals, food additives and personal use products. Integration with metabolomics, phenotype and disease measures enables mechanistic investigation of individual chemicals as well as complex chemical mixtures.

S 1156 How Sex, Dose, and Diet Shape Liver Responses to POPs

B. Wahlang. University of Louisville, Louisville, KY.

Polychlorinated biphenyls (PCBs), dioxins and organochlorine pesticides, the original "dirty dozen", are well-associated with steatotic liver disease and metabolic disruption. However, epidemiological studies investigating links between levels of these legacy persistent organic pollutants (POPs) and liver/metabolic disease outcomes were concentrated mostly on chemical and agricultural workers who were mostly men. Experimental and toxicological studies have also largely been carried out predominantly on male rodent models as they were considered less messy. Critical knowledge gaps exist in the context of pathogenesis and disease progression, and more importantly mechanisms driving toxicity outcomes pertaining to POPs and women's liver and metabolic health. There is also emerging evidence that exposures to these POPs, often dictated by socio-economic status, is accompanied by poor nutritional status and comorbidities such as obesity. This presentation will focus on how exposures to mixtures of these legacy POPs, tailored to simulate current human exposure patterns, in conjunction with hypercaloric diets, can lead to sex-specific liver and metabolic toxicity. Notably, the presentation will emphasize on the significance of environmentally relevant doses, and how distinct mode-of action(s) including xenobiotic receptor activation and altered estrogen signaling modulate sex-chemical-diet interactions to consequently impact liver disease outcomes.

S 1157 From Bench to Community-Based Interventions: Translational Insights into PFAS and MASLD Pathogenesis

L. Chatzi. University of Southern California, Los Angeles, CA. Sponsor: J. Beier

Per- and polyfluoroalkyl substances (PFAS) are persistent environmental contaminants that accumulate in the liver and are increasingly recognized as modifiable risk factors for metabolic dysfunction-associated steatotic liver disease (MASLD). This translational research framework integrates findings from human population studies and mechanistic *in vitro* models to illuminate how distinct PFAS compounds contribute to MASLD pathogenesis and inform targeted intervention strategies. In adolescents with severe obesity, elevated plasma concentrations of perfluoroheptanoic acid (PFHpA) were significantly associated with increased MASLD risk, severity of steatosis, and fibrosis. Multi-omics analyses revealed PFHpA-induced disruption of lipid metabolism, oxidative stress, and immune pathways. In parallel, advanced single-cell transcriptomics of 3D human liver spheroids exposed to PFHpA, PFOA, PFOS, PFHxS, or PFNA uncovered compound- and sex-specific mechanisms of hepatic injury. PFOA and PFHpA promoted lipid accumulation via upregulation of *de novo* lipogenesis and PPARα signaling, while PFNA and PFOS triggered oncogenic and immunosuppressive pathways. Notably, PFNA-exposed hepatocytes displayed strong cancer-like transcriptional signatures. Integrating omics data across platforms identified PFAS-related proteomic signatures predictive of MASLD risk, offering potential biomarkers for early detection. Collectively, our findings bridge molecular insights from the bench with population-level observations, advancing precision prevention strategies and informing community-based interventions to reduce PFAS-related liver disease burden.

S 1158 From Mechanisms to Medicine: Translating the Science of Metabolism Disruption into Clinical Practice

R. Sargis. University of Illinois at Chicago, Chicago, IL. Sponsor: J. Beier

The burgeoning metabolic disease pandemic has laid bare our failure to address its underlying drivers. While genetic predisposition coupled with excess energy intake and insufficient energy expenditure are undoubted drivers of metabolic dysfunction, these factors alone fail to explain the magnitude and rapidity with which metabolic health has deteriorated in the U.S. and globally. As such, other factors are likely at play and need to be addressed. Indeed, the last two decades have witnessed the emergence of robust data linking exposure to environmental toxicants to metabolic dysfunction. Our own cell-based, animal, and population-based studies corroborate the general literature that implicates exposure to a variety of environmental toxicants in the worsening of metabolic health. Furthermore, data indicate that many exposures, analyzed as single chemicals or mixtures, promote reinforcing cardiometabolic derangements that likely synergize to augment the risk of cardiovascular disease, the leading cause of mortality. Despite the strength of data across the

evidentiary spectrum, clinical medicine neglects the contribution of environmental exposures to metabolic disease risk. Our analyses of clinical practice guidelines confirms that the metabolic threat posed by environmental toxicants are largely, if not entirely, neglected in clinical medicine, a factor that impairs scientific progress, the development of public policy, and patient care. While policy interventions are critical for addressing these emerging health threats and promoting health equity, it is critical that the knowledge of environmental metabolism-disrupting chemicals leave the laboratories and enter the medical mainstream. Herein we discuss how our current environmental science can be applied to improve metabolic disease care.

S 1159 AHR-Mediated Dose Dependent Spatial and Temporal Metabolic Disruption in TCDD-Elicited Hepatotoxicity

T. Zacharewski. Michigan State University, East Lansing, MI.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) induces dose-dependent hepatotoxicity and the progression of simple, reversible steatosis to steatohepatitis with fibrosis in C57BL/6J mice. The integration of computationally identified putative dioxin response elements (pDREs) with ChIPseq, bulk RNAseq, snRNAseq, spatial transcriptomic and metabolomics datasets as well as complementary histopathology in wild-type and a hepatocyte-specific, inducible aryl hydrocarbon receptor (AHR) null model has identified the dose dependent disruption of a number of metabolic pathways following the persistent of the AHR activation by TCDD. This includes (i) the ablation of circadian regulated carbohydrate, lipid and amino acid metabolism, (ii) the functional organization of metabolism across the liver lobule, and (iii) the accumulation of toxic intermediates such as secondary bile acids, acrylyl-CoA, ammonia and dicarboxylic acids. Persistent AHR activation by TCDD also elicited changes in the levels of acetyl-CoA, beta-hydroxybutyrate, ammonia and glutathione. This resulted in alterations in the levels of post-translation modification by acetylation, beta-hydroxybutylation, carbamylation and S-glutathionylation, respectively. These changes not only compromised cellular bioenergetics and antioxidant defenses but also altered the levels of intracellular messengers such as hydrogen sulfide and cobalamin. Overall, our results suggests that the hepatotoxicity of TCDD and related compounds goes beyond changes in gene expression and likely is due to the cumulative burden of metabolic disruption resulting from persistent AHR activation by TCDD and related compounds.

W 1160 Integrating In Vitro Data and PBK Modeling for Exposure-Led Risk Assessment

N. Kramer. Wageningen University, Wageningen, Netherlands.

Exposure-Led risk assessment uses estimates of human exposure as a primary driver to evaluate health risks, determine testing needs, refine hazard information, and support regulatory decisions. In this context, the integration of new approach methodology (NAM)-derived *in vitro* data with physiologically based kinetic (PBK) modeling is increasingly relevant. Together, these approaches provide a practical and mechanistic means to increase human relevance, reduce reliance on animal testing, and support more transparent and predictive exposure assessments. When combined through *in vitro-in vivo* extrapolation (IVIVE), these approaches help link *in vitro* bioactivity data with internal and external exposure estimates in humans. They can be applied in reverse dosimetry, deriving external doses corresponding to *in vitro* effect levels, and forward dosimetry, predicting tissue concentrations from expected exposures to compare with *in vitro* bioactivity data. Because IVIVE-PBK approaches are based on mechanistic understanding of human biology, they allow refinement of traditional risk assessments by accounting for biological and population variability. This session will emphasize both scientific and regulatory perspectives on implementing IVIVE-PBK in real-world contexts, with speakers from Europe and North America representing academia and industry. Case studies will draw on recent initiatives such as the European Food Safety Authority (EFSA) ADME4NGRA project, the ONTOX project, VHP4Safety, and the Partnership for the Assessment of Risks from Chemicals (PARC). Applications will span environmental chemicals, consumer products, nanomaterials, and sensitive endpoints, including developmental and reproductive toxicity (DART). The session will explore how *in vitro*-derived parameters, such as metabolic clearance, protein binding, and transporter activity, can be used to parameterize PBK models. Both reverse and forward dosimetry applications will be covered, with a focus on deriving external doses aligned with *in vitro* effect concentrations and comparing predicted internal concentrations with thresholds of biological activity. The session will also address the use of virtual populations and probabilistic modeling to characterize interindividual variability and inform regulatory decision-making. Finally, we will review current best practices for documenting model assumptions, selecting and sourcing input parameters, and communicating uncertainty, with reference to international guidance. Audience engagement will be encouraged to discuss challenges, evolving best practices, and strategies for ensuring transparency, reproducibility, and regulatory relevance in exposure-led risk assessment.

W 1161 Using *In Silico* Modeling to Support *In Vitro* ADME Parameterization of Physiologically Based Kinetic Modeling

N. Kramer. Wageningen University, Wageningen, Netherlands.

Next generation risk assessment (NGRA) encourages the use of *in silico* and *in vitro* tools as opposed to *in vivo* kinetic studies to parameterize physiologically based kinetic (PBK) models for quantitative *in vitro-in vivo* extrapolation (QIVIVE). In this study, we examine the applicability domains of *in vitro*-based absorption, distribution, metabolism, excretion (ADME) assays parameterizing PBK models for NGRA. It draws on experience from four ongoing European research projects: (1) the European Food Safety Authority (EFSA) ADME4NGRA project, (2) the EU Horizon 2020 ONTOX project, (3) the Dutch National Research Organization Virtual Human Project (VHP4Safety), (4) the European Partnership for the Assessment of Risks from Chemicals (PARC). The study describes how *in vitro* distribution kinetic modeling can explain and extend the chemical applicability domain of *in vitro* ADME assays, including rat and human stem cell based intestinal models for assessing the role of extrahepatic metabolism in absorption, new methods for determining plasma protein binding, human hepatic models of increasing complexity, from microsomes to microphysiological systems, for assessing hepatic clearance, and human proximal tubule models for describing transporter mediated excretion. The relevance of model outputs, such as internal dose metrics for comparing with *in vitro* bioactivity thresholds, will be discussed alongside uncertainties in assay extrapolation. Regulatory considerations, including how these model outputs can inform exposure-led risk assessment decisions, will also be addressed. This talk will emphasize practical strategies for incorporating these approaches into exposure-led regulatory decisions.

W 1162 Advancing NGRA for Developmental Neurotoxicity and Developmental and Reproductive Toxicity: Integrating *In Vitro* and *In Silico* Strategies

M. Moreau. ESQlabs GmbH, Durham, NC.

Developmental and reproductive toxicity (DART) evaluations are increasingly relying on New Approach Methodology (NAM) and IVIVE-PBK models to interpret effects without *in vivo* testing. This presentation will focus on how gestational kinetic models contribute to the interpretation of developmental and reproductive toxicity (DART)-related NAM data, particularly during pregnancy. Here, PBK models must consider dynamic physiological changes during gestation, including placental function and fetal development. Current maternal-fetal modeling platforms and methodologies for parameterizing placental chemical transfer will be presented, along with the existing knowledge gaps. To demonstrate the practical application of these models, case studies using quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) from developmental neurotoxicity (DNT) research will be discussed, comparing approaches that can be used in tiered risk assessments and evaluating their respective strengths and limitations. We will also discuss model uncertainties and opportunities to enhance regulatory confidence through transparent parameterization and variability analysis using virtual populations. This presentation directly supports the session themes by addressing complex endpoints, NAM integration, and decision-relevant model outputs.

W 1163 Integrating *In Vitro* Data and *In Silico* Predictions to Develop and Apply PBK Models to Support Risk Assessments of Chemicals and Nanoparticles

Z. Lin. University of Florida, Gainesville, FL.

Integrating *in vitro* experimentation and *in silico* modeling is an important approach for the 21st century toxicity testing paradigm for chemicals and nanoparticles. While many studies have successfully applied *in vitro* data and *in silico* predictions to build PBK models for small molecular drugs and environmental chemicals, very few studies have been able to build PBK models for nanoparticles solely based on *in vitro* and *in silico* data. This presentation will discuss the similarities and differences in building PBK models for small molecular chemicals and nanoparticles. Recent studies will be presented to illustrate how *in vitro* dissolution, uptake, and retention data can be used to parameterize PBK models and predict internal concentrations in humans *in vitro*. In addition, the presentation will introduce how to apply PBK models to support toxicity and risk assessments of chemicals and nanoparticles by predicting human equivalent doses and risk ranking based on *in vitro* data. We will also address uncertainties in nanoparticle modeling and discuss how PBK outputs inform exposure limits in the absence of *in vivo* data, in connection with regulatory expectations for NAMs and risk-based decisions.

W 1164 Building Confidence in Bottom-Up PBK Model Development for NGRA Without Support of *In Vivo* Data

A. Punt. Unilever, Arnhem, Netherlands. Sponsor: N. Kramer

Bottom-up physiologically based kinetic (PBK) models use *in vitro* and *in silico* data to estimate internal chemical concentrations in humans. Evaluating PBK models typically requires *in vivo* data, but such data are often unavailable. This presentation will describe how confidence in PBK predictions can be achieved without *in vivo* data, through analog-based performance assessment, parameter sensitivity analysis, and software comparisons. We will illustrate how predicted plasma concentrations from bottom-up models are compared to *in vitro* thresholds to support point-of-departure derivation in regulatory submissions. Methods to transparently document assumptions, address uncertainty, and support model credibility will be discussed. This presentation supports the session themes of model transparency, reproducibility, and practical application of IVIVE-PBK in regulatory risk assessment. The results show that bottom-up PBK models can provide protective plasma concentration predictions for next generation risk evaluations (NGRA).

W 1165 Toxic Insights Unlocked: Leveraging Machine Learning and Advanced Image Analysis to Transform the Science of Toxicology

A. Green. Sciome LLC, Research Triangle Park, NC.

Advancements in imaging technologies and machine learning algorithms have revolutionized chemical safety assessment. Traditional methods, like manual evaluation of cellular assays or histological slides, are insufficient for addressing the growing influx of environmental and pharmaceutical compounds requiring examination. Three-Dimensional cell culture models and early developmental stage model organisms, such as zebrafish embryos, yield more physiologically relevant data but present new challenges with data complexity and throughput. To bridge this gap, the community has adopted scalable, automated procedures driven by bespoke deep-learning architectures and cloud-native infrastructures. In this session, attendees will discover how the latest imaging and computational advances are transforming chemical safety assessment into a faster, more reliable, and more predictive science. The session will explore how automated image-analysis workflows can turn thousands of raw images, whether whole organisms or cell cultures, into clear, quantitative readouts, overcoming long-standing challenges of data volume and human bias. Attendees will see how these approaches unlock subtle biological responses to chemical exposures at unprecedented scale, helping both academic researchers and industry practitioners generate high-quality data with confidence. The session also will highlight how integrated experimental platforms, 3D tissue models to high-content live-cell assays, are being bridged to traditional toxicology endpoints, speeding regulatory acceptance and strengthening mechanistic insights. Attendees will learn why packaging each step of the analysis into portable, end-to-end workflows is critical for reproducibility and collaboration across labs. Finally, the session will share general strategies for building robust analysis pipelines that stand up to real-world variability in staining, lighting, and sample preparation. Whether you're focused on environmental monitoring, drug safety, or regulatory science, this session will leave you equipped with the practical vision and inspiration to harness these innovations for your research programs.

W 1166 Advanced Scalable Screening of Phenotypic Responses to Chemicals

L. Truong. Oregon State University, Corvallis, OR.

This presentation will delve into our latest advancements in the scalable screening of phenotypic responses to chemicals using early life stage zebrafish models. We will discuss our cutting-edge techniques that enhance throughput and sensitivity in detecting potential toxicants, crucial for environmental and pharmaceutical safety assessments. We will discuss the state of scalable screening and automated imaging and analysis technologies that increase the efficiency and precision of phenotypic assays. We will discuss how data from phenotypic screenings are integrated and analyzed using advanced computational methods using new case studies. This presentation is intended for researchers, practitioners, and policymakers in toxicology, pharmacology, environmental sciences, and related fields. It will also benefit regulatory agencies and industry scientists interested in the latest methods for safety assessment. We hope to illustrate the practical applications of these techniques in real-world chemical assessments and to foster collaboration and discussion on improving chemical safety evaluations through advanced scientific techniques.

W 1167 Imaging of Microphysiological Systems for Toxicologic Pathology

S. Ferguson. NIEHS, Research Triangle Park, NC.

The needs of drug and environmental chemical safety research have inspired an array of new approach methods (NAMs) that mechanistically inform our understanding of chemical-biological interactions. However, most NAMs, to date, have limited

capacity to integrate biological response pathways to feed forward into recognizable toxicologic pathologies. Significant progress has been made with various types of microphysiological systems (MPS) that emulate tissue-like cellular architectures, hallmarks of tissue functionality, and human responses to drug and environmental chemical exposures. Surprisingly, MPS have had limited adoption for regulatory use, a consequence of historical approaches that rely on toxicologic pathology (e.g., clinical pathology, histopathology) in animal models and human clinical studies for decision-making and MPS developer's limited understanding of the needs of toxicologic pathology. To bridge these translational gaps, cellular imaging of MPS, in tandem with clinical pathology biomarkers and mechanistic assay systems, has the potential to serve as a 'Rosetta Stone' to relate these disparate fields and facilitate regulatory adoption of NAM-based approaches. Furthermore, due to their enhanced longevity and fidelity to model hallmarks of tissue functionality, MPS platforms hold the potential to model sub-chronic toxicity and disease. In this presentation, we describe case studies for the successful development and application of MPS to model human liver and kidney (i.e., proximal tubule) toxicity in response to drug and environmental chemical exposures that leverage imaging-based assay approaches. These include examples with exposures to PFAS, botanicals, and drug substances that reveal phenotypic changes consistent with hepatocellular injury, hepatic steatosis, cholestasis, and nephrotoxicity. In combination with biomarker and mechanistic data, imaging-based approaches with MPS using longer-term exposures hold the potential to serve as translational threads for the prediction of human health effects that collectively sew a growing quilt of drug and environmental chemical solutions for adoption in next-generation risk assessments.

W 1168 Automated Image Analysis Pipelines for High-Throughput Toxicological Screening

A. Tandon. Sciome LLC, Research Triangle Park, NC. Sponsor: A. Green

Advances in computational image analysis are changing toxicological screening by enabling automated, high-throughput measurement of morphological traits across multiple biological models. In this presentation we discuss customized deep learning pipelines for image analysis and segmentation of whole organism assays, histopathology slides, and cellular images to increase repeatability and efficiency in screening procedures. In our approach, we developed and deployed automated imaging and analysis pipelines for various assay formats, including 3D spheroid cultures, whole organism zebrafish embryos, high-resolution histopathology slides, and cell-based *in vitro* assays. We will discuss the deployment of a 3D U-Net convolutional neural network to analyze confocal z-stack images of liver spheroids inside a fully parallelized framework. Followed by the creation of a multi-view CNN system for checking early life stage zebrafish using dorsal and lateral projections, allowing for a combined method to identify more than twenty morphological features and segment essential organs with minimal user involvement. Lastly, we will present a computer vision approach for the assessment of cellular monolayers and tissue sections, incorporating focused loss algorithms and considerable data augmentation to address class imbalance and staining variability. This presentation concludes by discussing the benefits of containerizing each analysis pipeline to automatically orchestrate image preprocessing, inference, and post-analysis metric extraction to significantly increase throughput and reproducibility across screening campaigns.

S 1169 Understanding the GRAS Loophole: History, Science, and Legal Challenges

B. Mahadevan. Brincor Associates LLC, Corvallis, OR.

Recent discussions have centered on the potential for closing the generally recognized as safe (GRAS) loophole for food ingredient approval and strengthening US FDA oversight of food ingredients, leading to governmentally recognized as safe. The Human and Health Services Secretary has directed the US FDA to explore eliminating the self-GRAS pathway. This change could lead to premarket approval for food additives, potentially impacting the availability of some currently used ingredients and delaying the entry of future ingredients. It will create more work for the US FDA and increase costs for food manufacturers. Is rule-making to enforce US FDA-notified GRAS as mandatory possible? Potential legal hurdles and challenges will be illustrated. The history of GRAS, its current status, and related controversies will be discussed. Self-GRAS pros include streamlined approval, faster innovations, focus on established safety, and the role of qualified experts, while self-GRAS cons potentially include weakened oversight, lack of resources, potential for harm, lack of transparency, conflict of interest, and public health concern. These points must be considered and discussed before changing the current GRAS regulation. In recent months, the food regulatory landscape has been intensely scrutinized, with particular focus on the mechanisms and policies governing food ingredient approvals. This scrutiny has been sparked by growing public and scientific concern over the adequacy of current oversight practices.

S 1170 Mechanisms of Persistent Neurotoxicity: Changes Seen Throughout the Lifespan

D. Kendricks. NIEHS, Research Triangle Park, NC.

Early life exposures to many environmental contaminants may induce changes in nervous system development and function. Many such compounds induce neurobehavioral and physiological disruptions that arise shortly after exposure but are absent years after exposure has ended. However, there are many others that induce permanent changes in nervous system function that result in persistent, observable detriments throughout the lifespan, well into aging. These persisting effects are of paramount importance due to implications in neurodevelopmental disease onset, elevated risk of later-life disease manifestation, and heightened risk of neurodegenerative disease. Metals, pesticides, flame retardants, and many others remain a common risk in human populations throughout the world and have varied impacts on nervous system development and degeneration. However, current observations of the effects of these compounds have drifted away from investigating those mechanisms that persist throughout the lifespan. The goals of this Symposium are to shed fresh light onto the importance of understanding and considering long-term consequences of exposures and to provide further evidence of mechanisms associated with persistent effects seen following developmental exposure. In this Symposium, speakers will provide a framework that highlights essential considerations in models assessing long-term impacts of exposures and provide various examples of different models used to assess potential mechanisms underlying persistent neurotoxic effects. These discussions will include frameworks for understanding neurobehavioral outcomes linked to persistent neurotoxicity, biological variables to be considered, and examples of persistent neurotoxicity linked to exposure to heavy metals such as MeHg or ambient ultrafine particulate matter. Given the essential interaction between the time course of exposure and manifestation of neurotoxic effect, this Symposium will provide discussions highlighting exposures across various developmental timepoints. The speakers include experts from academia and government. The Symposium will begin with an introduction that will set the framework for understanding the importance of assessing risk of persistent neurotoxicity and current approaches for understanding potential mechanisms. The first speaker will provide a framework of the importance of understanding long-term disease risk associated with early life exposures. The talk will provide an overview of the importance of modeling behavioral outcomes resulting from early life exposure to showcase the importance of behavioral phenotype as an essential endpoint in the etiology of persistent disease. The second speaker will further elaborate on essential considerations in models addressing early life exposures. The talk will provide focus on incidence of neurodevelopmental disease risk and implications of sex as a biological variable. The third speaker will provide a discussion of potential mechanisms of persistent neurotoxicity associated with developmental exposure to the heavy metal methylmercury. The talk will address the relationship between chronic methylmercury neurotoxicity and the persistent decline in a dopamine-related behavioral phenotype using *Caenorhabditis elegans*. The final speaker will close out by discussing the persistence of behavioral effects resulting from developmental exposure to concentrated ambient ultrafine particulate matter and the potential relationships between these effects and altered levels of brain neurotransmitters in mice. The Symposium will conclude with a Q&A with all speakers to provide additional information and discussion on the importance of persistent effects of exposure. Together, this Symposium will provide valuable insights into the potential mechanisms of persistent neurotoxicity resulting from exposure to various environmental toxicants, which may advance the development of more effective prevention and intervention strategies to attenuate later-life disease risk.

S 1171 Long-Term Alterations in Behavior Resulting from Early Developmental Toxicant Exposure

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Toxicant exposure during the process of brain development can cause long-term alterations in neurobehavioral function that are expressed throughout the rest of life. In a great many studies, with a variety of toxicants including heavy metals, pesticides, marine toxins, flame retardants and polyfluorinated compounds, across species from zebrafish to rats to monkeys, we and others have found that developmental toxicant exposure causes long-term behavioral changes in sensorimotor function, emotional response and cognition. Several overarching conclusions can be drawn from these studies. Even relatively brief or low-level toxicant exposure during brain development can cause long-term behavioral alterations. Long-term effects are not necessarily predicted by short-term effects. Cognitive impairment from developmental toxicant exposure can emerge during older adulthood. Typical sex differences in behavior, particularly with locomotor activity and cognitive function, have been found to be diminished or reversed by developmental neurotoxicity. Therapeutic treatments can counter the imitation of developmental neurobehavioral toxicity or attenuate the expression of its behavioral consequences later in life. There is a plethora of interacting factors that play key roles in determining whether developmental toxicant exposure causes long-term neurobehavioral changes. Translational animal models can provide the complexity of an evolved integrated organism to test the long-term consequences of developmental neurotoxic exposure on neurobehavioral function. Species with shorter life courses can provide a look ahead at potential risks that may develop over the many decades of the human life span. The long-term func-

tional behavioral effects of developmental neurotoxicity can be a starting point to track back the interacting mechanisms that give rise to the impairment, meeting the forward mechanistic studies beginning with incipient effects to increase our understanding of the more complete causative series of events that result in long-term neurobehavioral dysfunction resulting from developmental toxicant exposure.

S 1172 The Timing Makes the Poison: Critical Windows, Chemical Exposures, and Neurotoxicity Across Brain Development and Maturation

H. Patisaul. NIEHS, Research Triangle Park, NC.

Heightened attention on the precipitous rise in the prevalence of multiple neurodevelopmental disorders (NDDs), such as autism spectrum disorder (ASD), has renewed interest in environmental factors as potential contributors, and how to better screen for developmental neurotoxicity (DNT). While most of the work seeking to understand how chemical exposures may contribute to NDD risk has focused on prenatal or early life exposure, significant aspects of human brain development occur long after birth and continue into early adulthood. Accounting for these windows is poised to significantly enhance the capacity to identify neurotoxicants and their modes of action. The post-natal brain has windows of rapid change and maturation currently not considered in animal or non-animal testing platforms. Those myriad processes include brain sexual differentiation, synaptic refinement, myelination, and neuroendocrine system maturation. Additionally, although all mental health disorders, including ASD, have a sex bias, many studies and research efforts, including emerging NAM platforms, still largely fail to incorporate sex as a biological variable let alone age, endocrine influence, or cellular or genetic diversity. Finally, although ASD and other NDDs share many socioemotional deficits, including sociality, the neuroendocrine systems underlying those behaviors remain absent from testing platforms despite the availability of tools from the neurosciences that could do so. This presentation will provide an overview of brain critical periods across pre- and post-natal life, how those windows align with NDD risk, and the processes and systems undergoing maturation and refinement during those key periods along with the imperative to account for them in any DNT testing platform as well as sex differences.

S 1173 Latent Alterations in Swimming Behavior by Developmental Methylmercury Exposure Are Modulated by the Homolog of Tyrosine Hydroxylase in *Caenorhabditis elegans*

M. Aschner. Albert Einstein College of Medicine, Bronx, NY.

Silent neurotoxicity is defined as persistent change to the nervous system that does not manifest as overt toxicity unless unmasked by experimental or natural processes. Silent neurotoxicants are challenging for risk assessors, as the multifactorial experiments needed to reveal their effects are seldom conducted, and they are not addressed by current study guidelines. Previous studies have shown that developmental exposure to MeHg can damage brain functions, which may only become apparent after years or decades. However, the underlying mechanisms of the latent neurotoxicity associated with exposure to MeHg are not yet fully understood. Using *Caenorhabditis elegans* (*C. elegans*), we investigated the latent toxicity of developmental MeHg exposure. Synchronized L1 stage worms were exposed to MeHg (0, 0.05, 0.5, and 5 μ M) for 48 hours. We analyzed the swimming speeds of adult worms that had been exposed to MeHg during their early larval stages. Worms exposed to MeHg developmentally showed a significant decline in swimming speed at the adult stage on day 10, but not on day 1 or day 5, despite mercury levels in worms exposed to 0.05 or 0.5 μ M MeHg being below the quantification limit on day 10. Day 10 adult worms treated with MeHg exhibited a significant decrease in bending angle and bending frequency during swimming. Additionally, their reduced swimming speeds tended to increase during the 300-second swimming experiment. Dopamine signaling is known to regulate worms' swimming speed. Therefore, we assessed the swimming speed of worms with cat-2 (mammalian tyrosine hydroxylase homolog) mutation or dat-1 deletion on day 10 of adulthood. The cat-2 mutant worms did not show a decline in swimming speed, body bends, or bending angles during swimming on day 10. Analysis of the swimming speeds of worms with dat-1 deletion revealed that their speeds were further reduced after MeHg exposure. However, the effects of MeHg and dat-1 deletion were not synergistic, as the interaction between these parameters did not reach statistical significance. In summary, our results suggest that developmental MeHg exposure induced a latent reduction in swim speed that was less pronounced with deficient dopamine synthesis. These findings highlight the pivotal role of dopamine and its metabolism in the latent, and likely the persistent neurotoxic effects of MeHg.

S 1174 Persistence of Neurochemical and Behavioral Changes Following Developmental Exposures to Concentrated Ambient Ultrafine Particulate Matter Air Pollution in Mice

D. Cory-Slechta. University of Rochester Medical Center, Rochester, NY.

Air pollution (AP) exposures are associated with neurodevelopmental disorders and neurodegenerative diseases, raising the question as to whether neurotoxic effects of developmental exposures persist and contribute to subsequent vulnerability for neurodegenerative diseases. Our prior studies in mice show that developmental exposures (postnatal days 4-7 and 10-13) to inhaled ambient ultrafine particulate (UFP) matter produce behavioral deficits and brain neurotransmitter system alterations. Subsequent studies have examined the impact of developmental exposures to inhaled nanoparticulate iron oxide (alone (Fe) or with SO₂ (FeS)), the most prominent AP metal contaminant as a potential determinant of UFP effects at 3 time points post exposure. Fe is critical to brain growth and development, but also redox active. At PND14, marked changes in brain frontal cortex and striatal neurotransmitter systems were observed, particularly in response to FeS as compared to Fe only, in glutamatergic and dopaminergic systems that were of opposite directions by sex. By PND 357, it was found, as expected, that behavioral experience markedly altered baseline levels of neurotransmitters as well as transsulfuration markers. Corresponding changes did not occur in developmentally FeS-exposed males. In contrast, females showed persistent neurochemical effects of developmental exposures to Fe alone at PND 215, as well as increased homocysteine levels in frontal cortex and striatum. Behavioral experience markedly increased correlations across frontal cortical and striatal glutamatergic, dopaminergic and serotonergic systems in control brains at PND357 (FeS) or 215 (Fe), and these correlations were significantly attenuated by developmental exposures to FeS and Fe, particularly in males. Collectively, the findings indicate a persistence of air pollution-related neurotoxicity with the trajectory of adverse changes modified by behavioral experience.

R 1175 Do We Need a Number, a Pathway, or Both? Applying Transcriptomics for Chemical Hazard Assessment

J. Rogers. Merck & Co. Inc., West Point, PA.

Gene expression profiling has gained considerable traction across the toxicology community. Beyond the original promise of "mechanistic interpretation," transcriptomics data is increasingly used for chemical prioritization and early derisking and to support alternatives to traditional toxicity testing (such as grouping and read-across). Transcriptomics platforms have been shown to provide a health-protective point of departure for provisional risk assessments, and gene expression biomarkers have been validated for well-studied hazards such as genotoxicity, liver injury, and endocrine disruption. Expansion to other hazards and their related mechanisms of action could expand the utility of these data beyond quantitative point-of-departure estimation. These readouts, whether from *in vivo* or *in vitro* studies, are often integrated with other experimental data into multidimensional datasets, thus meeting the long-standing need for "phenotypic anchoring." As the volume, speed, and complexity of gene expression data continue to grow, it's time to critically assess their accuracy and define clear contexts for their use. This session aims to highlight practical applications of toxicogenomic data in regulatory science. Speakers will explore several interconnected themes: a review of current and emerging gene expression profiling and interpretation methods in toxicology; case studies applying toxicogenomic data toward hazard characterization in regulatory contexts; and strategies to make these data more interpretable and actionable for decision-makers. By highlighting recent developments from pharma, government, and academic sectors, this session will further build consensus for how transcriptomics can directly support informed decision-making in toxicology.

R 1176 Toward a New Paradigm: Can Mechanistic *In Vivo* Studies Replace Traditional DART Guideline Studies?

S. Mitchell-Ryan. HESI, Washington, DC.

Current regulatory frameworks rely heavily on a battery of animal-intensive OECD test guideline studies to assess reproductive and developmental toxicity for various sectors, like agrochemicals and industrial chemicals. However, there is increasing recognition that short-term studies *in vivo* that employ high-dimensional biological metrics derived from 'omics technologies (e.g., transcriptomics) can be used to derive a point of departure (POD) that is based on a concerted molecular change. It has been suggested that this molecular POD-based approach potentially offers a more efficient, health protective, and animal-sparing alternative to traditional guideline studies. This Roundtable will explore the feasibility of converging toward a single *in vivo* study design that encompasses all critical windows of susceptibility and captures key developmental and reproductive endpoints while leveraging molecular and mechanistic data to ensure human health protection. Experts will discuss how traditional developmental and reproductive toxicology (DART) studies (e.g., OECD 414, 421, 416, and 443) are currently used and will present emerging designs that integrate gene expression and new approach methods to enhance predictive power and generate health-protective POD values while reducing/refining animal use. Special

attention will be given to how these newer approaches compare to current methods, including whether they can identify responses across all sensitive life stages and detect potential developmental and reproductive toxicants that might otherwise be missed. Challenges in interpreting gene expression data will be discussed, along with the role of kinetic data, cross-sector needs, and region-specific regulatory expectations. A case study presentation will illustrate comparisons between 'omics PODs and apical outcomes and highlight some specific efforts to increase reproducibility and standardize 'omics analyses and applications. Through collaborative discussion with the audience, panelists will work toward defining what an optimal, protective, and streamlined study could look like—one that reduces animal use, spans regulatory sectors, and is grounded in established mechanistic science. By identifying scientific and regulatory gaps, this session will help chart a path toward innovative testing strategies that are both ethically and scientifically robust.

R 1177 Mice with a Mission: Optimal Use of Humanized Transgenic Mice as an "FDA Innovative Platform"

T. Papenfuss. StageBio, Frederick, MD.

Humanized transgenic mice (HuMs) are a tool with potential to replicate human-specific physiology and pathology and provide a translational *in vivo* model for drug development. The recent US FDA Roadmap to reducing animal testing in preclinical safety studies has recognized the utility of HuMs as an innovative platform for refining current animal requirements. With this in mind, thoughtful guidance as to the proper application of HuM models to current drug development paradigms, technical considerations for their use, and discussion on their strengths and weaknesses for human translatability in comparison to traditional animal models is necessary. In particular, there are no consistent approaches to the technical and translational considerations of different HuMs for various disease models or how immune and pathology study endpoints are incorporated or applied for study interpretation or human risk assessment. The goals of this workshop will be to (1) provide an overview of the technical and translational applications of various HuM models currently available, (2) outline key considerations in study design and selection of immunotoxicology and pathology endpoints in safety versus efficacy studies, and (3) describe the practical use and application of HuMs data within industry to inform on drug development. At the beginning of the Workshop, attendees will be introduced to the US FDA "Roadmap to Reducing Animal Testing in Preclinical Safety Studies" and the concept of how HuMs can serve as an innovative platform as a refined *in vivo* method (for transition). Brief points to consider on how to optimize the use, application, and translational considerations of HuMs for reducing animal testing will be introduced prior to the presentations by the speakers. Presentations will provide technical and translational considerations for HuM models and a contextual framework on how and when HuMs should be employed (and for what purposes) that will serve as a foundation for the roundtable discussion. The first speaker will begin the discussion on the technical and translational applications of various HuM models that are currently available, the use of HuMs, and expectations for the qualification of use in drug discovery/validation versus nonclinical safety and efficacy. The second speaker will present study design considerations, such as how to leverage HuM models and immune concepts to answer key scientific questions and the preferred study data endpoints (i.e., immune, pathology) for answering these questions. Reference to designing a study with the "end-in-mind" and the different considerations of how efficacy versus safety studies will be emphasized. The last speaker will discuss how data generated from HuM models are currently incorporated into drug development programs for human risk assessment and opportunities/challenges with HuM models versus traditional large animal models. Following the presentations, there will be a roundtable discussion among presenters and audience members on how to optimize the use of HuMs to refine current animal testing practices while ensuring preclinical safety studies maintain the highest quality of translational relevance.

EC 1178 Navigating Your Scientific Career: The Power of Transforming Networking into Mentoring

C. McGinnis. Center for Toxicology and Environmental Health, Little Rock, AR.

Most of us have had the opportunity to experience a mentoring relationship at least once in our scientific careers, typically in an academic setting or as an early career scientist. What can be more challenging is to find and build a mentoring relationship outside of traditional hierarchies, such as supervisors or collaborators. It is not uncommon to attend networking events where we have opportunities to connect with peers and like-minded people. Perhaps we are connecting with new contacts at Specialty Section or Regional Chapter events. But what happens when there is interest in expanding surface-level networking into a more formalized mentoring relationship? This session will equip attendees with practical tools to build meaningful mentoring relationships by exploring best practices, strategies for setting effective goals, and addressing the current needs of participants. This session explores scenarios and avenues to establish mentoring relationships for all career stages and will start by asking the attendees to reflect on a time they needed mentorship. This starting reflection will be facilitated by a poll where participants submit one-to-two-word examples of a scenario where they needed mentorship (i.e., tenure, career change). The session will proceed with the first speaker who will provide an over-

view of mentoring-related resources available through SOT. The second speaker will explore mentoring at different career stages and share experiences in fostering successful mentoring relationships. The third speaker will explore opportunities to build a mentoring relationship through SOT Special Interest Groups and Regional Chapters. The session will transition to a breakout-group (BOG) style of discussion that will enable audience participation by sharing their experiences in mentoring relationships across career levels. The BOG will be facilitated by members of the SOT Career Advancement, Mentoring, and Networking (CAMAN) Committee; the SOT Graduate Student Leadership Committee (GSLC); and the SOT Postdoctoral Assembly (PDA) of varying career levels to provide some context on how mentoring relationships may vary throughout one's career. Prompting questions and scenarios will be used to encourage attendees to consider best mentoring practice, how to effectively communicate the goals of the mentoring relationship, and how to develop a mutual learning dynamic. Questions will include "how do I identify the right mentors for me," "what works or does not work in maintaining a strong mentoring relationship," "how does mentoring evolve throughout different career stages," and "how do you establish clear objectives of a mentoring relationship?" Scenarios will be used to illustrate ways to initiate and clearly communicate the goals, duration, and responsibilities of the mentor and mentee. Scenarios will be career-dependent to help facilitate the formation of mentorship relationships at all career stages.

S 1179 In Vitro Testing for Skin Sensitization of Mixtures: Where Science Stands Today

S. Farahmand. The Honest Company Inc., Los Angeles, CA.

The assessment of skin sensitization potential in complex mixtures, such as cosmetic products, medical devices, and industrial formulations, presents unique scientific and regulatory challenges. Such an endeavor also provides an avenue for applying the established scientific framework of the adverse outcome pathway (AOP) to enhance risk assessment methodologies for products and devices (mixtures) that we encounter in daily life. Traditional *in vivo* methods and many NAMs were primarily developed for individual chemicals and often require adaptation for effective application to mixtures. This session explores recent advancements in nonanimal approaches tailored to address these challenges, supplemented by detailed case studies demonstrating their practical implementation in risk assessment frameworks. Presentations will showcase NAM approaches that evaluate the key events (KE) in the skin sensitization AOP and methods to derive potency for use in quantitative risk assessment. Reconstructed human epidermis models, such as those enabling surface-area-based dosing and realistic exposure scenarios, are particularly effective for poorly soluble and complex substances. Advances in genomics-based platforms and machine learning models will also be discussed, emphasizing their ability to predict sensitization potency and derive points of departure (PODs) that support quantitative risk assessment without animal or human confirmatory testing. Additionally, the session will address the integration of exposure-based waiving thresholds and dermal absorption models that account for formulation- and skin-specific parameters, further strengthening risk assessment frameworks. Case studies will highlight the application of these methods to finished cosmetic products and medical device extracts, including adaptations that enable both hazard identification (addressing several KE) and potency estimation. The presented methods and their use in risk assessment are the outcome of several years of collaboration between industry, academia, and government agencies. This session highlights the achievements of this endeavor and the practical use by experts in a variety of sectors as a tool used to ensure consumer safety. Attendees will gain (1) a comprehensive understanding of the scientific and regulatory challenges in evaluating skin sensitization of mixtures, with a focus on risk assessment; (2) insight into validated and emerging *in vitro* models suited for complex formulations; (3) practical examples of how NAMs can be applied in product development and regulatory submissions; (4) knowledge of potency prediction methods and how PODs can be derived without animal or human testing; and (5) an overview of how integrated strategies and modeling tools can enhance risk assessment accuracy for mixtures. Collectively, these efforts represent a significant step forward in the development and regulatory acceptance of NAMs for complex mixtures, offering scientifically robust and ethically sound alternatives to traditional testing.

S 1180 Advancements Toward a Mixture-Specific NAMs-Based Framework for Skin Sensitization Risk Assessment

D. Allen. International Collaboration on Cosmetics Safety, Durham, NC.

Existing sensitization testing methods, including traditional *in vivo* approaches as well as new approach methodologies (NAM), have primarily been developed for individual defined chemicals and may not always be fully compatible with the testing of mixtures. Due to differences in their composition and sample characteristics (e.g. form, physical and chemical properties) testing and resulting interpretation can be challenging. Therefore, there is a pressing need to evaluate and amend the existing NAMs-based risk assessment framework so that it considers better the specific characteristics of mixtures. This adapted framework should incorporate exposure-based waiving thresholds, optimized NAM applications, as well as effective integration of data, to enable more accurate and reliable skin sensitization risk assessment of

mixtures. This presentation will discuss ongoing efforts that involve NAM development and evaluation activities, to address these challenges to ensure the safe use of cosmetic products, meeting the growing demand for natural and sustainable options while prioritizing consumer safety that includes such complex mixtures. Attendees will learn about novel *in vitro* method development as well as evaluation and optimization efforts directed towards expanding an existing next generation risk assessment framework to incorporate these types of substances.

S 1181 Application of the EpiSensA for Skin Sensitization Assessment of Mixtures

A. Islas-Robles. Institute for In Vitro Sciences Inc., Gaithersburg, MD.

Keratinocyte activation, identified as Key Event 2 in the skin sensitization Adverse Outcome Pathway, is a critical biological step that can be evaluated using *in vitro* methods to support non-animal hazard identification. The Epidermal Sensitization Assay (EpiSensA, OECD TG 442D) is the first *in vitro* skin sensitization assay validated using three-dimensional reconstructed human epidermis (RhE), providing a biologically relevant model that closely resembles native human skin. This tissue architecture enables surface-area-based dosing and more physiologically realistic exposure scenarios, making it particularly well-suited for the assessment of complex mixtures. The EpiSensA measures gene expression markers associated with keratinocyte activation, delivering a mechanistic and human-relevant endpoint for identifying sensitizers. This presentation will highlight a literature-based analysis that examines the potential to expand the applicability domain of the EpiSensA across diverse chemical spaces, with a particular focus on complex and difficult-to-test mixtures. The EpiSensA was applied to three agrochemical formulations and two surfactant-based mixtures with available *in vivo* data (Mizumachi, 2020). Both surfactant mixtures were accurately classified as sensitizers, and the sensitization potential of the agrochemicals was correctly identified, although one was a false positive. Similarly, a study evaluating various medical device extracts found that EpiSensA successfully detected sensitization potential in devices previously classified as sensitizers through *in vivo* tests (Okamoto, 2024). Notably, positive responses were observed for the non-polar extract, aligning with animal test outcomes. These findings highlight the potential of the RhE-based model to identify skin sensitizers across diverse mixtures, including those with challenging characteristics for *in vitro* testing. Further research is still needed using larger datasets, including the evaluation of other mixture categories such as cosmetics, to better demonstrate the assay's practical utility, performance attributes, and regulatory potential within defined approaches and integrated testing strategies.

S 1182 Use of the SENS-IS Transcriptomic Reconstructed Epidermis Assay in the Skin Sensitization Testing of Mixtures

H. Groux. Immunosearch Labs SARL, Grasse, France. Sponsor: S. Farahmand

The assessment of skin sensitization potential in complex mixtures, such as cosmetic products and medical device extracts, presents distinct scientific and regulatory challenges. While validated *in vitro* methods have significantly advanced for individual substances, their application to mixtures requires careful consideration of formulation effects, irritancy, and potential chemical interactions. The SENS-IS assay, based on a transcriptomic approach using reconstructed human epidermis, offers a robust tool for addressing these complexities. A catch-up study supporting the use of a newly developed in-house 3D epidermis model is currently under evaluation by the OECD, further reinforcing the assay's relevance for regulatory use. Our published work (2019) demonstrated that SENS-IS can identify a variety of mixture-related effects, including vehicle-dependent modulation of sensitization potency, irritant-induced enhancement, additive or synergistic interactions at low concentrations, and positive responses in finished consumer products. Since then, the assay has been applied to a wide range of mixtures, supporting its applicability across different regulatory contexts. This presentation will focus on recent case studies involving cosmetic products, illustrating how formulation components can significantly influence the sensitization profile of active substances. To expand the assay's relevance, we are also evaluating a complementary full-thickness skin model that incorporates a dermal compartment. This system more closely mimics native skin architecture and offers new insights into mixture effects involving deeper skin layers. Together, the epidermal and full-thickness models enhance the utility of the SENS-IS assay in mixture testing, contributing valuable data for risk assessment frameworks. This work underscores the importance of advanced *in vitro* models in the ongoing transition toward non-animal testing strategies in regulatory toxicology.

S 1183 In Vitro Assessment of Complex Mixtures Using a Genomics and Machine Learning-Based Approach

T. Lindberg. SenzaGen AB, Lund, Sweden. Sponsor: S. Farahmand

Recent advances in New Approach Methodologies (NAMs) have led to *in vitro* methods that assess skin sensitization with predictive performance comparable to animal models, offering greater ethical and human relevance. However, testing complex

mixtures and finished formulations remains challenging due to solubility, compositional variability, and interference from non-sensitizing components. Even when technically compatible with validated assays, these substances often require protocol adaptations, and interpreting results can be difficult. GARD (Genomic Allergen Rapid Detection™) technology, combining gene expression profiling with machine learning, is one such NAM. Based on a dendritic cell-like line and included in OECD Test Guideline 442E (Key Event 3), GARDskin has demonstrated potential for evaluating mixtures. This presentation reviews the scientific principles, performance characteristics, and limitations of GARDskin in the context of mixture testing. Data from peer-reviewed studies and industry collaborations highlight its applicability to personal care products, finished formulations, UVCBs (Substances of Unknown or Variable Composition, Complex Reaction Products, or Biological Materials), and hydrophobic mixtures (Log P: 3.5). The GARDskin Dose-Response adaptation will also be discussed, showcasing its ability to predict human NESIL values and UN GHS/CLP subclassifications—key for regulatory risk assessment. Technical considerations, including solvent compatibility with both polar and non-polar substances, are addressed as they are critical for handling hydrophobic ingredients and complex medical device extracts. In summary, GARDskin provides a robust and adaptable *in vitro* solution for assessing skin sensitization hazard and potency in complex mixtures. These advances further support the regulatory relevance of NAMs as scientifically valid alternatives to animal testing.

S 1184 Replacing Human Testing with NAMs: Deriving Points of Departure for Quantitative Risk Assessment of Natural Complex Substances and Mixtures

I. Lee. Research Institute for Fragrance Materials, Woodcliff Lake, NJ.

Quantitative risk assessment (QRA) for dermal sensitization is critical in establishing safe use levels of sensitizing ingredients in consumer products. Traditionally, QRA relies on the No Expected Sensitization Induction Level (NESIL) as the point of departure (PoD), often determined through human testing via the Confirmation of No Induction in Humans (CNIH) repeat patch test. However, with the advancement of New Approach Methodologies (NAMs) and their integration into Next Generation Risk Assessment (NGRA) frameworks, industry is moving toward not only non-animal, but also non-human testing strategies for PoD derivation. Significant progress has been made in applying NAMs to discrete chemicals, but challenges remain in evaluating natural complex substances (NCS) and other mixtures due to their multi-constituent and variable nature. In the fragrance industry, many NCS contain known discrete components, enabling a component-based risk assessment approach. However, this strategy is not universally applicable, prompting exploration of NAM-based methods for direct PoD derivation. This presentation highlights the use of several quantitative potency-based NAMs, which may include Bayesian models like SARA and SARA-ICE, the GARDskin dose-response assay, and OECD *in vitro* regression models, to support risk assessment of NCS and mixtures. Within the NGRA framework, these methods allow for the derivation of a NAM-based PoD, which can be adjusted using uncertainty factors to estimate a predicted NESIL (pNESIL). This value is then used to calculate Acceptable Exposure Levels (AELs) for consumer products such as deodorants and bar soaps, which are compared against estimated Consumer Exposure Levels (CELs) to determine the margin of safety. Case studies will be presented to demonstrate how currently available NAMs can be effectively used in NGRA to assess NCS, offering a scientifically robust alternative to traditional animal and human testing for QRA.

S 1185 NAM In Vitro Assessment of Intestinal Metabolism to Inform PBK Modeling of Bioavailability and Systemic Exposure

J. Wambaugh. US EPA, Research Triangle Park, NC.

Next-Generation chemical risk assessment (NGRA) aims to replace and expand on traditional toxicity testing with new approach methodologies (NAMs). One central aspect in NGRA is the assessment of toxicokinetic properties to estimate the bioavailable concentration of the dosed compounds and/or potentially toxicologically relevant metabolites. This question is also relevant to address interspecies differences or route-to-route extrapolation. The development of NGRA framework needs a joint effort, and several projects are advancing this field like ASPIS (Animal-Free Safety Assessment of Chemicals), the EFSA ADME4NGRA and PARC (Partnership for the Assessment of Risks from Chemicals). This session will highlight recent advances from these projects to fill methodological, as well as knowledge, gaps. A current knowledge gap is generating data on intestinal pre-systemic metabolism and metabolite formation. Advanced models for the study of intestinal metabolism in primary human cells and organoids will be presented along with studies on metabolism by the intestinal microbiome. The kinetics need to be integrated with toxicodynamic evidence to allow the derivation of a point of departure for hazard and risk assessment. The ASPIS project cluster is developing a framework for NGRA, which is termed ASPA (Animal-Free Safety Algorithm).

S 1186 Advanced *In Vitro* Models for the Assessment of Intestinal Metabolism

P. Lundquist. Uppsala Universitet, Uppsala, Sweden. Sponsor: B. Islam

Newly developed *in vitro* NAM models for intestinal metabolism and permeability, such as primary human enterocytes and 3D-cultured human adult stem cell derived intestinal organoids will be presented and discussed in the context of absorption of orally ingested xenobiotics. The models show pronounced activity of phase I and II metabolizing enzymes, including CYP450:s, UGT:s and SULT:s. Metabolism measured in these system have been modeled by PBK yielding human exposure estimates in good agreement with human studies. Additionally, activity ADME relevant transporters could be demonstrated. Global proteomics are used for in depth assessment of protein expression in the *in vitro* models enabling comparison to *in vivo* human intestinal physiology and expression of ADME proteins. Isolated human enterocytes and 3D cultured intestinal organoids from the jejunum and colon were found to show proteins expression levels in good agreement with the human tissue of origin, in contrast to cell line derived models. These novel intestinal *in vitro* models show promise to generate human relevant data on intestinal metabolism of toxicants, drugs, and xenobiotics.

S 1187 Rat and Human Intestinal Organoid Models to Assess Species Differences in Intestinal Absorption of Chemicals

J. Wang. Wageningen University and Research, Wageningen, Netherlands.

Traditionally, rats are used in toxicokinetics and toxicology studies. However, species differences in the processes driving the absorption, distribution, metabolism and excretion (ADME). For example, there are marked differences in transporter and biotransformation enzymes in enterocytes that affect a chemical's bioavailability. Human *in vitro* cell models have been well established as a means to estimate gut permeability. However, a comparative rat cell model is currently lacking. This presentation we present a rat jejunum organoid-derived model. We evaluate human and rat jejunum organoid-derived monolayer models in comparison with the conventional Caco-2 cell monolayer model for studying xenobiotic metabolism and transport. Key focus areas include profiling the expression of metabolic and transporter genes, highlighting both species-specific differences between humans and rats, and model-specific differences between organoid-derived and Caco-2 cell-derived monolayers. Tacrolimus, a clinically relevant CYP3A5 and P-glycoprotein (P-gp) substrate, will be used as a case study to illustrate the impact of intestinal pre-systemic metabolism and transporter expression on overall toxicokinetic. The resulting data will be integrated into a physiologically based kinetic (PBK) model of tacrolimus to support quantitative *in vitro*-to-*in vivo* extrapolation (QIVIVE) for nephrotoxicity.

S 1188 Advances in Assessing Chemical Biotransformation by the Gut Microbiome and Its Incorporation into Physiologically Based Kinetic Models

G. Aichinger. ETH Zurich, Zurich, Switzerland. Sponsor: B. Wetmore

The gut microbiome is emerging as a key contributor to chemical metabolism. We optimized and harmonized strategies to assess chemical biotransformation kinetics in anaerobic fecal fermentations and to include respective parameters for facilitating microbiome-competent PBK modeling. The application of this methodology for predicting pharmaco-/toxicokinetics of selected chemicals revealed a highly individual impact of gut microbiota on chemical bioavailability and facilitated quantitative extrapolation from *in vitro* toxicity characterization of microbial metabolites to their *in vivo* bioactivity.

S 1189 Evaluating Contribution of Intestinal Toxicokinetic Features on Bioavailability Estimation

B. Wetmore. US EPA, Research Triangle Park, NC.

Bioavailability is an important factor in physiologically-based toxicokinetic (PBTK) models estimating internal dose following oral exposure. In higher-throughput models designed for new approach methodology (NAM) application, a minimum set of TK inputs may be considered, with other parameters set to conservative assumptions (for instance, no consideration of extrahepatic metabolism). Strategic identification of key modulators of intestinal bioavailability may assist by either identifying priorities in selecting promising NAM models or in identifying key model inputs that could be leveraged for more immediate application in generic PBTK models. This presentation will summarize findings from an experimental 11-chemical case study that employed *in vitro*-*in vivo* extrapolation (IVIVE) - PBTK modeling and a meta-analysis designed to evaluate the contribution cytochrome P450 (CYP) 3A4, the most abundant intestinal CYP isozyme, along with the role of other intestinal enzymes, transporters, and physicochemical properties in modulating bioavailability. Findings show that incorporating CYP3A4 metabolic contribution to estimate

intestinal metabolism aligns with *in vivo* derived oral bioavailability estimates and far outweighs the contribution of intestinal absorption or other metabolic or disposition pathways. These findings should prove informative as development of TK NAM experimental and computational modeling approaches evolve. *This abstract does not necessarily reflect the views of the US EPA.*

S 1190 Integration of *In Vitro* ADME Parameters and Biokinetics Data in PBPK Modeling and Chemical Risk Assessment

B. Islam. Certara UK Limited, Sheffield, United Kingdom.

One of the steps in chemical risk assessment is converting toxic concentrations into an external dose using physiologically-based kinetic (PBK) modelling. Developing a PBK model requires information on the absorption, distribution, metabolism and excretion (ADME) properties of the compound, which can be obtained through various *in silico* and *in vitro* methods. Within the European Union Horizon2020 project Riskhunt3R, state-of-the-art methods have been used to characterize ADME parameters of the compounds and incorporate them in a tiered assessment framework. These include intestinal absorption and metabolism assessment. The influence of these parameters on PBK modelling and their implications for chemical risk assessment were systematically evaluated. In addition, biokinetic modelling of the compounds in *in vitro* systems was performed to account for losses due to binding to plastic surfaces, as well as partitioning into air, cells, and media components. Integrating data from *in vitro* assay-derived points of departure, *in vitro* biokinetics, and PBK modelling represents a critical component of *in vitro* to *in vivo* extrapolation and supports more accurate and mechanistic chemical risk assessment.

S 1191 New Frontiers in Reproductive Toxicology: Emerging Contaminants and Novel Mechanisms of Toxicity

A. Veiga-Lopez. University of Illinois at Chicago, Chicago, IL.

Pregnancy represents a uniquely sensitive window in which environmental exposures can exert lasting effects on maternal and fetal health. While certain environmental contaminants are well-studied, many contaminants of emerging concern—such as novel industrial compounds, dietary toxicants, microplastics, and next-generation PFAS—remain data-poor despite widespread human exposure. These emerging contaminants may not exhibit overt toxicity at high doses but can interfere with the development of critical biological systems, leading to adverse reproductive and developmental outcomes. This Symposium brings together a multidisciplinary panel of scientists representing a diversity of career stages and expertise, including exposure science, reproductive toxicology, and molecular epidemiology, to highlight cutting-edge research on emerging contaminants and novel mechanisms of reproductive toxicity in the context of pregnancy. Studies presented will utilize multiple approaches, including longitudinal pregnancy cohorts, *in vivo* animal models, and mechanistic *in vitro* systems, providing translational insight across biological levels and exposure contexts. The session will dive into the methods used to prioritize and identify emerging contaminants, focusing on the innovative tools and screening platforms that are reshaping how we understand these risks. The session will explore how emerging contaminants are identified and prioritized by federal agencies, with a focus on the innovative tools and screening platforms reshaping our understanding of environmental risks. Chemical profiling techniques and high-throughput screening will be highlighted, demonstrating how advanced *in vitro* systems are being used to assess bioactivity and toxicity in new and emerging contaminants. Dietary mycotoxins will also be a focal point, with research detailing their impact on placental barrier integrity and fetal development, integrating both cohort data and mechanistic insights. Next, the session will examine the reproductive risks posed by next-generation PFAS, exploring challenges such as endpoint selection, mixture toxicity, and the long-term effects on fertility and pregnancy outcomes, with an emphasis on sex-specific effects. The growing concern around microplastics will be addressed through their effects on placental function and transport, revealing how exposure to microplastics may alter fetal health outcomes. Finally, research will delve into new mechanisms of toxicity for legacy chemicals including PBDEs, UV stabilizers, and dioxins, exploring how they interfere with key placental signaling pathways, such as the epidermal growth factor, and challenge long-standing assumptions about their relevance to maternal and developmental health. A 20-minute panel discussion will conclude the session, providing a platform for cross-disciplinary exchange on research priorities, regulatory needs, and strategies for protecting maternal-child health in the face of a changing chemical landscape.

S 1192 New Frontiers, Hidden Threats: Introducing Emerging Contaminants in Reproductive Health

A. Paquette. Seattle Children's Research Institute, Seattle, WA.

The session will begin with a brief introduction of how emerging contaminants are identified and prioritized by federal agencies.

S 1193 Leveraging In Vivo, In Vitro, and In Silico Approaches to Accelerate the Discovery of Novel Reproductive Toxicants

P. Allard. University of California Los Angeles, Los Angeles, CA.

The identification of environmental chemicals that impair reproduction is complex due to the intricacies of the reproductive process which spans multiple organ systems and developmental/life stages. Traditional testing methods are not scalable to screen the approximately 350,000 chemicals in use globally, highlighting the need to address the significant public health issue of infertility which affects about 1 in 6 people worldwide. We will describe an integrated strategy combining *in vitro* and *in silico* methods used to assess the reproductive toxicity of 133 physiologically-relevant chemicals. Following a primary high-throughput screen leveraging the tractability of *C. elegans*, we validated the hits by a secondary reproductive assay also in *C. elegans* focused on embryonic viability. In total, 13 chemicals were classified as reproductive toxicants with the top hits being Quaternary Ammonium Compounds (QACs), commonly used as disinfectants but with limited reproductive toxicity data. Further mining of the ToxCast data revealed no significant differences in the bioactivity or in the average potency (AC50) between the top and bottom chemicals. However, chemicals with greater biological activity (higher Z-scores) showed an over-representation of steroid hormone targets, suggesting potential reproductive toxicity mechanisms. The presentation will conclude by emphasizing the need to prioritize the assessment of QACs' impacts on reproduction.

S 1194 Emerging PFAS: Ultra-Short Chain and Polyether Carboxylate Compounds Display Similar Maternal, Fetal, and Neonatal Effects

J. Robinson. University of California San Francisco, San Francisco, CA.

Perfluorooctanoic acid (PFOA), a legacy per- and polyfluoroalkyl substance (PFAS), remains widespread in humans, including pregnant women, despite efforts to phase out its use. The placenta is a suspected target of PFAS toxicity; however, the pathways by which PFAS disrupt placental development and function remain poorly defined. Placental cytotrophoblasts (CTBs) are essential for implantation, uterine invasion, and vascular remodeling, and their dysfunction underlies common pregnancy complications. In culture, primary human villous CTBs differentiate into an invasive phenotype modeling the CTB-to-extravillous trophoblast (EVT) transition. Primary human CTBs were isolated from second-trimester placentas and exposed to sub-cytotoxic concentrations of PFOA (0.1-25µM) for 48h. Proteomic profiling using SWATH-mass spectrometry detected 3,240 proteins, of which 274 were differentially expressed following PFOA exposure. Pathway enrichment analyses revealed overrepresentation of proteins involved in immune defense, lipid metabolism, and amino acid biosynthesis. Integration of proteomic and transcriptomic datasets identified regulatory nodes linking PFOA exposure to altered immune signaling and placental functional pathways. Molecular docking and molecular dynamics simulations predicted direct interactions between PFOA and structurally related PFAS with peroxisome proliferator-activated receptors (PPARA/G) and immune transcriptional regulators (STAT and IRF family members). Benchmark concentration modeling indicated that proteomic alterations occur at concentrations overlapping reported human serum levels and those associated with placental toxicity in animal studies. These findings provide systems-level mechanistic evidence that PFOA perturbs placental metabolic and immune networks through interactions with key transcriptional regulators. Targeting of shared regulatory pathways by PFOA and select PFAS may represent a mechanistic basis for PFAS-induced placental dysfunction.

S 1195 From Popcorn to Placenta: How Mycoestrogens Impact Development

L. Aleksunes. Rutgers, The State University of New Jersey, Piscataway, NJ.

As global temperatures rise and humidity increases, concerns about hidden toxins from fungal contaminants in our diet are also growing. Warm, humid conditions promote the overgrowth of *Fusarium* fungus and the release of mycotoxins, including zearalenone, into a wide range of foodstuffs, including baby formula, grains, cereals, popcorn, pasta, and oatmeal. Structurally similar to 17β-estradiol, zearalenone and its metabolites interact with estrogen receptors and are classified as a 'mycoestrogen.' Mycoestrogens enter and cross the rodent and human placenta, although a fraction undergoes active efflux back to the maternal circulation by the BCRP/ABCG2 transporter. This presentation will cover the latest research regarding how mycoestrogens adversely impact pregnancies and offspring development by targeting the placenta. We have identified the Q141K variant in ABCG2 as an

important determinant of how mycoestrogens reduce fetoplacental ratios and birth weight in healthy pregnancies. Moreover, we will discuss how mycoestrogens disrupt placental steroidogenesis in a sex dependent manner. Mechanistic studies suggest that zearalenone and other mycotoxins alter trophoblast migration and other pathways, yielding novel clues to their potential impact on pregnancy complications such as preeclampsia. As the field advances, it will be essential to identify factors that diminish BCRP-mediated protection to safeguard vulnerable populations at higher risk of perinatal mycoestrogen toxicity.

S 1196 Micro- and Nanoplastic Exposures During Pregnancy: Effects on the Placenta

P. Stapleton. Rutgers, The State University of New Jersey, Piscataway, NJ.

Micro- and nanoplastic particles (MNP) have been identified as a novel and emerging contaminant. Their deposition in human placental tissue has been reliably detected, with temporal increase in the last 20 years. This causes concern for the influence that MNP exposure may have on placental development including trophoblast invasion of the spiral arteries, maternal/fetal surface area, and intraheam distance, ultimately influencing fetal health. Our laboratory has established a whole-body rodent inhalation model, using polyamide (e.g., nylon) MNP to assess exposure throughout pregnancy. Using this model, we have identified that MNP particles migrate from the mother's lung to the placenta, breach the placental barrier, deposit within fetal tissues, and culminate in reduced fetal growth. Morphometric analyses revealed impaired placental development and maturation at gestational day 12, 16, and 20, after exposure to MNP aerosols during pregnancy. These outcomes are evidenced by poor trophoblast invasion and reduced maternal/fetal surface areas limiting nutrient delivery and waste exchange. In this session, we will discuss the placenta as a target organ of concern for maternal MNP exposure. Particle deposition elicits physiologically relevant responses, which directly affect fetal health and the developmental onset of adult disease.

S 1197 The Epidermal Growth Factor Receptor Serves as a Cell Surface Sensor for Environmental Chemicals: Relevance for Reproductive Toxicity?

T. Haarmann-Stemann. IUF—Leibniz Research Institute for Environmental Medicine, Düsseldorf, Germany.

The epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase of utmost importance for proper embryonic development and tissue integrity. Humans born with a loss-of-function mutation in the EGFR gene have shortened life expectancy, and EGFR-deficient mice die early after birth due to epithelial immaturity and multiorgan failure. EGFR is activated upon binding of growth factors to its extracellular domain (ECD), resulting in receptor dimerization and activation of kinase activity, which ultimately regulates cellular processes, such as proliferation, differentiation and migration. We and others have shown that several environmental contaminants, including dibenzo-p-dioxins, polychlorinated biphenyls, phenolic benzotriazoles and others, can interfere with the growth factor-triggered activation of EGFR in human cells. Using *in silico* docking analyses, receptor internalization assays, immunoblotting and mutational amino acid exchange experiments, we have observed evidence of direct binding of these chemicals to the EGFR ECD, leading to displacement of bound growth factors and an inhibition of DNA synthesis and other downstream events. Since EGFR and its ligand EGF play a critical role in regulating placental and fetal growth, and epidemiologic studies suggest that exposure to EGFR-interacting chemicals correlate with changes in birth weight or gestational age at delivery, further in-depth investigation of this novel mode of action could help improve risk assessment.

S 1198 Take a Deep Breath ... Hold It! Rising Cardiopulmonary Toxicity and Regulatory Concerns of Nontobacco-Derived Nicotine and CBD Vaping Products

A. Noël. Louisiana State University, Baton Rouge, LA.

The recent emergence of new alternative nontobacco-derived vaping products, including synthetic nicotine, nicotine analogs, and cannabidiol (CBD), raises concerns as their use becomes more widespread, the health risks are largely unknown, and regulations of these substances being either nonexistent or evolving. In the United States, in 2023, more than 17 million adults used vaping products, which is in addition to youth vaping which represents ~1.6 million individuals. Further, within the adolescent population, among current e-cigarette users, 21.3% have previously vaped CBD. In the US, the vaping products market was valued at \$11.2 billion, and in 2024, the estimated market shares for freebase nicotine were 77%, nicotine salt 10%, synthetic nicotine 2.2%, and CBD/hemp 0.04%. While tobacco-derived nicotine vaping may present fewer health risks than smoking, vaping is not without its own potential harms, particularly with prolonged use and with regard to the bioactive molecule (nicotine, synthetic/analog nicotine, CBD) present in the e-liquid. E-cigarette aerosol

toxicity is driven by several factors primarily linked to the chemicals found in the aerosols, and these factors could potentially be subjected to regulation. These key factors include (1) nicotine forms (free base, salt, synthetic, analog) or CBD and their concentrations in the e-liquids, which affects addiction and cardiopulmonary toxicity—nicotine analogs, which are distinct, but have a chemically similar structure to nicotine, may lead to different health risks and potential adverse effects are understudied; (2) flavoring chemicals, including vanillin and benzaldehyde, sweeteners, and solvents, which can cause irritation, oxidative stress, and enhance the cardiopulmonary toxicity of the other components; and (3) heavy metals (e.g., chromium, lead, nickel, iron and copper), which can leach from the heating coils and metallic parts of the devices to the e-liquid and then transfer to the e-cigarette aerosol, which is inhaled into the lungs. These metals can cause both carcinogenic and noncarcinogenic effects. Overall, vaping toxicity is influenced by a combination of factors and chemicals with oxidative potential and can directly damage the cardiopulmonary system through mechanisms involving cytotoxicity, inflammation, and oxidative stress. The regulation of vaping products depends on the substance being vaped and the country or state. In the US, the US FDA regulates nicotine vaping products, including e-cigarettes, as tobacco products, whereas nicotine analogs are not under the purview of the US FDA. This represents a loophole for which no premarket authorization (PMTA) is required. While in the US, there is no limit on nicotine concentration for vaping products, Canada and the UK established a nicotine concentration limit of 20 mg/mL. This diverse landscape of regulation and the already-documented health effects underscore the importance of further research and regulation in the vaping and cannabis space. This Symposium will discuss how specific components of either the e-liquid or the e-cigarette aerosol (e.g., nicotine form, nicotine analogs, metals, CBD) impact cardiopulmonary toxicity and elaborate on why each of these factors is crucial when establishing a regulatory framework for vaping products. The first speaker will discuss pulmonary effects of nicotine chemical forms, analogs and synthetics, in comparison to known benchmarks associated with the impact of freebase nicotine on respiratory health using an *in vivo* model. The second speaker will present on the development of biomarkers of both exposure and cardiovascular harm of new vaping products containing nicotine analogs (6-methyl nicotine). This will be followed by a presentation on the impacts of 6-methyl nicotine, sweeteners and solvents on *in vitro* and *in vivo* toxicity, with a focus on margin of exposures and safety thresholds. The fourth speaker will discuss the presence of metals in e-cigarette aerosols and their blood levels found in e-cigarette users as well as their potential health effects. The last speaker will talk about another bioactive vaping molecule of concern, CBD, with regard to the pulmonary effects in the context of regulatory screening work. Overall, the toxicological research needs for new alternative nontobacco-derived nicotine vaping products requires a consolidated effort regarding standardization of testing methodologies and reporting of outcomes to inform the development of regulatory frameworks to protect public health. This engaging Symposium on a timely matter will highlight research on the cardiopulmonary health impacts of nontobacco-derived nicotine vaping products to help better understand the short- and long-term health effects of those emerging products and initiate a conversation on how these data could be incorporated into a regulatory framework.

S 1199 The Source of Nicotine Matters: Influence of the Source of Nicotine, Tobacco-Derived Versus Nontobacco-Derived, in Electronic Nicotine Delivery System Aerosols on Pulmonary Responses in Mice

A. Noël. Louisiana State University, Baton Rouge, LA.

Nicotine can be found in several chemical forms, including: unprotonated (free base) present in cigarettes and in first- to third-generation electronic nicotine delivery systems (ENDS); protonated (nicotine salt) found in fourth-generation ENDS; and non-tobacco derived nicotine (synthetic), racemic mixtures of S- and R-nicotine enantiomers or up to 99% 'purified' S-nicotine. Despite their identical physicochemical characteristics, each nicotine enantiomer has distinct toxicokinetic and toxicodynamic profiles, which may result in different biological responses. Thus, the advent of new ENDS products using both S- and R-nicotine raise serious public health concerns, given that little is known about the pulmonary health effects and toxicity of R-nicotine or of the racemic mixture in humans when present in ENDS aerosols alongside heated propylene glycol, glycerin, and flavoring chemicals. We used a well-characterized mouse model of inhalation exposures to establish the difference in pulmonary toxicity between ENDS aerosol containing either tobacco-derived free base nicotine or non-tobacco-derived synthetic nicotine. In male mice, following 4 weeks of exposure, lung function testing revealed a significant decrease in the minute volume of mice from both groups exposed to nicotine-containing ENDS aerosols compared to air controls suggesting that inhaling nicotine-containing ENDS aerosols alter lung function. At the molecular level, the ENDS aerosol containing free base nicotine dysregulated the expression of genes associated with biotransformation and oxidative stress responses, whereas synthetic nicotine only down-regulated the expression of Nqo1, although to a greater extent than free base nicotine. These results suggest that nicotine from both sources can influence the antioxidant response element signaling pathway, with synthetic nicotine inducing a stronger suppression. While we showed that the inhalation of ENDS aerosols containing either tobacco-derived or non-tobacco derived nicotine induced similar pulmonary responses, in terms of effects on lung function and inflammation, unique respiratory impacts of ENDS aerosols containing synthetic nicotine compared to those contain-

ing tobacco-derived nicotine were noted at the molecular level. Thus, the source of nicotine, in addition to its chemical form, are critical factors impacting molecular lung toxicity.

S 1200 Tracking Biomarkers of Exposure and Biomarkers of Cardiovascular Harm of a Novel Non-nicotine Analog

D. Conklin. University of Louisville, Louisville, KY.

A new synthetic nicotine analog, (S)-6-methylnicotine (2-methyl-5-[(2S)-1-methylpyrrolidin-2-yl]pyridine; 6MN; aka Metatine), is present in an Electronic Nicotine Delivery System (ENDS) called SPREE BAR with the intent to circumvent FDA regulatory authority on nicotine containing products. However, neither 6MN (Metatine) metabolism nor its toxicity has been published. We sought to develop biomarkers of exposure and biomarkers of cardiovascular harm to track both exposure to and potential injury of 6MN exposure. For this, mice were injected with Metatine or inhaled Spree Bar or PG:VG with 6MN, and urine collected over 24h. Two human E-cig users also vaped Spree Bar Blue Razz Ice and urine was collected 2 h post-exposure. Our method identified: 6-methylnicotine and eight metabolites: 6-methylnor-nicotine; 6-methylnorcotinine; 6-methylcotinine; 6-methylnicotine-1'-N-oxide; 6-methyl-3'-hydroxycotinine; 6-methylcotinine-N-oxide; 6-methylnicotine-N-glucuronide; and 6-methylcotinine-N-glucuronide. To evaluate the metabolic fate of MNIC following inhalation exposure, we measured urinary levels of MNIC-derived metabolites in mice exposed to aerosols either of PG:VG+6MN or of Spree Bar with 6MN and normalized LC-MS responses to creatinine for each metabolite. All nine metabolites were detected at elevated levels shortly after exposure (0-3h), with several remaining elevated at the later time point (3-21h). At the early time point, Spree Bar exposure resulted in nearly twice the metabolite levels observed in the PG:VG+6MN group, reaching concentrations comparable to those seen following the high dose injection. These results show that systemic exposure to 6MN via either injection or inhalation led to indistinguishable metabolism of 6MN. This supports that 6MN urinary metabolites are likely highly robust and useful biomarkers of real-world exposures to 6MN regardless of product consumed (e.g., oral or inhaled). Direct exposure to Spree Bar e-liquid significantly impaired aortic contraction and relaxation – an effect not replicated by 6MN, S-nicotine, not PG:VG suggesting another component of Spree Bar is highly toxic. Overall, we provide an analytical method useful for detecting biomarkers of 6MN exposure, and evidence that 6MN is toxic at high levels potentially achievable in users of SPREE BAR, which is currently an unregulated non-nicotine product.

S 1201 Behavioral and Toxicological Effects and Health Risk Assessment of 6-Methyl Nicotine, an Emerging Synthetic Nicotine Analog in E-cigarettes and Oral Pouches

S. Jabba. Duke University, Durham, NC. Sponsor: A. Noël

To evade federal and state regulations, manufacturers of electronic cigarettes and oral nicotine pouches recently introduced products in which nicotine is replaced by a chemical analogue, 6-methyl nicotine. Previous preclinical studies demonstrated that 6-methyl nicotine is more toxic than nicotine and binds to nicotinic receptors with higher potency. However, although 6-methyl nicotine products are freely available on the US market for consumption, the chemosensory and toxicological properties of 6-methyl nicotine remain unknown. Data will be presented from mouse behavioral experiments comparing the chemosensory aversive effects of 6-methyl nicotine and nicotine. These studies revealed that 6-methyl nicotine is more potent than nicotine in producing aversive behaviors, with younger adolescent mice showing heightened sensitivity. Choice drinking assays in mice revealed that sweeteners and the electronic cigarette solvent, glycerol, augmented preference and consumption of 6-methyl nicotine, more robustly than for nicotine. These effects were absent in mice deficient in the sweet taste receptor (Tas1r2^{-/-}). Toxicological and metabolic assays in oral mucosal and human airway cells and lung organoids demonstrated that 6-methyl nicotine is more toxic than nicotine. The impact of other product constituents, including flavors, sweeteners and solvent chemicals, on 6-methyl nicotine toxicity was also investigated. Resulting data were used to assess the acute human health risks from use of 6-methyl nicotine containing products, such as potential for convulsions and increase in heart rate. Exposure levels for 6-methyl nicotine from commercial product were several orders higher than the human acute reference dose (ARfD) accepted for heart rate increase and also significantly higher than the calculated safety threshold levels for producing convulsions. Together, 6-methyl nicotine has robust chemosensory effects that corroborate its higher potency compared to nicotine. Other product constituent such as sweeteners facilitate 6-methyl nicotine intake. Consumers using these products are exposed to 6MN levels that may exceed safety thresholds, potentially leading to toxicity and adverse health effects. Nicotine analogues should be urgently addressed by lawmakers and regulators, and FDA should be granted authority to regulate products containing them as tobacco products.

S 1202 Blood Metal Levels Differ by Electronic Cigarette Use Behaviors and Device Characteristics: Findings from the EMIT Study

A. Rule. Johns Hopkins Bloomberg School of Public Health, Baltimore, MD. Sponsor: A. Noël

Introduction: Electronic cigarettes (e-cigs) rapidly evolved from “MOD” devices mostly used by smokers as a risk reduction strategy to “POD” devices with higher nicotine content and marketed to young non-smokers. Metals, such as nickel and chromium, found in e-cig components, have been found in e-cig aerosols. We hypothesized that differences between device type and user behaviors impact metal exposure. **Goal:** Compare metal levels in blood of e-cig users and non-users and by e-cig use behaviors and device type. **Methods:** From 63 participants (18 MOD, 26 POD, and 31 non-users) we collected e-cig aerosol, blood, sociodemographic data, use behaviors, device characteristics. Blood and aerosol metals were analyzed by inductively coupled plasma - mass spectrometry. Chi-squared, ANOVA, and regression analyses were used to assess relationships between variables and groups. **Results:** Chromium (Cr) was higher in POD and MOD users compared to non-users ($p=0.016$). Nickel (Ni) was higher in MOD users compared to other groups ($p=0.003$). Zn was higher in PODs compared to MODs ($p=0.007$). Nicotine percentage was positively associated with Pb ($p=0.011$), and Zn ($p=0.028$). The amount of e-liquid consumed per week was positively associated with Ni (0.007). Using mint/menthol e-juice over fruit/candy flavors was positively associated with blood Zn ($p=0.028$). Earlier time to first vape in the morning was positively associated with Ni ($p=0.01$). The positive interaction between amount of e-juice per week and nicotine percentage was significant for Cd ($p=0.048$), Cr ($p=0.037$), and Sb ($p<0.001$). **Conclusions:** E-cig heating components, differences in power between devices, and different chemistry of flavorings within the e-juice help explain differences in metal exposures. Our results show that device type and behaviors determine exposure to metals and could indicate differential health risks for e-cig users given what we know about chronic, low-dose metal inhalation. This information can help inform potential interventions and public health strategies.

S 1203 Pulmonary Effects of CBD Vaping Products

M. Doyle-Eisele. Lovelace Biomedical, Albuquerque, NM.

Cannabidiol (CBD), a phytocannabinoid of increasing interest for its purported therapeutic effects, is primarily consumed via ingestion and inhalation. While the toxicology of orally administered CBD has been reported, little is known about the effects of CBD inhalation. Doses selected for these analyses allowed for evaluation of dose-response at concentrations >100-fold higher than typical human consumption levels. Rodent and canine inhalation studies, nose-only or face mask, resulted in dose-dependent inflammatory and necrotic responses in the nose. There were no histological findings in the extrapulmonary organs. Exposure-related clinical observations were related to respiratory distress and labored breathing that was recoverable in the large animal studies. Improved formulations, dry powders vs. aqueous solutions, have increased bioavailability of various forms of CBD opening new challenges within the drug development pathway. Complications associated with targeted delivery systems has created additional hurdles within the CBD market due to the physiochemical properties and formulations needed for the various routes of administration.

S 1204 Visionary Foresight: Pioneering Cell-Based Therapies

E. Budzynski. Akkeri, Deforest, WI.

Cell-Based therapies are rapidly transforming the treatment landscape for diseases with high, unmet medical needs, particularly within the realms of ocular disorders and neurodegenerative conditions. This Symposium will explore the nonclinical development, regulatory considerations, and translational challenges associated with bringing advanced cell therapies from bench to bedside. Presentations will highlight the strategic and scientific complexities of developing both autologous and allogeneic products, derived from embryonic stem cells or inducible stem cells. The session will emphasize regulatory expectations for toxicologic evaluations from first-in-human (FIH) to biologics license applications (BLA). Case studies spanning therapies for Parkinson's disease, ocular surface diseases, and geographic atrophy due to age-related macular degeneration (AMD) will provide real-world examples of how toxicology, biodistribution, tumorigenicity, and product characterization shape clinical readiness. The role of contract research organizations (CROs) in navigating preclinical development, as well as collaborative innovations across academia, industry, and regulatory sectors, will be discussed. By focusing on translational strategies and safety evaluations in relevant animal models, this session will equip attendees with a comprehensive view of the current landscape and future directions for cell therapy development in ophthalmology and beyond.

S 1205 Overview of Regulatory Requirements for Ocular and Neurological Cell Therapeutic Developments in Support of FIH and BLA

J. White. Blue Rock, Cambridge, MA. Sponsor: E. Budzynski

Cell therapy is an ever-changing field with innovative approaches that can lead to groundbreaking therapeutics to treat diseases with unmet medical needs. Owing to its rapidly evolving nature, there may be many program-to-program differences that can lead to numerous regulatory questions and ultimately challenges during the drug review process. This session will include an overview of the toxicology requirements for regulatory submissions from investigational new drug (IND) applications to biologics license application (BLA) submissions for stem cell therapy. This presentation will showcase a nonclinical case study highlighting the successful development trajectory of a therapeutic cell therapy product. Using an allogeneic stem cell therapy for the treatment of Parkinson's disease as a case example, we will explore its preclinical efficacy and safety data within the broader context of drug development.

S 1206 Enabling Breakthroughs in Ocular Disease: The Strategic Role of CROs in Cell Therapy Development

S. Kumar. Pharmaron, Carlsbad, CA. Sponsor: E. Budzynski

Cell-based therapies hold transformative potential for treating ocular diseases, offering innovative approaches for conditions that currently lack effective treatments. Their successful development, however, requires comprehensive and rigorous preclinical evaluation to ensure safety, efficacy, and alignment with regulatory expectations. Contract Research Organizations (CROs) play a pivotal role in supporting pharmaceutical and biotechnology sponsors throughout the nonclinical development pipeline. This presentation will highlight the strategic contributions of CROs in designing and executing ocular cell therapy studies that support safety and efficacy assessments—from early-stage planning through Investigational New Drug (IND)-enabling studies. Key focus areas include selecting appropriate and translational animal models, thorough characterization of the cell therapy product, precise surgical delivery techniques tailored for ocular administration, and advanced *in vivo* imaging modalities to monitor biodistribution, therapeutic effects and safety. The importance of adhering to Good Laboratory Practice (GLP) standards will also be discussed, as these are critical for ensuring data integrity, meeting regulatory requirements, and facilitating a smooth transition to first-in-human clinical trials.

S 1207 Toxicology Strategies and Challenges for Developing Subretinal Cell Therapeutics for Geographic Atrophy Treatment

R. Skalter. Cell Cure Neuroscience Ltd., Jerusalem, Israel. Sponsor: E. Budzynski

New treatment approaches are needed to address the retinal damage and vision loss associated with geographic atrophy (GA) secondary to dry age-related macular degeneration (AMD). Development of novel approaches, such as cell therapy, presents both advantages and challenges due to the unique aspects of ocular anatomy and physiology. OpRegen (RG6501), a clinical-stage allogenic pluripotent stem cell line-derived retinal pigment epithelium (RPE) cell therapy, is one example of such an approach. While a single subretinal administration of an RPE cell suspension offers the potential to reverse retinal damage and restore vision in patients with GA, toxicological strategies require chronic studies in relevant animal species to enable clinical transition. A case study of the completed comprehensive safety and toxicology evaluations to enable the first in-human study of OpRegen will be discussed.

S 1208 Developing an Autologous Cell Therapy for Age-Related Macular Degeneration

K. Bharti. National Eye Institute, Bethesda, MD. Sponsor: E. Budzynski

This presentation will provide an overview of a cell replacement autologous patch therapy for treating GA patients currently in clinical development testing. Patient induced pluripotent stem cells (iPSC) are differentiated into pure RPE cells and matured on a biodegradable polylactic co-glycolic acid (PLGA) scaffold. The presentation will cover the nonclinical safety evaluation strategy of the iPSC-RPE patch in a pig model of laser induced RPE ablation to support FIH studies. The Phase I/IIa trial for this work is currently ongoing at the National Eye Institute at NIH to test the safety and feasibility of an autologous iPSC-RPE patch in AMD patients with GA.

S 1209 Bridging Concept to Clinic: Preclinical Considerations for Stem Cell-Based Ocular Surface Repair

T. Ilmarinen. StemSight, Tampere, Finland. Sponsor: E. Budzynski

This session will focus on the nonclinical development of an allogenic iPSC-derived limbal stem cell therapy designed to treat severe ocular surface disorders, such as limbal stem cell deficiency or persistent epithelial defects. The preclinical toxicology strategy and general considerations to support FIH studies will be discussed. Key aspects addressed will include nonclinical strategy to support FIH, including demonstration of proof of concept, biodistribution, and tumorigenic and toxicologic potential in both rodent and large animal models. A key translational challenge was optimizing delivery to maintain cell viability and ensure engraftment across species. The program employed iterative testing *in vitro*, *ex vivo*, and *in vivo* systems, enabling refinement of the delivery method, dosing strategy, and potency assay selection. Iterative testing across *in vitro*, *ex vivo*, and *in vivo* platforms provided critical insights into product performance and safety, which in turn informed the design of FIH clinical trials and guided regulatory strategy.

W 1210 Cardiovascular Toxicity as a Globally Harmonized System Hazard Trait: The Evidence and the Path Forward

I. Rusyn. Texas A&M University, College Station, TX.

While cardiovascular disease is the leading cause of death worldwide and adverse cardiac effects are a top reason for the attrition of pharmaceuticals, the potential cardiovascular risks from environmental pollutants are not yet recognized as a stand-alone hazard trait. There is growing evidence that environmental chemicals pose a significant and often underrecognized threat to cardiovascular health. Both human and animal studies show that many industrial and environmental chemicals, such as heavy metals, solvents, and endocrine disruptors, can adversely affect the heart and blood vessels. These substances may induce oxidative stress, inflammation, and endothelial dysfunction, potentially leading to hypertension, arrhythmias, and heart failure. Despite these risks, current chemical safety assessments often overlook cardiovascular endpoints. This session will first review the evidence used to support the evaluation of the state-of-the-science for recognizing the cardiotoxicity of chemical substances under the OECD Working Party on Hazard Assessment. Next, key characteristics of cardiovascular toxicants and new approach methodologies (NAMs) that can provide experimental evidence for evaluating potential hazards will be presented. Finally, a framework will be discussed for integrating clinical, animal, and NAMs data to support decisions regarding potential cardiovascular toxicity.

W 1211 Evidence in Support of Classifying Cardiotoxicity as a GHS Hazard Trait

C. Tsitsimpikou. General Chemical State Laboratory of Greece, Athens, Greece. Sponsor: I. Rusyn

Human health hazards and related risks from chemicals are well regulated internationally. However, in many regulatory frameworks, cardiotoxicity is not recognized as a stand-alone hazard, nor are there harmonized criteria for classifying substances as cardiotoxic. To substantiate the concern of cardiotoxicity as an emerging hazard, a regulatory roadmap should be adopted. To support the development of this roadmap, an OECD working group was established with the following objectives: to define cardiotoxicity; describe established modes of action at the cellular and tissue levels; assess the ability of existing toxicity test methods to identify cardiotoxic hazards; validate the use of existing animal data by introducing acceptance criteria; and evaluate new regulatory approaches and novel methods (such as NAMs and omics technologies) for identifying cardiotoxicity. This presentation will describe the background evidence linking environmental exposures to cardiovascular health effects and provide an update on the recently endorsed OECD working group progress.

W 1212 Key Characteristics of Cardiovascular Toxicants: An Organizing Principle for Collection and Evaluation of the Hazard Data

M. Smith. University of California Berkeley, Berkeley, CA.

A group of experts representing different areas of mechanistic and regulatory toxicology developed 12 key characteristics (KCs) of cardiovascular toxicants to better identify and assess chemicals that may harm the heart and blood vessels. These KCs were established through expert consensus and organized by effects on the heart, blood vessels, or both. They include impairments in heart rhythm, contractility, vascular function, mitochondrial activity, and hormone signaling, as well as induction of inflammation, oxidative stress, and dyslipidemia. The KCs aim to improve understanding of cardiovascular toxicity and guide more systematic evidence maps from existing data as well as prospective testing of environmental chemicals and pharmaceuticals.

W 1213 From Assay to Dose: Integrating ToxCast Data with IVIVE and PBPK Modeling to Predict Cardiotoxicity of Environmental Chemicals

S. Krishna. Physicians Committee for Responsible Medicine, Durham, NC.

High-throughput screening (HTS) data from ToxC21/ToxCast offers a powerful, human-relevant platform for identifying environmental chemicals with cardiotoxic potential. In this study, we identified 314 HTS assays associated with molecular and cellular events relevant to six cardiovascular failure modes, covering 40 cardio-relevant targets. Using the Toxicological Priority Index (ToxPi) for visualization and ranking, 1,138 chemicals were prioritized for their potential to disrupt cardiovascular function. To enhance translational relevance, bioactive concentrations were converted to human equivalent administered doses (EADs) using *in vitro* to *in vivo* extrapolation (IVIVE) combined with physiologically based pharmacokinetic (PBPK) modeling. These EADs were compared against exposure predictions and *in vivo* points of departure from toxicological studies to refine hazard characterization. The integration of IVIVE and PBPK modeling revealed chemical classes—such as organotin, bisphenols, flame retardants, and pesticides—likely to induce cardiotoxic effects at human-relevant exposure levels. This multiscale approach supports the use of NAMs in cardiovascular risk assessment and identifies priority chemicals for further investigation.

W 1214 iPSC-Derived Cardiomyocytes and Endothelial Cells as Robust NAMs for Studies of Chemical Effects on Cardiovascular System

I. Rusyn. Texas A&M University, College Station, TX.

Human-induced pluripotent stem cell (iPSC)-derived organ-specific cells have rapidly become a key tool in biomedical research and preclinical safety testing. With respect to cardiovascular system both cardiomyocytes and endothelial cells are available from human iPSCs and have been used widely in studies with drugs and environmental chemicals. This presentation will highlight their use in assessing cardiovascular toxicity hazards, characterizing risk, and modeling population variability. These cell-based models improve the accuracy and efficiency of identifying cardiovascular toxicity from drugs and environmental chemicals. While promising, challenges remain, such as improving cell maturation and expanding their application to structural cardiotoxicity and effects on contractility, as well as broader risk assessment.

W 1215 Translating Pharmaceutical Cardiovascular Safety Frameworks to Environmental Hazard Assessment: Opportunities and Challenges

J. Valentin. UCB Biopharma SRL, Brussels, Belgium.

Pharmaceutical development has long invested in structured frameworks to identify, characterize, and mitigate cardiovascular (CV) safety risks across the product lifecycle. These efforts integrate mechanistic understanding, translational models, and evolving regulatory expectations to reduce attrition and protect patients. As environmental exposures increasingly emerge as contributors to cardiovascular disease, lessons from pharmaceutical CV safety can inform environmental hazard identification and risk assessment. This presentation will explore how principles such as weight-of-evidence integration, safety margin estimation, use of human-relevant biomarkers, and tiered testing strategies can be adapted to the environmental context. Particular attention will be given to challenges in modeling combinatorial exposures, translating findings from alternative methods (NAMs), and ensuring relevance to human disease pathogenesis. Bridging these domains offers a unique opportunity to advance cardiovascular risk assessment across sectors, enhance public health protection, and reduce reliance on animal testing.

R 1216 Does Toxicology Need a Reformation? A Debate About Scientific Integrity

C. Borgert. Applied Pharmacology and Toxicology Inc., Gainesville, FL.

Much has been written about scientific revolutions, but periodically, fields of human inquiry may need to ask whether reformation is more appropriate than revolution. Reformation implies a reaffirmation of the fundamental principles that have allowed the field to advance and to ask whether a field is losing integrity in its quest for novel ideas, fresh theories, and surprising discoveries. A few practitioners of toxicology and related fields have asked questions regarding the erosion of scientific integrity for more than a decade, but these warnings went largely unnoticed or were ridiculed. Recently, however, words such as “crisis” and “failure” have been used to describe the status of the life sciences, again begging the question whether toxicology needs a broad reformation toward fundamental scientific principles. For the past decade, the Society of Environmental Toxicology and Chemistry (SETAC) has attempted to address this void in annual sessions on issues of scientific integrity (SI). Those sessions have been highly attended and well supported by scientists with diverse and often countervailing viewpoints. In 2022, the SOT Ethical, Legal,

Forensic and Societal Issues (ELFSI) Specialty Section and the SOT Regulatory and Safety Evaluation Specialty Section (RSESS) partnered with the Institute for the Advancement of Food and Nutrition Sciences (IAFNS) to sponsor a series of three online webinars on SI issues, all of which were well-attended. This session on SI partners with and builds upon the SETAC sessions. The session will consist of three brief talks to outline the topic and provide context and will be followed by a substantive panel discussion that encourages audience participation in the form of questions, comments, and debate about whether toxicology needs a reformation. The session will begin with a general talk on what constitutes SI and why it is essential for toxicology if toxicology is to continue to advance as a discipline and provide benefit to society. Examples of the ill-consequences of science that lacked integrity will serve to illustrate the issue and importance to public and environmental health. The second talk will argue that toxicology needs a reformation, as evidenced by the broad range of topics that have been covered and debated during annual SETAC sessions. An organizer of those sessions will provide a collective perspective, including an example of how toxicology has lost public trust and how failing to address the erosion of SI in toxicology may negatively affect SOT as well as SETAC. A final talk will provide the opposite perspective and will argue that toxicology is a field that has maintained high integrity and should serve as an exemplar to other scientific specialties that contribute expertise in the arena of public and environmental health protection. The final talk will offer suggestions on how to best communicate the high integrity of toxicology and toxicologists to the public. The debate framed by these talks will kick-start an interactive panel discussion with audience participation.

IS 1217 **Guardians of Public Health: The State We Are In**

D. McCant. Texas Commission on Environmental Quality, Austin, TX.

State toxicologists play a vital role in applying scientific evidence to protect public health and the environment, especially since each state has a unique set of priorities and challenges. Given the current uncertainties related to federal regulatory agency organization and priorities, the work of state agencies is even more important for identifying solutions and developing state-of-the-science assessments protective of human and environmental health. This session will focus on the unique role of state toxicologists and how they apply toxicological principles, the risk assessment framework, and risk communication to help inform practical decision-making and guide state-specific services, policy, and regulatory actions. Speakers from several states with a range of perspectives will discuss their state's approach to current toxicological and risk assessment issues. Each speaker will discuss tips and lessons learned on communicating risks and health information to the public in their states. One common goal among the different states is to build trust and empower communities. The session will examine strategies employed to effectively communicate complex toxicological concepts and risk information to varied stakeholders—ranging from the general public and community groups to industry representatives and policymakers. The session will conclude with a panel discussion with invited guests from private industry, a federal agency, and selected presenters to engage in a dialogue around a set of preapproved questions and relevant issues currently shaping the field. They will share their insights on toxicology at the state level and their experiences with state regulations and recommendations.

EC 1218 **Earning Trust and Driving Impact: Lessons in Communicating Risk to Diverse Audiences Amid Widespread Science Skepticism**

C. McLoughlin. Enhesa, Schuyler, VA.

Growing public distrust in science has been fueled in large part by the rapid spread of information without corresponding access to the expertise needed for critical interpretation. As a result, confusion—and often concern—about the safety of everyday products and the environment has taken hold, despite robust research and regulatory efforts. Meanwhile, misleading buzzwords like “chemical-free” have taken root, with marketing increasingly shaping public perception in ways that sideline scientific understanding. At the same time, the broader value of research and scientific expertise has come under growing scrutiny. This makes the need for effective science communication more urgent than ever. Scientists and advocates must adopt creative, audience-centered approaches to build trust and cut through the noise. That means not just simplifying complex topics but also demonstrating their relevance to people's everyday lives. Tools like engaging storytelling, relatable examples, visual content, and the use of diverse media platforms can help make science more accessible and compelling. In this climate of skepticism, researchers and communicators must hone strategies for meaningful public engagement. By fostering deeper understanding and addressing common misconceptions, they play a critical role in connecting scientific insight with public awareness. The session will open with practical guidance on tailoring scientific messages to different audiences, from students to the general public. The speaker will highlight the importance of understanding audience needs, reducing jargon, and adjusting complexity to improve clarity and engagement. The second speaker will share their approach to making toxicology relatable, highlighting strategies for connecting with regulatory and policy relevant audiences. The third speaker will highlight best practices and opportunities for engaging with policymakers and contributing to informed regulatory discussions. The final speaker will reflect on how external experts most effectively assisted in decision-making, offering an inside view into successful scientist-regulator commu-

nication. Following the individual presentations, a panel discussion will bring the speakers together to compare perspectives, explore common challenges, and answer questions from attendees. This session aims to equip participants with practical insights and tools for clear, impactful communication. Drawing on the expertise of our panelists, attendees will explore a range of strategies to counter misinformation and promote science-based policies. Real-World examples and interactive discussions will reinforce the importance of clarity, relevance, and trust in public outreach. Together, these perspectives will provide attendees with a strong toolkit for engaging nonscientific audiences, contributing to policy conversations, and advocating for the value of research. Whether you're speaking to your local community, preparing for a US Capitol Hill visit, or responding to a public comment period, this session will help sharpen your message and maximize your impact.

S 1219 **Human Health Risk Assessments of Biopesticides: Evaluating Safety for Agricultural Use**

L. Zorrilla. Bayer Crop Science, Chesterfield, MO.

Global interest in biological-based products for agriculture is growing rapidly, including products based on micro-organisms. This is an expanding market globally worth \$12 billion with a 15% compound annual growth rate, and conservative estimates put this market worth \$27 billion by 2027. As such, regulatory agencies all over the world are now reviewing data packages for microbial-based pesticides. However, there are no globally harmonized guidelines for human safety assessments, and in some countries, no regulatory paradigm exists to evaluate potential hazard and risk from these types of products. While regulatory frameworks exist for evaluation of synthetic pesticides, the frameworks are not fit-for-purpose to use for microbials, and even less so for genetically modified micro-organisms. Human safety assessments for biopesticides should be focused on specific strategies suited to the unique characteristics of the test material and not on using irrelevant testing strategies validated for small molecules or proteins. Some of the areas to be considered to address areas of potential human safety concern for microbial biopesticides are: (1) is the natural substance a potential human pathogen or capable of causing infection; (2) does the natural substance have toxic or genotoxic properties; (3) does the natural substance have the capability to produce clinically relevant antibiotics or contain clinically relevant antibiotic resistance markers; and (4) what additional safety information is critical for modified micro-organisms, or is that scientifically irrelevant? In addition, considering all the validation efforts for new approach methodologies (NAMs) for chemicals, could those be tailored to suit microbials, which could potentially support eliminating animal testing requirements? Critical to these efforts is global harmonization of testing strategies to reduce the number of *in vivo* tests to accurately predict potential human risk for these types of products. This session will provide a short background on microbes and their recent uses in agriculture, perspectives on human health testing strategies and challenges from industry and regulators, updates from the Organisation for Economic Co-operation and Development (OECD) Expert Group on Biopesticides (EGBP), challenges with developing NAMs and some ongoing efforts to validate micro-organisms, and considerations for testing strategies for modified micro-organisms. The attendees should come away with a basic understanding of the current human health risk assessments for micro-organisms, updates on challenges from the regulatory and industry perspectives, and the state-of-the-science updates on ongoing projects, including the OECD working groups where all stakeholders are working to modernize and align the best scientific practices for regulatory purposes.

S 1220 **Setting the Stage for the Current Status of Human Health Risk Assessments for Biopesticides**

L. Zorrilla. Bayer Crop Science, Chesterfield, MO.

Requirements to register microbial pesticides in the United States were first established by the Environmental Protection Agency (EPA) in 1983, revised in 1996, and since then, have become the basis and only guidelines for the hazard identification and risk assessment of microbial pesticides globally. The EPA's Office of Prevention, Pesticides, and Toxic Substances (OPPTS) 885 series of guideline studies evaluates whether a microbe can cause infection, disease, or toxicity through various routes of administration. In addition, standard acute “6-pack” studies, which are required for registration of conventional pesticides, are also required for biopesticide formulations. These studies aid in categorizing hazard potential for label safety details and assure safe use of the product. Since the EPA guidelines were issued, many advances in new approach methodologies (NAMs) have been developed to reduce the need for animal testing. However, biopesticides have not been included in these validation efforts, and no updates based on the current state of the science have been made to date. This introduction will provide a short history and outlines the opportunities for fit-for purpose human health risk assessments for biopesticides.

C. Pestana. Federal University of Paraná, São Paulo, Brazil.

Microbial pesticides are generally regarded as less toxic alternatives to synthetic pesticides. However, like their chemical counterparts, microbial pesticides are subject to regulatory approval before marketing. In addition to the focus on the toxicity of traditional plant protection products, microbial pesticides should also be assessed for infectivity, pathogenicity, and the effects of potential metabolites. Therefore, toxicological endpoints should be adapted and harmonized to achieve adequate confidence levels, particularly in the context of reducing or eliminating animal testing. Whilst New Approach Methodologies (NAMs) may hold significant promise for assessing microbial toxicity, several limitations remain. The potential value of NAMs in overcoming such challenges in assessing the sensitization hazard of microbial pesticides has been discussed to inform proposals for NAM-based strategies. A deeper understanding of the sensitization processes could pave the way for the development of NAMs, thereby improving the assessment of microbial pesticides, one of the most challenging toxicological endpoints in this field. Among NAMs, *in silico* methodologies could be particularly valuable for screening and for guiding further investigations into the hazard potential of secondary metabolites. Case studies illustrating the challenges and opportunities of *in silico* assessment of low and high-molecular-weight secondary metabolites, with a particular focus on cyanobacterial metabolites, are discussed. While traditional *in silico* tools are suggested to be useful for screening, prioritization, and elucidating potential mechanisms of action of genotoxicity and carcinogenicity of small molecules, they are less effective for larger macromolecules, which may fall outside the applicability domain of many models and lack reliable physicochemical descriptors. To address these limitations, multiple *in silico* tools covering diverse toxicological endpoints were integrated into a weight-of-evidence (WoE) approach, combined with read-across approaches and NAM existing mechanistic data to evaluate tumor promotion effects of microcystins (cyanobacterial metabolites). By explicitly accounting for uncertainties in the outcomes, this integrated approach is proposed as a viable alternative for assessing large and complex molecules.

S 1222a **The EU Regulatory Framework on Pesticides: An Introduction to the New Endocrine-Disrupting Hazard and the Registration Challenges**

M. Sachana. OECD, Paris, France. Sponsor: L. Zorrilla (L. Zorrilla in italics)

Microbial pesticides are part of the most scientifically and regulatory challenging categories of biopesticides, yet they are considered valuable alternatives to conventional pesticides. Their application is increasingly encouraged within integrated pest management approaches or in isolation, and they are recognised as one of the important pillars of sustainable agriculture. Under the auspices of OECD, the Expert Group on BioPesticides (EGBP) has, since 1999, coordinated international efforts to advance and facilitate the registration of microbial pesticides by supporting governments and industry with guidance documents and test methods and by harmonising and modernising testing and risk assessment approaches across countries. This presentation will outline past outputs and current activities under the EGBP that address human health considerations in the risk assessment of microbial pesticides. These include published guidance, for example, on microbial contaminants and anti-microbial resistance, and consensus documents on microorganisms strains. The audience will also be informed about ongoing work to develop an internationally recognised OECD Test Guideline that covers toxicity, pathogenicity and infectivity for testing microbial pesticides, using as a base an existing USEPA test and an applicable risk assessment framework for microbial pesticides that applies problem formulation and pathways for human health-related protection goals and hazards. Findings from a recent international survey will be presented to shed light on challenges and solutions captured with the aim to inform existing projects and shape future EGBP activities. Survey results highlight challenges and potential solutions to improve *in vivo* exposure scenarios and interpretation of microbial clearance findings and data. Respondents expressed interest in applying weight of evidence approaches to risk assessment that integrate whole genome sequencing data, bioinformatics, existing scientific literature and alternative methods. In this respect, preliminary work explored the application of new approach methodologies (NAMs), such as *in vitro* OECD Test Guidelines. A new project is expected to start under the EGBP to develop guidance and adapt these NAMs that could potentially replace the standard acute "6-pack" studies, starting with *in vitro* eye irritation test methods and expanding to other endpoints such as skin and respiratory sensitization. Ultimately, the presentation will highlight the coordinated efforts under the OECD to reduce redundancy of testing and accelerate the availability of innovative biological pest control solutions in the market by promoting scientific and regulatory exchange, developing guidance and introducing tools to improve and increase efficiency and harmonization in regulatory decision making, while protecting human health.

S 1223 **Challenges with Developing NAMs for Microbes**

P. Marx-Stoelting, and K. Wend. Bundesinstitut für Risikobewertung (BfR), Berlin, Germany. Sponsor: L. Zorrilla

The current guidelines for testing microbial pesticides face several challenges, primarily because these products consist of living microorganisms, which require different consideration from chemical pesticides. Guidelines for chemical pesticides focus on toxicity, but microbial pesticides also assess infectivity, pathogenicity, and the effects of potential metabolites. Unfortunately, microbial research to identify these effects is lacking, which makes safety assessments complex. One issue is defining "clearance" in microbial toxicity studies. The clearance of microorganisms from the body or tissues is often undefined, leading to uncertainties in interpreting the results. Moreover, spore-forming microbes, which have slow clearance rates, add complexity. The current test methods also struggle with the variability in microbial growth under different conditions. For example, temperature thresholds that could potentially exclude infectivity studies are not well-established, making it hard to apply a standardized approach. Additionally, many test guidelines lack clear instructions on the use of positive or negative controls. Without guidance, interpretation of results can be delayed or compromised, hindering the registration of microbial pesticides. Another challenge is related to microbial dose concentrations. When Colony-Forming Units (CFU) concentrations in tests exceed those in actual formulations, adverse effects may be misinterpreted as pathogenic, making hazard assessments difficult. Additionally, discrepancies in physical properties of microbial test materials can complicate the accurate preparation of test substances. Regulatory submissions in the EU and US demand Good Laboratory Practice (GLP)-compliant studies, but early development studies may be non-GLP, raising questions about their reliability. There is also increasing interest in non-animal methods (NAMs) for microbial pesticide testing, and integrating these methods could address some challenges in safety assessments. The development of NAMs and the use of technologies like whole-genome sequencing could identify microbial hazards without relying on animals, thus advancing microbial pesticide regulation globally. Ultimately, harmonizing testing methods and leveraging new technologies is crucial for ensuring the safe use of microbial pesticides while adhering to modern regulatory standards.

S 1224 **The Ever-Evolving World of Microbes: The Current State of Microbial Taxonomy, Genome Evolutionary Dynamics, and the Impact on Future Microbials Risk Assessment**

K. Karberg. Bayer Crop Science, Chesterfield, MO. Sponsor: L. Zorrilla

Risk assessment frameworks for plant agricultural biotechnology products have been in place for decades, focused on the evaluation of living biotechnology products created through genetic engineering. These products contain genetic material from outside the breeder's gene pool, which is often from different taxa or represents "novel combinations of genetic material". These products are typically considered to be "genetically modified" (GM) organisms in regulatory jurisdictions. However, in the microbial world, particularly among Bacteria and Archaea, the rapid expansion of genome sequence databases shows that natural microbial innovation primarily occurs through the natural exchange of genetic material from various sources, even from different taxa. This means that many microbes can be considered naturally occurring GM organisms. This raises the question of whether labeling a microbe as GM is always scientifically relevant for risk assessment. In most regulatory frameworks, being classified as GM significantly impacts the registration path, especially for microbes intended for environmental release. A more effective and science-based regulatory approach would assess the actual functions of a microbe rather than relying on the uncertain classification of its genetic material. This would benefit regulators, developers, and society by promoting the use of microbial technologies for agricultural use. The current state of microbial taxonomy, knowledge on genome evolutionary dynamics, and the relevance of this knowledge to regulation of agricultural biologicals intended for environmental release will be presented.

S 1225 **Advancing Skin and Respiratory Sensitization NAMs for Microbial-Based Pesticides**

A. Forreryd. SenzaGen AB, Lund, Sweden.

Microbial-based pesticides are a type of biopesticide that utilize living microorganisms, such as bacteria, fungi, viruses, or protozoa, to control agricultural pests. These agents act through mechanisms like infection, parasitism, or toxin production, offering high target specificity and minimum ecological disruption. They are increasingly adopted as sustainable alternatives to conventional chemical pesticides in integrated pest management. However, the human health risk assessment of microbial pesticides presents challenges and current safety evaluations lack globally harmonized guidelines. Allergenicity is one of the key health risk concerns, particularly in occupational settings when microbial spores or components may be inhaled or come into contact with the skin of workers. This presentation will introduce the GARD® technology, a genomics and machine learning-based New Approach Methodology (NAM) and explore its potential to support the human health risk assessment of microbial-based pesticides for the endpoints skin and respiratory sensitization.

GARD@skin, included in OECD Test Guideline 442E for skin sensitization testing, has demonstrated broad applicability across diverse chemical groups and can currently be tested with microbial pesticides. GARD@air, the first commercially available *in vitro* assay for respiratory sensitization, provides a novel solution for identifying and ruling out potential respiratory sensitizers in candidate substances, supporting safer product development and occupational health risk management. Recent scientific data from research and industrial collaborations showcase how GARD@skin and GARD@air address current challenges in skin and respiratory assessment, including: (1) Testing of “difficult-to-test” samples; (2) Skin sensitization potency assessment, including UN GHS/CLP subclassification and the prediction of safe dose levels or Point of Departure for Quantitative Risk Assessments; and (3) Respiratory sensitization assessment of chemicals and proteins. This presentation will explore the potential applicability of GARD@skin and GARD@air to microbial-based pesticides, addressing current knowledge gaps and future directions in non-animal safety testing for this emerging class of products.

S 1226 Mechanism of Metal Neurotoxicity in the Era of ‘Omics

A. Bowman. Purdue University, West Lafayette, IN.

Metals such as lead (Pb), iron (Fe), manganese (Mn), mercury (Hg), arsenic (As), and cadmium (Cd) have long been recognized for their toxicity in humans, with significant implications for neurological health. Exposure to these metals, whether through environmental sources, occupational hazards, or contaminated food and water, has been linked to various neurodegenerative and cognitive disorders, including Alzheimer's disease, Parkinson's disease, and developmental impairments in children. Heavy metals exert their neurotoxic effects through multiple mechanisms. They can disrupt neuronal function by generating oxidative stress, impairing mitochondrial activity, and inducing neuroinflammation. Metal ions interact with cellular structures, leading to alterations in enzyme activities, protein misfolding, and disruption of calcium signaling. Furthermore, these metals can interfere with neurotransmitter systems, which play a critical role in brain function and behavior. Despite advancements, there are still many uncertainties surrounding the mechanisms of neurotoxicity induced by these metals. Additionally, the interplay between genetic susceptibility and environmental factors complicates the identification of at-risk populations. Continued research into metal neurotoxicity is essential for developing preventive strategies, improving diagnostic methods, and informing public health policies to mitigate these risks and safeguard neurological health. This session will focus on the latest advancements in understanding the mechanisms of metal neurotoxicity, particularly through the lens of ‘omics technologies, such as epitranscriptomics, proteomics, metabolomics, multi-omics, and single-cell transcriptomics. With the growing prevalence of metal exposure and its associated neurotoxic effects, there is an urgent need to explore the molecular pathways and biological systems that are disrupted by metals. ‘Omics approaches offer a powerful tool set for uncovering these mechanisms and providing new insights into the cellular and molecular basis of neurotoxicity, enabling the development of novel diagnostic biomarkers and therapeutic strategies. Key objectives of the session are (1) to explore how ‘omics technologies are providing deeper insights into the molecular pathways of metal-induced neurotoxicity; (2) to discuss the mechanistic understanding of metal toxicity in the context of gene-environment interactions, epitranscriptomics, and their influence on neurodevelopmental and neurodegenerative diseases; (3) to evaluate the role of specific molecular networks, including oxidative stress, neuroinflammation, and mitochondrial dysfunction, in the pathophysiology of metal neurotoxicity; and (4) to assess the potential of ‘omics-derived biomarkers for early detection of metal exposure and neurotoxicity and their application in public health initiatives. This session is designed for researchers, clinicians, and public health professionals with an interest in toxicology, neurology, environmental health, and ‘omics technologies. It will also be of value to those focused on disease mechanisms, biomarker discovery, and novel therapeutic approaches. Participants will gain a deeper understanding of the molecular and cellular mechanisms underlying metal neurotoxicity, enhanced by the application of ‘omics technologies. By bringing together leading diverse experts in the field, the session will foster collaboration and stimulate future research aimed at mitigating the effects of metal exposure on neurological health. Additionally, it will highlight the potential for personalized medicine approaches in managing metal-induced neurological disorders.

S 1227 Multi-omics Tool Development for Exposure Signature Discovery and Regulatory Pathways Leading to Adverse Outcomes

R. Spurbeck. Battelle, Columbus, OH. Sponsor: S. Sarkar

The epigenome is a complex system of regulatory mechanisms which respond to exposures such as diet, chemicals, infections, stress, and other environment factors causing changes to the person's biology that can impact their health. The epigenome includes DNA methylation, histone modifications, chromatin accessibility, regulatory RNAs and transcription. Through study of integrated epigenetic signatures encompassing the whole epigenome, we can determine affected biological systems in response to different occupational and environmental exposures and develop diagnostic assays to identify individuals who have been impacted by these exposures. Furthermore, as some epigenetic modifications can be reversed, epigenetic signatures identified could become targets for therapeutics to treat harmful effects of

exposure. Here, the development of two tools for better understanding toxic exposures and regulatory pathways which lead to adverse outcomes will be described. First, the Epigenetic Signature Discovery Algorithm (ESDA), a computational model to identify diagnostic exposure signatures will be discussed. Second, we will describe the use of a platform which combines a microphysiological system with single-cell RNAseq and ATACseq, methylation, and hydroxymethylation sequencing. These tools combine into an end-to-end system to discover human exposure signatures and adverse outcome regulatory pathways which can enable therapeutic and diagnostic development.

S 1228 Multi-omics Identify Novel Molecular Networks in Metal-Induced Epitranscriptomic Dysregulation Underlying Chronic Neuroinflammation

A. Kanthasamy. University of Georgia, Athens, GA.

Astrocytes and microglia respond dynamically to environmental neurotoxic chemicals to protect and support neuronal health. When exposed to neurotoxins, glial cells undergo changes in their morphology and function, becoming reactive glia. While this reactive response aims to limit damage and maintain homeostasis, chronic activation of astrocytes and microglia leads to uncontrolled inflammatory responses and contributes to various neurodegenerative conditions. Nevertheless, the precise molecular networks that regulate the pro-inflammatory state of these guardian cells under chronic neurotoxic conditions remain enigmatic. Emerging epitranscriptomic research reveals that N6-methyladenosine (m6A), the most abundant RNA modification in eukaryotic cells, plays a crucial role in regulating pro-inflammatory mediators including cytokine and chemokine production. However, how these m6A mRNA modifications regulate glia-driven chronic neuroinflammation in metal neurotoxicity is completely unknown. This presentation will highlight the power and versatility of multiple multi-omic approaches such as RIP-seq and m6A-seq in identifying epitranscriptomic networks associated with chronic neuroinflammation during neurotoxicant exposures in glial cells. Specifically, our studies reveal manganese exposure selectively downregulates the m6A reader protein YTHDF2 to activate the SEK1-JNK-CJun signaling network, which serves as a molecular switch to sustain a proinflammatory state in astroglia. Results from our other studies highlight how manganese can upregulate and mislocalize the m6A mRNA reader protein METTL3 in microglia to induce a neuroinflammatory response. Collectively, multi-omic technologies are integral to deciphering cell- and stimulus-specific molecular events underlying chronic neuroinflammation associated with environmentally linked neurodegenerative diseases such as Parkinson's and Alzheimer's diseases. *Supported by NIH grant ES R0103624.*

S 1229 Single-Cell RNA-Sequencing Enables Sensitive Detection of a Persistent Neurotoxic State

A. Bowman. Purdue University, West Lafayette, IN.

Methylmercury (MeHg) is a neurotoxin that indiscriminately disrupts multiple homeostatic pathways. Its neurotoxic outcomes include latent effects that persist long after exposure has subsided. We report on experiments to test the hypothesis that persistent effects of MeHg are self-perpetuating via interdependent relationships of key biological pathways that sustain and regulate neurological function. We developed a human induced pluripotent stem cell (hiPSC) model of differentiating cortical glutamatergic (GLUergic) neurons to assess persistent MeHg-induced neurotoxicity. Cultures were exposed continuously for ~6 or ~12 days during developmental stages to *in vivo* relevant MeHg concentrations (0.1 and 1 μ M). Following exposure, cortical GLU neuronal differentiation was continued till cultures reached mature stages. At day 40, 150 and 280 of differentiation cells were assessed for persistent effects using single cell RNA sequencing (scRNAseq). MeHg exposure during development resulted in a robust pattern of differentially expressed genes that play key roles in functional and biological pathways indicating a persistent toxic effect. Persistently impacted pathways include signaling pathways required for healthy aging, neurotransmission and metabolic/energetic homeostasis; and functional correlates of these changes have been observed. We will present ongoing studies aimed at identifying the basis of these persistent changes long-after exposure has ceased and MeHg is absent from the model system. Our data demonstrate that scRNAseq can detect persistent effects of developmental exposures to MeHg, that these effects can be reproduced by proteomic approaches, and that persistence manifests as an altered -omic and functional state that differs across distinct cell identities in hiPSC-derived cortical cultures. *Supported by NIH R01 ES007331.*

S 1230 Using New Approach Methods and Multi-omics to Study Metal Toxicity Mechanistically

S. Sarkar. University of Rochester Medical Center, Rochester, NY.

Epidemiological studies have implicated the critical role of environmental factors in driving neurodegeneration in various diseases like Parkinson's (PD), Alzheimer's disease (AD), and others. Manganese (Mn) overexposure has been linked to the development of a neurodegenerative disorder similar to PD called Manganism. Further, Mn has also been shown to induce aggregation of α -synuclein, a PARK gene, demonstrating the potential role of Mn exposure in sporadic PD. We took a bottom-up approach and performed untargeted high-resolution mass spectrometry proteomics and metabolomics on the brains of flies exposed to Mn. Pathway enrichment analysis on the multi-omics dataset identified peroxisomes as a key altered pathway in Mn toxicity. Peroxisomal markers were downregulated *in vivo* and *in vitro*, further confirming the importance of this organelle in Mn-toxicity. We identified peroxisomal biogenesis and contact peroxisomal transport as key regulators using a fly model. We further used a pharmacological drug to enhance peroxisomal biogenesis and demonstrated that the drug was able to rescue Mn-induced toxicity in patient-derived iPSC-derived midbrain culture. The peroxisomal biogenesis drug also improved mitochondrial health, suggesting a crosstalk between peroxisomes and mitochondria. High-resolution live cell microscopy revealed that Mn reduced the physical contact between peroxisome and mitochondria by downregulating the tethering protein Mitofusin (Mfn). Finally, the lipidomic analysis identified carnitines, a key lipid involved in the peroxisomal transport of lipids, to be upregulated in Mn-exposed flies. Previous studies in human patients suggested that stearate treatment can reduce acylcarnitine levels. In humans and flies, the stearate level can be regulated by Elovl7, a fatty acid elongase. Interestingly, Elovl7 is also a PD risk factor. Our preliminary data further suggested that increasing stearate through diet or genetically by regulating Elovl7 reduced Mn-induced toxicity and mitochondrial dysfunction. In summary, we demonstrated that the Peroxisomal biogenesis and reduced membrane contact sites between the peroxisome and mitochondria (PerMit) regulated Mn-induced neurotoxicity.

S 1231 Leveraging High-Resolution Mass Spectrometry to Decipher Molecular Mechanisms of Metal-Induced Toxicity

Y. Lai. Columbia University, New York, NY.

Metal exposure remains a significant public health concern, with both essential and non-essential heavy metals and/or metalloids contributing to a range of toxicological outcomes across organ systems. While the mechanistic understanding of metal-induced toxicity has advanced, the complexity of downstream biochemical alterations remains poorly characterized, requiring more sophisticated analytical assays and/or approaches that confer high sensitivity, coverage, and precision. High-resolution mass spectrometry (HRMS) has emerged as a transformative tool in toxicology, enabling the untargeted detection and *de novo* structural annotation of hundreds of thousands of endogenous and exogenous molecules within biological matrices. This study/proposal highlights the development and application of HRMS-based approaches in elucidating the molecular fingerprints associated with metal exposure, particularly heavy metals such as cadmium, arsenic, manganese, and lead. By integrating HRMS with advanced bioinformatics and exposomics frameworks, researchers can trace metal-induced perturbations on a much broader scale across metabolic, lipidomic, and xenobiotic pathways with high flexibility. HRMS also allows for discovering reactive metal species, altered redox metabolites, and metal-binding biomolecules that critically mediate toxicity. In cellular and animal models, longitudinally, time-resolved HRMS analyses provide insight into early biomarkers of exposure and injury, while in human cohort studies, non-targeted profiling supports exposure-wide association studies (ExWAS) for chronic metal burden. Together, these applications demonstrate how HRMS enables a systems-level view of metal toxicity, uncovering key pathways and molecular signatures that traditional approaches likely overlook. Future directions include coupling HRMS into spatial omics (i.e., metabolomics/lipidomics/exposomics MS imaging), ion mobility, and machine learning to further unravel the dynamic interplay between metal exposures and biological responses across wide-ranging toxicological and etiologic contexts.

S 1232 Metal Toxicity Meets Stem Cell Innovation: Early Stage Scientists Forging New Frontiers in Environmental Health

S. Medina. University of New Mexico, Albuquerque, NM.

Metals accumulate in the environment and are commonly found in soil, water, and living organisms, as well as in everyday consumer products. Many metals, including arsenic, mercury, cadmium, and lead, are toxic even at low exposure levels and have been linked to developmental disorders, increased cancer risk, neurological damage, and organ failure. Understanding the toxicological effects of metals on human health remains a critical scientific challenge. In recent years, stem cell-based models, particularly those utilizing human-induced pluripotent stem cells (hiPSCs), have become powerful tools for toxicological research. These systems allow modeling of specific human cell types at various developmental stages and can capture

donor-specific genetic variability, supporting personalized risk assessment. This session highlights early career scientists who are using innovative stem cell technologies to investigate how metals affect human health across multiple organ systems. The session will begin with an overview of stem cell-based platforms for toxicity testing, followed by presentations examining (1) persistent neurotoxic outcomes of developmental methylmercury and lead exposure in hiPSC-derived cortical neurons; (2) mechanisms of arsenic-induced hematotoxicity in primary mouse and human hematopoietic stem and progenitor cells; (3) chamber-specific impacts of lead exposure on calcium dynamics in iPSC-derived atrial and ventricular cardiomyocytes; (4) transcriptomic and functional disruption of intestinal epithelial differentiation caused by uranium-bearing particulate dust in human colonic organoids; and (5) cell-type and tissue-specific responses to chronic arsenic exposure using heterogeneous primary epithelial cultures derived from human ovarian and fallopian tube tissues. The session will conclude with a moderated panel discussion and audience Q&A designed to synthesize key insights, explore cross-cutting themes, and stimulate dialogue across disciplines. This session will provide a valuable platform for early career scientists to showcase their research and expertise, while also fostering meaningful interaction with established investigators to encourage feedback, mentorship, and potential collaborations. Collectively, these presentations and discussions will highlight the expanding role of stem cell models in metals toxicology and their potential to advance mechanistic insight and improve human health outcomes.

S 1233 Introduction to Stem Cell-Based Models for Toxicity Testing

S. Medina. University of New Mexico, Albuquerque, NM.

This talk will present a brief introduction to the use of stem cell models for toxicity testing and recent advances in these stem-cell based *in vitro* models, topics which will be further elaborated by subsequent speakers in the session.

S 1234 Developmental Methylmercury or Lead Exposure of hiPSC-Derived Cortical Cultures Induces a Similar Persistently Altered State

M. Strom, and A. Tukker. Purdue University, West Lafayette, IN.

It is becoming increasingly apparent that neurodegenerative diseases (NDs) are not just diseases of aging. The Developmental Origins of Health and Disease theory hypothesizes that early life exposure to environmental toxicants can create long-term lasting effects that can increase vulnerability for later-life chronic diseases. Here we investigate the hypothesis that developmental exposure to heavy metals can establish a persistently altered neurotoxic state early in life that can render an individual more susceptible to NDs in later life. Developmental exposure to heavy metals was modeled using human-induced pluripotent stem cell (hiPSC)-derived cortical glutamatergic neuronal cultures. Developing cultures were exposed for six days to 0.1-100nM methylmercury (MeHg) or 0.3-300nM of lead (Pb). 100+ days after the exposure was removed, microelectrode array (MEA) and Seahorse XF technology were used to assess persistent changes in spontaneous neuronal activity and cellular bioenergetics. Electrophysiological analysis revealed that developmental exposure to MeHg or Pb persistently alters neuronal network patterns in a similar manner. Notably, cultures exposed to lower concentrations of MeHg (0.1, 1nM) and Pb (0.3, 3nM) have an increase in overall neuronal activity compared to control cultures, whereas exposure to higher concentrations (10, 100nM and 30, 300nM, respectively) decrease overall activity. To understand the underlying molecular changes that cause these persistently altered network patterns, single-cell RNA sequencing was performed. Transcriptomic analysis revealed that pathways relating to NDs and oxidative phosphorylation are significantly altered, which led us to investigate changes in ATP production. Cultures exposed to lower concentrations of MeHg and Pb increase in overall ATP production, whereas cultures exposed to higher concentrations have decreased ATP production. We conclude that early developmental exposure to MeHg and Pb neurotoxic agents create a similar persistently altered state that affects major metabolic pathways involved in cellular health, aging, and neurodegeneration, rendering individuals more vulnerable to development of NDs. We plan to use hiPSC lines derived siblings with and without ND-related impairments to further explore gene x environment interactions in early life exposure and later life risk for NDs.

S 1235 Defining Mechanisms of Arsenic-Induced Hematotoxicity Using Mouse and Human Hematopoietic Stem and Progenitor Cell Models

L. Heine. University of New Mexico, Albuquerque, NM.

Hematopoietic stem and progenitor cells (HSPCs) are essential for maintaining blood system homeostasis by generating all mature blood cell lineages. Disruption of HSPC function can impair lineage specification and red blood cell differentiation, contributing to the development of anemia and other blood disorders. Our work over

the past several years has focused on characterizing how chronic environmental exposures to arsenic perturb HSPC fate decisions and transcriptional regulation. Using both primary mouse and human CD34+ bone marrow-derived HSPCs, we have demonstrated that inorganic arsenic (arsenite, AsIII) disrupts early erythropoiesis by targeting zinc finger transcription factors critical for erythroid lineage commitment, including GATA-1. Building upon these findings, we are now investigating how arsenic biotransformation influences hematotoxicity, with a particular focus on the methylated AsIII metabolite, monomethylarsonous acid (MMAIII). Together, these studies define novel mechanisms by which arsenic and its metabolites interfere with hematopoietic differentiation pathways, impair red blood cell development, and increase anemia risk in exposed populations.

S 1236 Chamber-Specific Impacts of Lead Exposure on Calcium Dynamics in Human iPSC-Derived Cardiomyocytes

I. Melis. University of Michigan Medical School, Ann Arbor, MI.

Lead (Pb) is a widespread environmental toxicant linked to increased cardiovascular risk, yet its direct effects on cardiomyocyte function remain incompletely understood. In this study, we investigated the chamber-specific impacts of Pb on calcium dynamics in human-induced pluripotent stem cell-derived atrial (aCMs) and ventricular cardiomyocytes (vCMs), focusing on dose- and time-dependent effects. Atrial and ventricular cardiomyocytes were differentiated from iPSCs using published small molecule-based protocols and purified to over 98% purity. Cells were exposed to environmentally relevant Pb concentrations in acute and sub-chronic models, and calcium dynamics, including beat frequency, calcium transient amplitude, and conduction velocity, were assessed using high-resolution optical mapping. Baseline measurements revealed that aCMs exhibited significantly higher beat frequency and conduction velocity, while vCMs displayed greater calcium transient amplitude. Pb exposure induced dose-dependent effects, with aCMs showing increases in calcium transient amplitude and upstroke slope at 500 μ M Pb, while vCMs experienced significant reductions in these parameters. Time-course analysis at 5 μ M Pb revealed dynamic, chamber-specific responses: baseline fluorescence in aCMs decreased after 1–12 hours of exposure but normalized by 72 hours, while vCMs showed an initial increase that returned to baseline by 6 hours. Calcium transient amplitude in aCMs peaked at 3 hours and remained elevated at 72 hours, whereas vCMs exhibited sustained decreases. Preliminary RNA sequencing analyses of these time-series samples revealed that the number of differentially expressed genes (DEGs) decreased over time, consistent with the temporal patterns observed in the calcium imaging data. These transcriptomic findings support the functional results and provide insight into the molecular mechanisms driving Pb-induced cardiac effects. Together, these results highlight the importance of chamber identity in toxicant susceptibility and lay the groundwork for identifying targets to mitigate Pb-related cardiovascular risks.

S 1237 Heavy Metal-Laden Particulate Dust Disrupts Intestinal Epithelial Differentiation in Human Colonic Organoids

J. In. University of New Mexico, Albuquerque, NM.

Over 160,000 abandoned mine waste sites are concentrated in the western United States and continue to degrade the environment with elevated heavy metals penetrating the surrounding sediment and groundwater. The sedimentary dust and water located near abandoned uranium mine (AUM) sites often exceeds the maximum contamination level for uranium (U, EPA max level 30 μ g/L). Chronic exposure to environmental toxins and heavy metals has been linked to intestinal inflammation, increased susceptibility to pathogen-induced diseases, and higher incidences of colorectal cancer, all of which have been steadily increasing in prevalence for the past 40 years. The negative effects of heavy metals on the intestinal microbiota, barrier permeability, and inhibition of intestinal epithelial healing have been described; however, transcriptomic changes within the intestinal epithelial cells are largely unknown. Thus, our goals are to characterize U-bearing dust (UBD) as an environmental toxicant that damages the intestinal epithelia and determine the molecular changes that develop as a response to this injury. We have previously characterized human intestinal organoids (HIOs) under homeostasis and pathogen infected conditions, to establish HIOs as a relevant intestinal pathophysiological model. Particularly in the secretory lineage of intestinal epithelial cells, there is divergence between human and rodent digestive physiology. Using HIOs, we seek to understand how UBD directly affects the stem and proliferative cells within the intestinal deep crypt and leads to transcriptional changes in the mucus-producing goblet cells and hormone-producing enteroendocrine cells. Understanding the mechanistic changes within the epithelia will help us understand if these changes have peripheral impact from the gut.

S 1238 Modeling Chronic Arsenic Exposure in Heterogeneous Human Primary Cultures: Insights into Cell-Type Specific and Tissue-Specific Mechanisms of Toxicity

D. Azzam. Florida International University, Miami, FL.

Understanding the long-term effects of environmental toxicants like arsenic requires model systems that reflect the cellular diversity of human tissues. In our study, we utilize primary human cultures—specifically OCE1 and FNE1—derived from the ovarian surface and fallopian tube epithelium of the same donor. These cultures comprise heterogeneous populations, including epithelial, immune and stem/progenitor cells, and offer a unique human-relevant platform to examine how chronic arsenic exposure impacts distinct cell types within a shared microenvironment. Our findings demonstrate that prolonged exposure to environmentally relevant levels of arsenic lead to significant cellular, molecular, and morphological alterations. These include the emergence of giant and/or multinucleated cells exhibiting phagocytic activity, DNA damage associated with nuclear remodeling, upregulation of cancer stem cell markers and activation of the NF- κ B inflammatory signaling pathway. These responses suggest coordinated cellular adaptations to chronic stress that may contribute to malignant transformation. By employing primary heterogeneous cultures, this model provides new insight into cell-type specific and ovarian-specific mechanisms underlying metal-induced toxicity.

S 1239 Toward Sex-Differences Research in Toxicology: New Approach Methodologies for Assessing Sex as a Biological Variable

K. Sullivan. Institute for In Vitro Sciences Inc., Gaithersburg, MD.

There is arguably no other attribute that has a greater impact on mammalian phenotype, physiology, and disease than sex. Diseases with well-established environmental etiology, such as cardiovascular diseases, liver diseases, neurodegenerative diseases, diabetes, and cancer, exhibit marked sexual dimorphism, suggesting that sex-dependent effects of environmental toxicants may underlie some of the observed differences in disease pathogenesis between males and females. Sex-specific effects have been reported for a wide array of environmental exposures, such as heavy metals, per- and polyfluoroalkyl substances, air pollution, and plastics. Despite these findings, the molecular mechanisms underlying sex differences in the context of environmental exposures are poorly understood. This is in part because until recently, females have been systematically underrepresented in both clinical and basic science studies. Likewise, sex chromosome complement in cell culture studies has largely been ignored. In studies where both sexes are included, sex-based analyses of the data are often lacking. Improvements in assessing sex differences have been made since the National Institutes of Health “Considering Sex as a Biological Variable” mandate in 2016, but challenges and opportunities remain. Concerns and misconceptions persist about cost and increased experimental variability when both sexes are included, as well as historic beliefs that males and females differ only in their reproductive biology. An additional hurdle has been a lack of appropriate models and methods to address sex differences. Recently, the biomedical sciences community has made strides in this regard with the development of new approach methodologies (NAMs) that specifically address sex as a biological variable. Additionally, in 2025, the US Food and Drug Administration released updated guidance on evaluating sex differences in clinical investigations, highlighting, among other considerations, the importance of collecting pharmacokinetics data. A more robust consideration of sex differences using NAMs in the preclinical stage could help facilitate better clinical study planning. This Symposium provides an up-to-date discussion of the current understanding of the impact of sex differences on disease etiology. Presentations will highlight the ongoing challenges and opportunities presented by sex-inclusive research and examine some of the recent new approach methodologies currently being explored to assess sex differences in toxicology studies. Physiologically based kinetic models can become more relevant to female toxicologic responses by considering incorporation of female-specific ADME parameters and anatomic compartments. Liver spheroids can be developed to reflect sex- and species-related response diversity, which is especially relevant for pharmaceutical efficacy and toxicity testing. Induced pluripotent stem cell-derived cardiomyocytes from male and female donors provide an opportunity to understand the sex-impacted response potential and mechanisms after PFAS exposure. A panel and audience discussion will explore research needs and challenges to addressing sex differences in toxicology, especially in the context of apparent defunding of initiatives focused on women. Discussions will also consider identification of priorities for further model development, current and future regulatory initiatives or frameworks across regions and sectors, and practical solutions with the greatest potential for impact on consumer and patient protection.

S 1240 Incorporating Sex as a Biological Variable into Basic and Clinical Research Studies

J. Regensteiner. University of Colorado Anschutz, Aurora, CO. Sponsor: *L. Svoboda*

Sex is one of the most fundamental differentiators of human beings. Sex differences, widely present in risk factors and diseases, need to be identified and understood so that women and men are treated optimally with regards to health. However, these

differences are not well understood at this point in time. For example, among individuals with type 2 diabetes (T2D), cardiovascular disease (CVD) is the leading cause of morbidity and mortality and accounts for >75% of hospitalizations and >50% of all deaths. Although nondiabetic women have fewer cardiovascular events than nondiabetic men of the same age, this advantage appears to be lost in the context of T2D. In addition, women with diabetes experience significantly greater morbidity and mortality than men from CVD. One cause of this may be abnormal cardiac and vascular function which are more pronounced in women than men with T2D. We found that peak exercise capacity (highly correlated with cardiovascular mortality) was reduced in men with diabetes and even more so in women with diabetes compared to nondiabetic counterparts, potentially related to cardiac and skeletal muscle abnormalities which became evident, in premenopausal women and age similar men with uncomplicated T2D. However, the reasons for these sex differences are not well understood and need to be better understood so that both women and men receive optimal care for their diabetes. These data provide a human-relevant lens to launch our discussions on the overarching need to reflect biological differences across the preclinical and clinical model development continuum.

S 1241 Induced Pluripotent Stem Cell-Derived Cardiomyocytes as a Model for Examining Sex-Specific Cardiotoxic Effects of Developmental PFAS Exposures

L. Svoboda, T. Ishikawa, C. Clark, A. Tapaswi, A. Monteiro Da Rocha, E. Jimenez-Vazquez, D. Jones, and J. Colacino. University of Michigan, Ann Arbor, MI.

Sex and environmental exposures play critical roles in the etiology of cardiovascular diseases (CVDs), but how these factors interact, and underlying molecular mechanisms, are unclear. Perfluoroalkyl substances (PFAS) are a family of man-made chemicals that resist degradation and bioaccumulate, resulting in ubiquitous human exposure. PFAS exposures are linked to several CVDs, but sex-specific effects of developmental exposures on the human heart are unclear. In this study, we utilized human-induced pluripotent stem cell-derived cardiomyocytes (iPSC-CM) from 2 donors (1 male and 1 female) to investigate the effects of PFAS exposure on CM differentiation, transcriptome, and function. iPSCs were exposed to perfluorooctanoic acid (PFOA), a ubiquitous legacy PFAS (0, 0.5, 5, and 50 μ M) for 3 days. Viability and pluripotency were measured using live cell imaging and qRT-PCR for expression of OCT4, SOX2, and NANOG. iPSCs were then differentiated into ventricular CMs in the continued presence or absence of PFOA using an established small molecules protocol, followed by CM purification and maturation for 10 days in PFOA free conditions. CM differentiation efficiency was assessed using flow cytometry and staining for cardiac troponin T (cTnT). Calcium dynamics and voltage were assessed in CM using optical mapping. IKr, IK1, ICa channel currents were measured using patch clamping. PFOA had no significant effect on viability or pluripotency of iPSCs from either donor. Exposure of the male-derived iPSCs to 5 μ M PFOA resulted in fewer cTnT positive cells, suggesting reduced differentiation efficiency. Optical mapping studies in iPSC-CM showed that PFOA significantly altered several parameters (beat frequency, conduction velocity, action potential duration, IKr currents $p < 0.05$) in both cell lines, with effects that differed by dose and donor. PFOA altered pathways related to lipids and lipoproteins in the female line, and calcium ion regulation and heart morphogenesis in the male line. A key objective is to understand effects of chromosomal sex on PFAS cardiotoxicity, and ongoing studies are being conducted in iPSCs from additional donors. These results underscore the need for further investigation into the sex-specific developmental effects of these chemicals on the heart, and highlight the utility of iPSC-CM for this purpose.

S 1242 Species and Sex-Specific Liver Microtissues: A Set of Micro-physiological Systems to Assess Translational Hepatotoxicity in Drug Development

M. Nag, M. Vittoria Colombo, F. Wenz, L. Fäs, A. Pawlowska, and B. Filippi. InSphero AG, Schlieren, Switzerland. Sponsor: K. Sullivan

The hepatotoxic effects of certain drugs differ significantly between humans and animals due to species-specific biological mechanisms. Interestingly, expanding on that theme, there are certain classes of drugs that differ in the way they are metabolized in biological females versus biological males, thereby, possibly flagging different risks. Species and sex specific liver microtissues can accurately recapitulate these differences, providing reliable preclinical toxicity assessments. Liver microtissues were developed from parenchymal and non-parenchymal cells isolated from *Macaca fascicularis* (Cynomolgus monkey), *Canis familiaris* (Beagle dog), *Rattus norvegicus* (Sprague Dawley rat), and humans. The microtissues were exposed to two FDA-approved drugs—fialuridine and chlorpromazine—and evaluated for cytotoxicity and transcriptomic responses. Cytotoxicity assays showed fialuridine was significantly more toxic to human liver microtissues compared to animal counterparts. Chlorpromazine, with no known species-specific hepatotoxicity, exhibited comparable cytotoxicity levels across all tested species. This is in agreement with clinical data. No differential hepatotoxicity *in vivo* has been reported for chlorpromazine. Transcriptomic analyses revealed a distinct response to fialuridine exposure in human liver microtissues, including significant alterations in genes associated with DNA damage response pathways. Multiple gene sets, encompassing genes acting

in the DNA damage response cascade, are specifically upregulated in human liver microtissues treated with low concentrations of fialuridine. These findings demonstrate that species-specific liver microtissues effectively capture critical differences in drug-induced hepatotoxicity. Following the same experimental rationale, we decided to test a rank ordered list of twenty drugs from the FDA's DILIRank database that were hypothesized to have differential effects in males versus females. These drugs will be tested for hepatotoxicity at both the level of the individual donor as well as with pooled donors. We expect that the differences that we see at the functional and transcriptomics levels will give us more insight into the differential mechanisms involved in hepatotoxicity and lay the foundation for employing this approach in other areas of toxicology and safety pharmacology.

S 1243 Advancing Female-Specific PBK Modeling: From Sex-Specific Organs to Whole-Body Pharmacokinetics

S. Fischer-Holzhausen. ESQlabs, Oslo, Norway. Sponsor: K. Sullivan

Biological differences between males and females extend well beyond the reproductive organs and their endocrine regulation. Sex dimorphism is described across human biology including anatomy, metabolism, the cardiovascular and respiratory system, as well as physiological processes such as immune function. Consequently, biological sex can affect how chemicals are absorbed, distributed, metabolized, and excreted by the body and thus plays a key role in determining potential risks. Physiologically based kinetic (PBK) and Quantitative systems modeling are established approaches that can incorporate sex differences in their model structure and parametrization. Historically, however, PBK model models have been developed using male reference physiology, often overlooking sex-specific anatomical and physiological differences. Our work addresses this gap by developing and validating female-tailored models. Leveraging the open-source modeling ecosystem provided by Open Systems Pharmacology, we are developing model extensions to address female-specific challenges in drug and chemical development. One such advancement is the extension of PBK models to include the female reproductive tract (FRT) – a structure usually neglected in standard PBK models. For the FRT extension module, four tissue compartments (cervix, vagina, endometrium, and myometrium) and two fluid compartments (uterine and cervicovaginal fluid) were informed using publicly available data. We validated the model for levonorgestrel and metronidazole for different routes of exposure (e.g. oral and vaginal route) by comparing predictions to clinical data, using goodness-of-fit plots and pharmacokinetic parameters. With minor parameter adjustments most model predictions fall within the 2-fold deviation. By predicting FRT tissues and plasma concentration for several routes of exposure, this extension addresses a gap to enable female-specific decision-making. Looking ahead, integrating PBK models with detailed endocrine regulatory networks represents a promising avenue for advancing a comprehensive women's health modeling platform covering female-specific anatomical structures and beyond, to physiological and metabolic differences throughout the body. Such integrative approaches will support model-informed decision-making in drug development and chemical safety assessment.

W 1244 Endocrine Disruption Safety Assessment Approaches for Cosmetics

K. Fussell. Wella Company, Darmstadt, Germany.

Disruption of endocrine system homeostasis is a toxicological hazard, one which is under increasing regulatory scrutiny. Within the context of the EU Regulation for Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) and the US Endocrine Disruptor Screening Project, substances are systematically evaluated for endocrine disruption (ED) against three criteria: an endocrine mode of action, adverse effects in intact organisms or (sub)populations, and a biologically plausible link between the mode of action and the observed effect. This evaluation currently demands the use of animal models for determination of the second criterion of adversity. For cosmetic ingredients, which are managed in the US and EU within the regulatory contexts of REACH, the EU Cosmetics Directive, the US Modernization of Cosmetics Regulation Act, and the US Toxic Substances Control Act, such data generated after the EU 2013 ban on animal testing cannot be used to assess their safety; yet, *in vivo* data generated prior to the ban do not fully address these ED regulatory requirements. In this context, the incorporation of results generated with new alternative methods into an integrated safety assessment has become imperative for cosmetics. Next-Generation risk assessments (NGRAs) are a means to integrate historical *in vivo* findings and novel *in silico* and *in vitro* data into a single, transparent risk profile. The resulting weight of evidence (WOE) evaluation provides a comprehensive assurance of human safety, leveraging data from the new alternative methods to plug current data gaps without the use of new animal studies—in effect modernizing older data beyond their historical limitations. This is particularly critical for thyroid disruption, which under previous guidelines was only detectable as altered thyroid gland histopathology. This Workshop aims to address the scientific suitability of NGRA-based approaches to the safety assessment of cosmetic ingredients with potential endocrine activities. Importantly, perspectives of both regulators and regulated industries will be evenly represented, including both EU and US regulatory contexts and geographies. These diverse speakers will explore the application

of NAMs data to address real-world concerns due to data gaps. Several practical state-of-the-art NGRA cases involving potential thyroid-disrupting substances and their WOE for human safety decision-making will be discussed. The first presenter, as a subject-matter expert at several EU regulatory authorities, will use 4-methylbenzylidene camphor to discuss the EU regulatory body perspective, highlighting some current issues with NGRA acceptance and proposing some next steps. The second speaker, representing an EU-corporate perspective, will cover an assessment approach suitable for the EU regulatory environment, which uses toxicokinetic data to bridge between *in vitro* thyroid peroxidase measures and findings from 90-day rat exposures conducted under previous OECD 408 guidelines. The third presenter is a member of a US regulatory authority and will address potential thyroid disruption during the critical fetal development window using a first trimester maternal-fetal high-throughput *in vitro* to *in vivo* extrapolation model to simulate thyroid active exposures from ToxCast activities. The final industry speaker from a US-based, consumer-products multinational will present a NGRA for the use of fluoride in oral health products, taking into account its alleged thyroid-disruption potential. Following each presentation, time permitting, the audience will have the opportunity to ask one or two brief questions. Lastly, the four speakers will join a dedicated panel discussion session in which additional questions from the floor will be addressed to allow deeper engagement with the speakers, as well as a structured dialogue using polling, both to be moderated by the session Chair.

W 1245 NGRA for Cosmetics Use: What Next Steps Are Needed

N. Cabaton. INRAE Toxalim, Toulouse, France. Sponsor: K. Fussell

Next generation risk assessment for cosmetic product ingredients represents a major advancement in the European Union's regulatory approach. Faced with health security challenges and increasing transparency demands, the EU aims to integrate alternative methods to animal testing, such as *in silico* models and *in vitro* tests. However, several challenges have to be met, such as the scientific and regulatory validation of these new approaches which remains a long and complex process. International standard harmonization poses a significant obstacle to the implementation of these new methods. To overcome these challenges, the EU could strengthen collaboration between researchers, regulators and industry professionals to accelerate the validation and acceptance of alternative methods. A case study describing the risk assessment of 4-methylbenzylidene camphor will serve as an example to highlight the gaps that need to be filled, particularly for extrapolations to humans from *in vitro* or *in silico* test results, often focusing on a single organ or tissue for which existing models are not yet sufficiently efficient and does not always reflect what happens in a more integrated model.

W 1246 Hazard Assessment of Endocrine-Disruption Potential Using New Alternative Methods: Use of Toxicokinetic Data to Bridge Endocrine Activity, Endocrine Disruption, and Human Health Risk

K. Fussell. Wella Company, Darmstadt, Germany.

Endocrine Disruption (ED) is a toxicological hazard defined by three criteria, only one of which can currently be assessed using new alternative methods (NAMs). The others currently require the use of animal models to meet regulatory requirements. In an effort to avoid additional animal testing we introduce an integrated hazard assessment using NAMs-generated toxicokinetic and endocrine data. For this purpose, we determined the metabolic stabilities of the hair dyes resorcinol (Res), p-aminophenol (PAP) and 3-amino-2,6-dimethylphenol (AdMP) in by human skin keratinocytes and liver hepatocytes with identification of all significant metabolites. In addition, intrinsic hepatic clearance and fraction unbound in plasma were modeled using the ToxCast HHTK package for R and used to calculate the volume of distribution and the steady-state plasma concentration, i.e. the systemically available levels of each substance. QSAR modeling was then applied to predict the potential of Res, PAP, AdMP or their metabolites for (anti-)estrogenic, anti-androgenic, thyroid- or steroidogenic activities. Thyroid peroxidase (TPO) inhibition was identified as the main effect of concern. The relevant metabolites were then synthesized and tested for TPO inhibition activity using the *in vitro* tyrosine iodination assay. Although inhibition was observed in the sensitive assay, our data indicate that the systemically available levels of Res, PAP, AdMP and their metabolites do not inhibit TPO. This is supported by the results of 13-week OECD 408 studies in rats demonstrating a lack of thyroid effects, as well as a general lack of such findings in modern pharmacovigilance and cosmetovigilance reports. Together these findings indicate no relevant hazard from cosmetic use of Res, PAP and AdMP and suggest the validity of our NAMs-based safety assessment for the determination of ED for regulatory purposes.

W 1247 Interpretation of Thyroid-Relevant Bioactivity Data for Comparison to *In Vivo* Exposures: A Prioritization Approach for Putative Chemical Inhibitors of *In Vitro* Deiodinase Activity

K. Paul Friedman. UL Research Institutes, Research Triangle Park, NC.

Many ToxCast assay endpoints can be mapped to molecular initiating events (MIEs) within the thyroid adverse outcome pathway (AOP) network, but a framework for interpretation of thyroid-relevant bioactivity data across MIEs has been lacking. Using ToxCast data on the inhibition of deiodinase (DIO) enzymes, which convert thyroid hormones between active and inactive forms, substances most likely to inhibit DIO enzymes were identified. Data from 4 relevant cell-free *in vitro* assays for chemicals in multi-concentration screening were filtered to identify chemicals that demonstrated inhibition for each DIO enzyme less likely to be confounded by assay interference, refining the list of putatively active chemicals from 523 to 135. *In vitro* bioactivity data were then used to estimate administered equivalent doses (AEDs) using a novel high throughput toxicokinetic (HTTK) model for *in vitro* to *in vivo* extrapolation (IVIVE) of dose. To understand thyroid-disrupting activity in an appropriate dose context, we extended an existing human maternal-fetal HTTK model to allow for simulations involving the first trimester of pregnancy. For many chemicals, simulations of fetal tissue concentrations produced lower AED estimates than using modeled maternal plasma concentrations alone, at least partially due to conservative assumptions in our HTTK model of complete gestation. This extensible approach for MIE groups of thyroid-related bioactivity data from ToxCast may inform further screening or analyses for potential adverse outcomes during pregnancy and development. *This abstract does not reflect US EPA policy.*

W 1248 Application of Next-Generation Risk Assessment to Better Understand ED Risk: Bringing Fluoride into Focus

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Cited as one of the ten greatest public health interventions of the 20th century, fluoride has left an indelible mark for its role in both reducing dental cavities but also improving general oral health of adults and children since the inception of US water fluoridation. However, fluoride has come under recent scrutiny regarding concerns over potential neurodevelopmental and endocrine-related toxicities. Several reviews have been conducted by authoritative bodies such as the NTP and EFSA, who concluded with moderate/reasonable confidence that higher levels of fluoride exposure (ie those above the upper limit in drinking water of 1.5 mg/L) are associated with modest decrements in IQ in children. EFSA also determined that adverse effects on the CNS, including neurobehavior or thyroid homeostasis, are unlikely to occur in children at exposures below those associated with dental fluorosis, making this a protective critical effect to utilize in risk assessment for this population. Thyroid homeostasis is of importance, as this is hypothesized to be associated with the epidemiological observations on IQ. A key outcome of these reviews is the level of uncertainty regarding whether these modest findings are biologically plausible. To address this outage and better understand if fluoride exerts direct biological activity on molecular and cellular targets related to the EATS modalities, an NGRA approach consisting of receptor binding assays of thyroid and other hormone related targets, H295R Steroidogenesis, and Sodium-Iodide Symporter (NIS) assays were carried out using exposures at and well in excess to those in the general public. Results showed that at levels well in excess of typical exposures, fluoride did not alter hormone levels in the steroidogenesis assay or bind to classical targets such as aromatase, steroid 5 alpha-reductase, estrogen or androgen receptors. Additionally, no alterations in iodide uptake via the sodium-iodide symporter or binding to thyroid-related targets such as the thyroid receptor or thyroid peroxidase were observed, and binding with other related targets, including but not limited to glucocorticoid and constitutive androstane receptors, were also not observed. The negative results from this series of experiments provide key insights into the absence of effects on the studied targets well in excess of typical exposure scenarios.

W 1249 Optimizing Chemical Risk Assessment for Medical Device Biological Safety: Challenges, Progress, and Collaborative Opportunities

X. Dai. Abbott Laboratories, Saint Paul, MN.

To ensure the biological safety of medical devices, manufacturers comply with international standards (ISO 10993). Use of the ISO 10993 series of standards enables more consistent evaluation of devices and helps drive harmonization of safety requirements for global regulatory submissions. With ISO 10993-17:2023 recognition by the US FDA and ongoing work tackling analytical chemistry issues associated with implementation of ISO 10993-18, regulatory demands have significantly increased. Despite significant progress to develop and update consistent and effective methods, unique challenges remain on how to determine potential toxicological risks for chemicals released from devices during clinical exposure. Inherent complexities of medical device material composition and manufacturing processes place unique hurdles in the path to advance and harmonize regulatory acceptance of biological safety risk without compromising a rigorous evaluation of accumulated safety

evidence. Currently, regulatory submission strategies rely on general methodologies defined in ISO 10993 with regulatory emphasis on being “most protective.” This approach potentially results in overestimated chemical safety risks, which can delay patient access or require unnecessary implementation of safety controls. A technical gap to address an unmet regulatory need represents an opportunity for well-defined approaches that determine potential chemical harms from clinical exposure to patients and are applicable for medical device chemical hazard and risk assessment. Multidisciplinary collaboration is critical to determine more relevant analytical conditions to human exposure and to generate exposure data specific to the intended clinical-use condition for the ultimate purpose of protecting patients from potential chemical harm when treating conditions with medical devices. In this session, experts from regulatory, industry, and nongovernmental organizations will come together to discuss the need, challenges, progress, and opportunities for a consensus perspective on optimizing and gaining confidence in chemical risk assessment approaches to improve confidence in medical device biological safety. The first presentation will discuss the current state-of-art chemical characterization studies from scientific, regulatory, and international standards perspectives. Key analytical opportunities affecting data quality will be addressed, including simulating solvents, analytical evaluation threshold (AET), uncertainty factor (UF), quantification by non-targeted screening (NTS), etc. Finally, suggestions for improving chemical characterization studies without compromising data quality will be proposed to stimulate discussion and collaboration among scientists, regulators, and standards. The second presentation will provide an industry perspective and present case studies related to device extractables and leachables that aim to capture practical applications, while discussing current technical limitations and challenges, such as the considerations of specific and appropriate chemical characterization conditions and parameters. The third presentation will highlight the implementation of new ISO 10993-1 and ISO 10993-17 standards to facilitate risk-based framework for biocompatibility assessments, focusing on the toxicological risk assessment of medical devices to address clinically relevant toxicological risks in addition to lessons learned following the release of the revised standard. The fourth presentation will focus on reviewing recent advancement in computational power to predict human exposure and pharmacokinetic parameters of nanomaterials. This review will draw from academic research and explore potential practical applications for the biological safety evaluation of medical devices that contain or are composed of nanomaterials. The fifth presentation will share advancements in regulatory science tools developed by US FDA CDRH Office of Science and Engineering Laboratories with a goal to provide clinically relevant, yet still conservative, chemical patient exposure estimates using a physics-based transport model for polymeric systems. It further explores the application of this extraction model to current ISO 10993-18 standard updates. Attendees will be invited to a short Q&A and panel discussion to learn more perspectives on the evolving nature and how to further expand state-of-the-art approaches and to delve into collaborative engaging activities to advance further scientific development of innovative approaches to assure the safety of medical device. The Workshop serves well for those who desire advanced knowledge and learning in the field of medical device and combination products.

W 1250 Can Chemical Characterization Studies of Medical Devices Be Streamlined Without Affecting Their Safety Assessment Through Toxicological Risk Evaluation?

J. Li. Chemical Characterization Solutions LLC, Woodbury, MN. Sponsor: X. Dai

The toxicological risk assessment for medical devices, as outlined in ISO 10993-17, heavily depends on the precision and accuracy of chemical characterization results conducted in line with ISO 10993-18, especially in terms of quantification and identification. Despite the implementation of ISO 10993-18:2020 five years ago, the standard has not fully achieved its intended outcomes due to various scientific challenges. Consequently, there is considerable variability among many contract research organizations (CROs), and the accuracy of data regarding both exposure levels and chemical identities within the same laboratories can remain uncertain. This presentation is intended to cover the following topics: 1) A comprehensive overview of the essential role that chemical characterization plays within regulatory frameworks for the approval and market entry of medical devices; 2) Current chemical characterization: scientific, regulatory, and ISO standards perspectives; 3) Summary of key complexities in chemical characterization of medical devices; 4) Discussion on scientific opportunities in chemical characterization. The presentation will contain comprehensive information and data, making it suitable for toxicologists, chemists, and professionals involved in the biological evaluation of medical device safety. It also aims to encourage dialogue and cooperation among regulators, scientists, and standards bodies.

W 1251 Practical Consideration with Case Studies on Clinically Relevant Patient Exposure via Targeted Chemical Analysis and Simulated-Use Extraction

S. Zhang. Abbott Laboratories, Little Canada, MN.

Chemical characterization per ISO10993-18 to provide critical patient chemical exposure data for the risk assessment assure the biological safety evaluation of toxicological effects for medical devices. For long-term implantable devices, exhaustive extractions are recommended to produce a worst-case patient exposure. When any exhaustive extractable potentially presents a toxicological risk, a more physiological relevant chemical characterization study is necessary to understand the patient exposure clinically, i.e. a leachables study under simulated use conditions. However, due to the complexity of medical device contact nature, the extraction condition to simulate the clinical use of the device, and the correlation of extractables release under exhaustive and simulated use conditions are not well understood. This presentation will provide practical considerations with a case study on the extractables release kinetics of a cardiovascular device incorporating complex materials and manufacturing processes. The extractables released from exhaustive extraction solvents and the commonly recognized blood simulating solvent will be compared and the toxicological impact in light of the clinical use conditions of the device will be discussed. In additional, evaluation of solvent systems to simulate the blood contact of various polymeric materials used in implantable medical devices will be discussed.

W 1252 Navigating ISO 10993-17 and 10993-1: Evolving Standards, Practical Insights, and Regulatory Impacts

X. Dai. Abbott Laboratories, St. Paul, MN.

Recent updates to ISO 10993-1 emphasize a more dynamic, risk-based framework for biological safety assessments. Many of the proposed changes to Part 1, including expanded biological effects for various device categories, are recommended to address by chemical characterization and toxicological risk assessment (TRA). ISO 10993-17 revision in September 2023, retitled Toxicological Risk Assessment of Medical Device Constituents, reflects a shift toward a more systematic approach to evaluate toxicological risks. This includes new concepts to offer practical tools for streamlining TRAs and strengthening weight-of-evidence evaluations, particularly for devices undergoing exhaustive extractions (e.g., long term implantable devices). This presentation will explore the application of these tools, their integration into broader biological safety evaluations, and their role within the overall risk management framework. As these methodologies are adopted, limitations and uncertainties have emerged. For example, how should these tools be applied to devices used in young pediatric patients? What approaches are appropriate for constituents with suspected carcinogens? When should simulated-use extraction studies be initiated to reliably estimate maximum total quantity for patient exposure? These challenges will be discussed alongside scenarios where caution is warranted and regulatory feedback that may influence future applications of ISO 10993 standards. Attendees will gain insights into the evolving landscape of biological safety risk assessment, practical strategies for implementing new TRA tools, and lessons learned from applications.

W 1253 Technical Advancements in Computational Technologies to Support Biological Safety Risk Assessment of Nanomaterials

T. Puzyn. Uniwersytetu Gdański, Gdansk, Poland. Sponsor: X. Dai

The biological safety evaluation of medical devices made of nanoparticles presents unique challenges due to distinct physicochemical characteristics of nanomaterial, such as chemical compositions and physical properties, which are critical to toxicities. Nanomaterials in medical devices can be present as free or embedded nanomaterials, potentially releasing into the body, posing significant toxicological risks. The existing knowledge gap regarding the mechanistic information on toxicity potency of nanomaterials necessitates thorough data assessment using advanced methodologies for risk evaluation to ensure regulatory decision making and patient safety. Recent advancements in computational power, big data informatics, artificial intelligence assisted analysis, and machine learning models have significantly enhanced the integration of mechanistic data on chemical and physical properties with biological information. These technologies better predict toxicity parameters for nanoparticles. Especially aggregating extensive ongoing research and case studies from academia provides further insights into the continuous development of international ISO standard for medical device safety.

W 1254 Incorporating New Approach Methods into Regulatory Science Tools for Toxicological Risk Assessment of Medical Device Leachables

D. Saylor. US FDA/CDRH, Silver Spring, MD. Sponsor: X. Dai

Toxicological risk assessment (TRA) is a common tool used to address toxicological concerns associated with chemicals that can leach from medical device materials. TRA currently relies primarily on *in vitro* extraction testing to estimate the potential for patient exposure, but the clinical relevance of the test results is often ambiguous. Regulatory science tools incorporating New Approach Methods (NAMs) are being developed to improve the current TRA paradigm. We have developed physics-based mass transport models that can be used to supplement or in lieu of *in vitro* testing to provide more clinically relevant exposure estimates of medical device leachables. Moreover, we have created web-based applications, collectively known as Chemical RISK calculators (CHRIS), to facilitate the use of these models by the medical device community. This presentation will provide an overview of the mass transport models for both *in vivo* exposure estimation and *in vitro* extractables testing. Further, we will describe efforts to conservatively establish system specific model parameters and validate the model predictions. The potential benefits and limitations will be discussed, along with ongoing efforts to expand the applicability and improve the clinical relevance of the model predictions. Additionally, this session will highlight how these advancements may help to reduce reliance on animal testing and enhance the use of *in vitro*, *in silico*, and *in chemico* methods, thereby promoting more ethical, rapid, and accurate safety assessments.

W 1255 Toxic Air, Targeted Care: Innovations in Inhalation Injury and Intervention Through Early Career Perspectives

B. Baird. University of New Mexico, Albuquerque, NM.

The American Lung Association reports that nearly half of Americans live in areas with unhealthy air, and western states dominate the list of most polluted US cities. This session brings together a diverse array of studies that collectively deepen our understanding of how inhaled environmental pollutants—ranging from nanoparticles and ozone to woodsmoke—disrupt physiological systems and contribute to disease. Despite differing pollutants and experimental models, a central theme emerges: air pollution induces complex, multi-organ inflammatory responses that are profoundly shaped by biological context, including sex, metabolic health, diet, and neuroendocrine signaling. Across all six presentations, lipid mediators and immune signaling surface as key drivers of pollutant-induced pathology. Several talks highlight the role of lipid-derived molecules—such as prostaglandins, resolvins, and cholesterol derivatives—in either exacerbating or resolving inflammation, depending on the exposure and physiological background. Equally prominent is the recognition that underlying conditions like metabolic syndrome, dietary composition, and circulating hormones critically influence both the magnitude and duration of pollutant effects. A recurring mechanistic thread throughout the session is the disruption of barrier integrity—whether in the airway epithelium or the blood-brain barrier—leading to heightened susceptibility to immune cell infiltration and sustained inflammatory damage. These effects are compounded by sex differences and neurohormonal regulation, with emerging data pointing to receptor-mediated crosstalk between stress and sex hormone pathways as modulators of pollutant response. Collectively, these talks emphasize the importance of context—metabolic, hormonal, and nutritional—in shaping individual vulnerability to air pollution. They also offer promising avenues for intervention, including dietary strategies (e.g., omega-3 fatty acids, vitamin D), pharmacological modulation of signaling pathways (e.g., MAPK, Rho-kinase), and hormone receptor targeting. This session underscores a growing recognition that personalized, systems-level approaches are essential for protecting vulnerable populations and mitigating the long-term health consequences of air pollution. Collectively, these presentations showcase the multifaceted nature of inhalation research and potential interventions to optimize health outcome, stories all shared by early career scientists. This session aims to foster discussions, specifically among trainees, encouraging collaboration and further exploration into the complex world of inhalation toxicology and therapeutic interventions during a concluding Q&A panel.

W 1256 Beyond Smoke and Mirrors: Introducing Inhalation Toxicology Therapeutics

S. Phatak. University of New Mexico, Albuquerque, NM.

The World Bank estimates that health damages from air pollution cost the global economy approximately \$6 trillion annually, equivalent to about 5% of global GDP. Air pollution is a persistent and evolving global health concern, with growing evidence linking environmental exposures to a wide array of adverse outcomes across organ systems. This session explores the mechanistic underpinnings and modifiers of health effects induced by various inhaled pollutants—ranging from nano-sized particles and woodsmoke to ozone—focusing on pulmonary and neurological outcomes, systemic inflammation, and susceptibility factors. A critical component in understanding pollutant toxicity lies in the physicochemical characterization of inhaled nanoparticles, including parameters such as size, surface charge, solubility,

and chemical composition, which influence their biological interactions and health effects. To contextualize the material that follows, it is essential to understand that inhaled pollutants interact with biological systems not merely through local airway effects, but also by influencing lipid mediators, immune signaling, and neurohormonal pathways. These interactions can vary based on underlying conditions such as metabolic syndrome, diet, sex, and genetic background. Lipid mediators—such as prostaglandins, resolvins, and protectins—play key roles in inflammation initiation and resolution, and disruption of these pathways can tip the balance toward chronic inflammation. Equally important are the structural and immunological functions of airway epithelial cells, which act as the first line of defense but can be compromised following pollutant exposure, leading to heightened immune cell infiltration and impaired tissue recovery. The structure of the session will be described, including the sequence of research presentations and the format of the Q&A discussion, where trainees are particularly encouraged to engage.

W 1257 Inhalation of Nanomaterials Disrupts Inflammatory Lipid Mediators in a Sex- and Disease-Dependent Manner

A. Pitchai. Purdue University, West Lafayette, IN.

Nanosized particles pose health risks following inhalation exposure. Most evaluations of nanoparticle safety have focused on healthy models. However, epidemiology studies have demonstrated individuals with metabolic disease are more vulnerable to particulate air pollution. Our research has determined a metabolic syndrome (MetS) male mouse model exhibits specific alterations in lipid mediators involved in inflammatory regulation and increased lung inflammation after exposure to silver nanoparticles (AgNPs), compared to a healthy male mouse model. Sex differences exist in lipid regulation between males and females, which may influence susceptibility to nanoparticle inhalation exposures. Further, as with males the prevalence of MetS in females has risen drastically and may influence exposure-induced susceptibility. Our study hypothesized sex-dependent inflammatory processes would be modulated due to MetS following AgNP exposure via lipid dysregulation. C57BL/6J male and female mice were fed either a healthy diet or a high-fat Western diet for 14 weeks. Subsets of mice were exposed to 50 μ l of water (control) or 50 μ l of 20 nm AgNPs at 1 mg/ml via oropharyngeal aspiration. Endpoints of lung and systemic injury, inflammation, and lipid dysregulations were evaluated 24 hours post-exposure. Analysis of bronchoalveolar lavage fluid revealed an exacerbation of AgNP-induced neutrophilic influx due to MetS in both sexes compared to healthy, while female MetS mice demonstrated elevated neutrophilia compared to male MetS mice. The exacerbation of neutrophils in female MetS was related to AgNP modulation of gene expression of pro-inflammatory mediators IL-6, CCL-2, and CXCL-2 as well as lipid metabolizing enzymes such as COX-2 and ALOX-15. Assessment of lung lipid alterations demonstrated exposure to AgNP induces inflammatory responses through prostaglandin E2 (PGE2) pathways and disrupts the protectin D1 (PD1) resolution pathway in female MetS mice. Overall, notable sex differences were identified, particularly in the presence of MetS, indicating a sex-specific vulnerability linked to the underlying disease. This vulnerability may be influenced by sex-specific dysregulation of lipid signaling following inhaled particle exposures.

W 1258 Woodsmoke-Induced Airway Injury Is Preventable with MEK Inhibition

C. Cherry. University of California Los Angeles, Los Angeles, CA.

From the warmth of woodburning stoves or campfires to the devastation of wildfires, woodsmoke (WFS) permeates both our ambient and household environment, but the health risks from these exposures are not known. Upper airway samples from patients exposed to the Los Angeles wildfires demonstrate epithelial tissue damage, including loss of the airway epithelial barrier and thickened basement membrane. Consequently, transmission electron microscopy demonstrates intracellular localization of metals and ultrafine particulates, including within surface airway epithelial cells and tight junctions. To further understand this phenotype, we developed an environmental exposure system to directly expose primary human airway cultures to woodfire smoke (WFS) that closely replicates the chemical and particulate composition of WFS from ambient biomass combustion. Exposure of the human airway cultures to WFS shows transcriptional downregulation of cytoskeletal and microtubule binding genes. While minimal changes occur in protein abundance, phosphoproteomic analysis reveals dephosphorylation of cilia-related, tight junction, and cytoskeletal proteins that contribute to impaired ciliary function. Ultimately, these changes lead to loss of epithelial barrier integrity, as well as ciliary paralysis, which only partially recovers over time. Furthermore, we demonstrate that inhibition of the MAPK pathway during or after WFS-induced epithelial injury preserves tight junction integrity together with ciliary motility, allowing for maintenance of the epithelial barrier. Overall, this work offers mechanistic insights into WFS-induced airway injury observed both *in vitro* and *in vivo* and highlights a promising therapeutic strategy for mitigating this injury. These findings underscore the potential of this therapeutic approach to protect populations at high risk of wildfire smoke exposure, including communities in fire-prone regions, firefighters, individuals with occupational exposures, and military personnel exposed to burn pits, offering a strategy to mitigate airway injury and improve respiratory health in these vulnerable groups.

K. Schichlein. University of North Carolina at Chapel Hill, Chapel Hill, NC.

Ozone is a common respiratory irritant formed through photochemical reactions of nitrogen oxides (NOx) and volatile organic compounds (VOCs). Exposure is linked to increased risks of cardiopulmonary and neurological diseases, cancer, and mortality. In the respiratory tract, chronic ozone exposure can contribute to lung disease pathogenesis—including COPD and asthma—especially in adolescents, through mechanisms such as airway inflammation, hyperreactivity, reduced pulmonary function, increased infection susceptibility, and exacerbation of pre-existing conditions. Susceptibility to ozone's effects varies due to genetics, age, sex, comorbidities, and dietary factors. Our lab recently conducted an exploratory study examining how acute ozone exposure alters cholesterol biosynthesis derivatives in the lung and systemically. We found significant changes in cholesterol precursors and derivatives in plasma and sputum of asthmatic and non-asthmatic subjects. Notably, in non-asthmatics, the primary circulating form of vitamin D, 25-hydroxyvitamin D, positively correlated with lung function and sputum uteroglobin and negatively with sputum neutrophils, total cell count, and multiple cytokines—suggesting a protective role against ozone-induced inflammation and functional decline. Given these findings, we hypothesized that vitamin D supplementation could offer protection from ozone exposure. Although oral vitamin D has been proposed as a treatment for lung diseases such as COPD, asthma, influenza, and COVID-19, results have been mixed despite clear associations between vitamin D status and disease outcomes. We proposed that inhaled vitamin D may offer a more effective delivery route to protect the lungs from ozone damage. In this study, we expanded on prior findings by (a) examining vitamin D's effects on ozone-induced inflammation in airway epithelial cells, (b) evaluating the protective potential of nebulized apical vitamin D administration, and (c) investigating the cellular mechanisms underlying vitamin D's protective effects

W 1260 **Targeting Wildfire Smoke-Induced Neuroinflammation: Mechanisms of Immune Cell Infiltration and Emerging Interventions**

B. Baird. University of New Mexico, Albuquerque, NM.

Over the past four decades, wildfires in the U.S. have intensified, largely due to climate change. The resulting smoke poses serious health risks, including cardiovascular, respiratory, and neurological effects. A key neurological consequence is neuroinflammation, which may lead to both acute and long-term outcomes. This study investigates the immune timeline of woodsmoke-induced neuroinflammation and explores dietary and therapeutic interventions, including lipid mediators and Rho-Kinase inhibitors. Young female C57BL/6 mice were exposed to either filtered air or woodsmoke (500 µg/m³ particulate matter) for 4 hours/day on alternate days over 14 days. Exposure was generated using a smoldering temperature, creating a distinct particulate matter profile with unique physiochemical properties compared to complete combustion. Brains were examined at 1, 14, and 28 days post-exposure using high-dimensional flow cytometry to assess markers of peripheral immune infiltration (CD3, CD4, CD8, LFA-1), resident immune cells (CD11b, ACSA-2), and inflammation-related proteins. Two high-fat diets—a coconut oil diet and a Western diet—worsened immune cell infiltration and delayed resolution of inflammation. Additionally, the Rho-Kinase inhibitor fasudil was tested for its ability to stabilize the blood-brain barrier and reduce immune cell influx. The Omega-3 fatty acid Resolvin D1 (RVD1) was administered to assess its anti-inflammatory vascular effects. Results showed a diet- and time-dependent increase in CD4 T cells (CD3+, RORγ+, FOXP3-, CD44+) that may promote inflammation resolution. Immune markers such as LFA-1, ICAM, and VCAM peaked one day after the final exposure, indicating a sustained inflammatory response. Both high-fat diets prolonged this response beyond 28 days. Fasudil significantly reduced immune cell entry into the brain, suggesting that blood-brain barrier compromise contributes to neuroinflammation. RVD1 effectively countered the pro-inflammatory effects of the Western diet and accelerated recovery, shortening the inflammation timeline by two weeks. Overall, this study highlights the role of peripheral immune cells in CNS inflammation following woodsmoke exposure and demonstrates the potential of dietary and pharmacological interventions to mitigate these effects.

W 1261 **Air Pollution Derived Fe-Oxide Inhalation Produces Persistent Sex-Specific Behavioral and Morphological Deficits in Mice**

J. George. University of Rochester Medical Center, Rochester, NY.

New abstract to replace the EPA speaker's abstract (new abstract needed to remove the EPA references):

Iron-overload, a hallmark and potential contributor to neurodegeneration, comprises a large portion of ultrafine particulate (UFP) matter of air pollution, with especially high concentrations in US subway systems. Previous research demonstrated that

Fe-oxide nanoparticles can translocate along cranial nerves into the brain, form deposits within the brain, and associate with degenerating myelin. We hypothesize that the essential role Fe plays in myelination and the direct physical damage caused by Fe-oxide nanoparticle transport along axons can contribute to the sex-specific white matter dysregulation observed in neurodegeneration. To assess the impact of Fe-oxide nanoparticle inhalation and age-related increases in brain Fe content on white matter, two cohorts of 10-week-old C57BL/6 mice were randomly exposed to HEPA-filtered air or 1.5 ng/L iron-oxide UFPs for two hours daily for 20 days. An acute cohort was either acutely sacrificed followed by MRH or subjected to a behavioral battery. The other cohort aged undisturbed in their home cage until MRH, and a broad behavioral battery were performed at 12 and reassessed at 18 months. Micro-dissected corpus callosum from 18-month-old animals were sent for electron microscopy imaging. Acutely, Fe-exposure produced sex-specific diffusivity differences in olfactory bulb, with Fe-exposed females exhibiting increases in diffusion indices and males decreases. At 12 months, significant increases, not decreases, in fractional anisotropy in both sexes were observed in caudal regions along the olfactory circuit. In Novel Object Recognition, Fe-exposed female mice had significantly lower recognition indices acutely and at 12 months indicating a persistent, female-specific memory deficit. No locomotor or motor changes were observed in either sex at 12 months of age, however, at 18 months, Fe-exposed male mice exhibited significant hypoactivity on a locomotor assay and significantly more footslips on a 2.5cm and 1.25cm balance beam on days 2 and 3, representing an emergent male-specific motor deficit after early adulthood Fe-oxide exposure. Ultrastructural 18-month corpus callosum myelin analyses demonstrated Fe-induced female-specific decreases in g-ratio and increases in axon, fiber, and myelin area indicating thicker myelin sheaths. However, Fe-exposed female mice were also found to have significantly more split myelin sheaths indicating significant myelin dysregulation in Fe-exposed females. Contrastingly, Fe exposure significantly increased total fiber and myelinated areas in males without concomitant decreases to g-ratio or increases in axonal area. Similar to Fe-exposure in females, Fe-exposed males also had significantly more split myelin area. However, when considering geometry of the axons, significant sex-specific changes to circularity index were observed with Fe exposure reducing circularity index in females but increasing the index in males. This change in axonal geometry could potentially underlie sex differences in diffusivity. Early adulthood Fe-oxide nanoparticle inhalation produces sex-specific behavioral and morphological differences that persist and even, emerge, with age. The observed behavioral differences indicate that Fe-exposures produces sex-specific behavioral alterations with females exhibiting persistent short-term memory deficits and males exhibiting emergent motor deficits at 18 months. Fe-exposure produced acute sex-specific differences in white matter in the olfactory bulb and these diffusivity differences moved caudally with age. At the ultrastructural level, Fe-exposure produced sex-specific changes in g-ratio and axonal geometry which could potentially underlie diffusion differences. These data suggest that Fe-oxide inhalation can cause sex-specific white matter dysregulation with concomitant behavioral deficits.

IS 1262 **Advancing Risk Communication: Strengthening Collaborations Between Toxicologists and Exposure Scientists in the Face of a Changing Regulatory Landscape**

N. Erraguntla. American Chemistry Council, Washington, DC.

Clear and strategic risk communication among all stakeholders—including regulatory agencies at the state, federal, and international levels; industry; the scientific community; and the public—is essential to informed and effective decision-making. It enables all stakeholders to contribute as knowledgeable and trusted partners in conveying scientific evidence, operational practices, and exposure mitigation strategies. This collaborative communication fosters transparency; strengthens stakeholder trust; and supports the development of science-based, practical policies that protect public health and the environment. The 2016 amendments to the Toxic Substances Control Act (TSCA) ushered in a new era of comprehensive risk evaluation for high-priority existing chemicals and offered the opportunity to identify risk communication best practices among diverse stakeholders. The TSCA process provides an example of a complex regulatory process spanning chemical prioritization, risk evaluation, and risk management rulemaking. This process has created multiple layers of stakeholder engagement and information exchange. Key insights have emerged on how to successfully navigate and enhance these communication pathways to improve program outcomes. This session will examine effective risk communication strategies specific to the fields of exposure assessment, epidemiology, toxicology, and risk assessment. Regulatory experience has revealed common challenges and highlighted best practices for information clarity and stakeholder alignment. Refined methods for peer engagement—developed by the US EPA and stakeholders alike—are increasingly tailored to the goals of each communication effort. Research on selecting and optimizing these methods will be discussed. A critical lesson learned is the importance of a well-defined assessment purpose, clearly articulated through robust problem formulation. Case studies will illustrate how improved information flow supports US EPA analyses, particularly through comprehensive use-mapping that represents the full value chain. Product stewardship toxicologists are instrumental in providing context and clarity. Occupational toxicologists play an important role in contextualizing health risk values and fostering effective communication with workers and their representatives. Further, opportunities continue to emerge to more fully utilize exposure data to communicate risk characterization findings to communities adjacent to industrial facilities. As risk determinations give rise to

risk management rules, toxicologists serve a vital role in interpreting and conveying their implications to diverse audiences, including regulatory bodies, communities, and workers. Emerging technologies may further revolutionize how data and knowledge are shared in regulatory contexts. Toxicologists must develop proficiency in these evolving tools to remain effective communicators in the next generation of regulatory science. This session will highlight practical examples and innovative approaches to risk communication that are not only relevant to TSCA but also universally applicable across other regulatory frameworks in the toxicology field.

PL 2000 Multi-Task Neural-ODE Modeling of Nanoparticle Biodistribution in Tumor-Bearing Mice for Toxicological Risk Assessment

Z. Zhang, and Z. Lin. University of Florida, Gainesville, FL.

Background and Purpose: Understanding the biodistribution of nanoparticles in tumors and major organs is essential for advancing nanomedicine design, optimizing therapeutic efficacy, and minimizing off-target toxicity. Traditional pharmacokinetic (PK) models, while mechanistically interpretable, often struggle to generalize across heterogeneous nanoparticle formulations and tumor models. Conversely, purely data-driven machine learning methods can capture complex relationships but lack the temporal continuity and biological realism required for concentration-time prediction. Neural ordinary differential equations (Neural-ODEs) provide a promising framework by embedding differential equation solvers within neural networks, enabling flexible modeling of dynamic PK processes. We developed a multi-task Neural-ODE to predict nanoparticle biodistribution in tumors and six major organs of tumor-bearing mice, enabling zero-shot prediction for unseen formulations and providing a data-driven yet biologically grounded tool for nanomedicine development. **Methods:** This study compiled biodistribution data of nanoparticles from the Nano-Tumor Database reported by Chen et al. (2023, ACS Nano), which integrates 534 tumor datasets (2345 data points) and 1972 datasets (8461 data points) for five major organs (liver, spleen, lung, heart, and kidney). Categorical variables such as particle type and tumor model were represented through learned embeddings, while numeric features were log-scaled and standardized, with binary flags added for imputed values. A static context vector was formed by concatenating all embeddings and scaled features and was used to condition the Neural-ODE at three levels: the initial latent state after bolus dosing, the latent dynamics through feature-wise modulation, and the tissue-specific decoders. A multi-task learning framework was implemented by sharing one NODE backbone across all tissues, with organ-specific heads producing concentration-time profiles. Model training minimized mean squared error on log-transformed concentrations, with uncertainty-based loss weighting applied to balance tissue-specific prediction tasks. Feature screening was conducted within cross-validation using a consensus approach that combined LASSO regression, random forest permutation importance, and mutual information, retaining features consistently ranked among the most predictive. Data were split at the nanoparticle formulation level (80% training/validation set and 20% independent test set) to prevent information leakage across studies and to ensure generalization to unseen designs. Model evaluation was performed using a held-out test set, with performance assessed by coefficient of determination (R^2), root mean squared error (RMSE), and mean absolute error (MAE) on log-transformed concentrations. **Results:** The multi-task Neural-ODE model successfully captured the temporal dynamics of nanoparticle biodistribution across tumors and major organs. Cross-validation results demonstrated consistent performance across tissues, with mean R^2 values exceeding 0.70 for tumor and liver, and 0.60-0.68 for kidney, spleen, lung, and heart. The model effectively generalized to unseen nanoparticle formulations in the test set, achieving overall $R^2 = 0.65$, with root mean squared error (RMSE) $<0.25 \log(\%ID/g)$. Feature selection identified hydrodynamic size, zeta potential, targeting strategy, tumor size, and body weight as the most predictive covariates influencing biodistribution patterns. **Conclusions:** The development of a multi-task Neural-ODE framework that integrates nanoparticle physicochemical, tumor, and animal covariates has demonstrated strong potential for accurately predicting biodistribution time-courses across tumors and major organs in mice. Importantly, this framework provides a biologically grounded yet flexible approach for capturing both the therapeutic targeting of tumors and unintended accumulation in off-target organs, which are crucial for nanoparticle safety evaluation. This modeling strategy provides a powerful data-driven tool to support nanotoxicology assessment by predicting tissue-specific exposure patterns underlying efficacy-toxicity trade-offs. Future work may extend this approach to repeated dosing, species extrapolation, and mechanistic priors, advancing risk assessment and safer nanomaterial design.

PL 2001 RADisH: ROBOKOP Assisted Discovery of *in silico* Hypotheticals, A case study of mechanisms linking nuclear receptors to liver disease

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Background and Purpose: Adverse Outcome Pathways (AOPs) describe the cascade of biological events leading to toxic outcomes. An AOP is typically built through manual efforts of subject matter experts identifying empirical molecular or biolog-

ical evidence for each node in the chain beginning with Molecular Initiating Events (MIEs) and intermediary Key Events (KEs) that culminate in an Adverse Outcome (AO). Recent developments in biomedical information retrieval led to the emergence of biomedical Knowledge Graphs (KG), which integrate multiple elementary semantic triples, i.e., special 'subject-predicate-object' relationships between entities (such as 'chemical X causes hepatotoxicity') described in biomedical literature or databases. One KG, ROBOKOP (Reasoning Over Biomedical Objects linked in Knowledge Outcome Pathways), is a biomedical publicly available graph integrating 50+ databases to include over 9M nodes and 140M edges. ROBOKOP is a tool developed and maintained as a collaboration of the UNC's Molecular Modeling Laboratory and UNC's Renaissance Computing Institute (RENCI). **Methods:** ROBOKOP Assisted Discovery of *in silico* Hypotheses (RADisH) is a methodology for automated discovery of AOPs based on graph mining algorithms. We first evaluated the KG's ability to reconstruct known biological event networks, referencing Adverse Outcome Pathways (AOPs), by querying the KG against a reference list of well-supported AOPs and determining the portion successfully reproduced (recall). The recall metric, or the number of events from the reference that are identified by the KG divided by the total events identified, demonstrates the capacity of the KG to capture known biological mechanisms and validity of the tool to potentially propose hypothetical components. To test this hypothesis we consider the use case of nuclear receptor activation initiating events in the development of fatty liver disease by referencing the events described by the thirteen expert-defined AOPs in the AOPwiki.org. Queries to the graph are structured as (nuclear receptor → fatty liver disease) and the resulting list of genes is evaluated against the events described across all AOPs. **Results:** Of the approximately 50 unique events described across the thirteen AOPs in AOPwiki, KG queries considering just one nuclear receptor, AhR, resulted in 34 genes of which 26 were partial (i.e., LDLR is in the wiki but the result returned from ROBOKOP is LDLR variant LDLRAP2) or complete matches to AOP events implying a recall of ~70%. The remaining 8 genes not matched to existing AOP events, including SUMO2 and SOD2, were evaluated for plausibility within the context of fatty liver disease and investigated for potential applicability as hypothetical mechanistic event candidates. A comprehensive query considering all MIEs described in AOPwiki entries identifies nearly 160 genes, or some four times the number of explicitly identified genes or gene-products within AOPwiki entries for fatty liver disease. **Conclusions:** The use of novel hypothetical mechanisms identified through ROBOKOP mining can support future development of quantitative models predicting chemical toxicity. Furthermore, these hypothetical mechanistic events can be tested empirically by biomedical researchers using *in vitro* and *in vivo* tools to evaluate potential causality of the novel KEs and broaden the scope of biomedical knowledge. RADisH presents a methodology which may further enhance present applications of the AOPwiki database, and may contribute to the development of wholly computationally predicted AOPs (cpAOPs) by going beyond simple elaboration on events as identified in this work to instead suggest new chains of events in the AOP style.

PL 2002 From black boxes to chemical interpretability: explainable deep learning for mutagenicity prediction

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Background and Purpose: Deep learning models have become powerful tools for predicting chemical toxicity but often act as "black boxes", limiting trust among experimental scientists. Explainable artificial intelligence (XAI) addresses this by generating feature maps that highlight atoms or molecular scaffolds influencing toxicity predictions. Such interpretability helps users understand model behavior and refine models accordingly. However, the reliability of current XAI methods remains uncertain, as studies have reported inconsistencies across methods and with established chemical knowledge. Here, five widely used XAI techniques are benchmarked for their ability to identify molecular scaffolds and explain mutagenicity predictions. The structural motifs highlighted by these methods are compared with known toxicophores to assess interpretive value and limitations. Based on these insights, the model was refined by incorporating molecular descriptors: global physicochemical (e.g., HOMO-LUMO gap, logP), local electronic (Fukui indices), and geometrical (molecular shape) to improve predictive performance and clarify toxicological reasoning. **Methods:** Molecular structures were represented using SMILES strings. An encoder-decoder model was trained on ChEMBL to convert non-canonical to canonical SMILES. The pretrained encoder, with frozen weights, was then used to extract embeddings for scaffold or property prediction. These embeddings served as input to a Text-CNN trained on the Ames dataset (~7,000 molecules). The CNN used multiple parallel convolutional layers, followed by fully connected layers for multi-label (scaffold) or binary (toxicity) classification. Five explainability methods were evaluated: Integrated Gradients (IG), SHAP GradientExplainer, DeepLIFT, Occlusion, and Grad-CAM, representing gradient-based, backpropagation-based, and perturbation-based techniques. For properties requiring geometry optimization, molecules were protonated to their dominant forms at physiological pH (7.4) and converted into 3D structures using RDKit and custom scripts. Conformer ensembles were generated with CREST and further optimized via semiempirical and DFT calculations using ORCA. The resulting molecular descriptors were merged with CNN embeddings for the final prediction. **Results:** The model achieved a micro-averaged ROC-AUC of 1.0 for scaffold prediction, showing perfect discrimination, and 0.85

for Ames mutagenicity. Both models showed the same general tendency: attribution patterns from IG, DeepLIFT and SHAP were highly consistent, while Occlusion showed slightly larger deviations and Grad-CAM produced broader, more diffuse maps. Mean cosine distances between attribution vectors quantified method similarity, where lower values indicate better agreement. For Ames, mean cosine distances were $\sim 1.0 \times 10^{-4}$ (SHAP-IG), 7.0×10^{-4} (IG-DeepLIFT), 1.7×10^{-2} (Occlusion), and 1.8×10^{-1} (Grad-CAM). All methods correctly highlighted known toxicophores. Compared with toxicophore-based screening, the model reduced false positives by $\sim 51.3\%$, while false negatives remained nearly unchanged. After adding molecular descriptors, the Ames ROC-AUC increased to 0.88, and additional structural features, such as conjugated systems, were emphasized by XAI attributions. **Conclusions:** Explainable deep learning can achieve high predictive accuracy for chemical toxicity while preserving interpretability. Benchmarking multiple XAI methods showed that gradient- and backpropagation-based approaches yield consistent, chemically meaningful attributions aligned with known toxicophores. Integrating molecular descriptors with neural embeddings further improves both performance and interpretability, supporting more transparent identification of structural drivers of mutagenicity.

PL 2003 Leveraging Automated Data Mining and Machine Learning to Rank and Predict PFAS Toxicity

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Background and Purpose: PFAS are a large class of persistent chemicals. More than 1,400 have been identified in over 200 uses across 64 categories. Composed of carbon chains densely bonded with fluorine, these molecules possess exceptionally strong C-F bonds, which confer outstanding repellency to water and grease as well as remarkable resistance to environmental degradation. Although thousands of PFAS are widely used in commercial products, only a small fraction have been systematically studied for their toxic effects. Notably, two individual compounds, PFOA and PFOS, alone account for 21% and 39% of the entire dataset, respectively. While legacy PFAS such as PFOA and PFOS have been extensively studied, the overwhelming majority of PFAS remain largely untested, creating substantial data gaps that hinder comprehensive ecological risk assessments and effective regulatory decision-making. Despite recent advances, existing models remain constrained by **underutilization of qualitative non-animal-derived data, and reliance on relatively simple computational approaches** that fail to fully capture species-specific variability. Significant challenges remain in effectively addressing the PFAS toxicity data gap. **Methods:** we designed a framework that begins with systematic searches of databases such as PubMed, Web of Science, and EPA ECOTOX to identify relevant PFAS toxicity papers. We automatically retrieved publications from PubMed using Python scripts—via the official API, with a crawler based on requests and BeautifulSoup as a fallback. A multi-modal LLM (with the ChatGPT API) will then be applied to parse full texts and extract both quantitative endpoints and comparative ranking statements. A representative subset of the extracted data will be manually reviewed to verify the accuracy and validity of the ranking labels. Quantitative endpoints will be standardized into consistent units and harmonized across studies, while narrative comparative statements will be translated into relative ranking matrices. Uncertainty of the narrative comparison will be assigned based on a matrix of how comparable they are in literature. We then apply a pair-wise Learning to Rank framework to train machine learning models (e.g., Random Forest, XGBoost, LightGBM). **Results:** we demonstrated an automated Python extraction, pulling toxicity information from the PFAS-TOX database. To demonstrate applicability, we performed simple feature extraction and trained standard machine learning models (GAM, random forest, XGBoost) on a small PFAS-specific dataset. In parallel, we trained a Bayesian Matrix Factorization model on a larger dataset (PFAS-specific + ADORE) without feature extraction, considering only chemical-species comparison relationships. We compared the models on the same test dataset, models trained on the small dataset with limited feature extraction outperformed the larger dataset model despite having fewer samples. Moreover, Figure highlights carbon chain length as a key predictor of PFAS toxicity. Furthermore, we trained four machine learning models—random forest, SVM, MLP, and Naïve Bayes—within the pairwise learning-to-rank framework to demonstrate its feasibility. Figure shows the NDCG@5 comparison, with random forest achieving the best performance at 0.897. **Conclusions:** This approach directly advances the 3Rs by replacing animal testing with computational methods, potentially eliminating 10,000+ animal tests annually while providing regulators with urgently needed risk prioritization tools within weeks rather than years.

PL 2004 Deciphering exposure-driven biology through integrative multi-omics using tidyexposomics

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Background and Purpose: Human exposome research tackles the daunting task of integrating heterogeneous exposure, molecular and phenotypic data in order to understand exposure-response relationships. However, current analytical frameworks are fragmented, difficult to reproduce and lack standardization tools. To address these gaps, we developed tidyexposomics, an open-source R package that

provides a modular and reproducible framework for exposure assessment, multi-omics integration and downstream interpretation. **Methods:** The current implementation of tidyexposomics contains several modules. Data pre-processing functions allow for ontology-term assignment for interoperability of findings, missingness, imputation and data transformation assessment, sample clustering, and dimensionality reduction analysis. Exposome scoring enables exposure summarization to characterize the impact of multiple exposures. Association analysis can be performed between exposure, phenotype, and omics data. Network analysis modules can prioritize exposures with differential effects on omics features. Functional enrichment of exposure-responsive omics features supports biological interpretation. Several pivot functions enable the user to access data in a way to support custom analysis. Finally, pipeline tracking and result export features streamline transparent reporting. **Results:** We applied tidyexposomics to analyze exposomics and multi-omics data from children with and without asthma from a lower socioeconomic background from the ISGlobal Exposome Data Challenge 2021. Ontology categorization organized exposure variables into general groups (e.g. aerosols, polyatomic entities, and transition metals). Particulate matter and perfluorohexane sulfonic acid (PFHxS) exposure were correlated with the most principal components. Multi-omics integration identified several latent factors that jointly explained variation in asthma status. Omics features with high loadings on these factors were associated with exposures. Although no individual exposures were significantly associated with asthma status, the omics features linked to both asthma and exposure variation were enriched for biologically meaningful pathways. Gene expression features were associated with terms related to vesicle dynamics, while methylation features were associated with kinase activity and developmental processes. **Conclusions:** tidyexposomics provides a comprehensive and reproducible computational framework for exposome-omics research in toxicology. Overall, tidyexposomics enables toxicologists to systematically understand the potential molecular mechanisms of exposure effects and accelerates the translation of exposomics into actionable insights for human health.

PL 2005 Cross-Species Translatability Illuminated by Over 7,000 Marketed and Investigational Drugs: Modality and Species-Stratified Concordance Mapping Between Preclinical and Clinical Phenotypes

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Background and Purpose: Translating preclinical safety findings into actionable insights for human risk assessment remains a central challenge in drug development. Despite advances in data curation, previous studies have faced limitations such as biased drug selection, insufficient stratification by species and drug modality, inadequate exposure control, inconsistent analytical approaches, and restricted data access. In this context, the purpose of this work is to systematically evaluate the relationship between preclinical toxicity phenotypes and clinical safety outcomes, offering a more robust and transparent foundation for translational safety assessment. **Methods:** Animal and human safety endpoints were harmonized via MedDRA mapping by integrating PharmaPendium with OFF-X platform, enabling standardized, cross-species analyses across five major preclinical species (mouse, rat, rabbit, dog, monkey) and humans. Our analytic strategy prioritizes likelihood ratios to minimize prevalence bias and systematically extends beyond identical-term mapping by including semantically and mechanistically related, non-identical adverse event pairs. Analyses were further broken down by species, drug type, and adjusted for drug exposure. We also linked safety findings to drug targets and biological pathways to understand the underlying mechanisms. Finally, all results were made accessible through a user-friendly web app and an AI-powered multi-agent system, enabling scientists to explore and use the data for decision-making. **Results:** Our concordance analysis produced a wealth of insights into how animal toxicity findings relate to human safety outcomes, covering more than 3,000 distinct toxicity phenotypes. Moving beyond strict identical-term mapping, the inclusion of semantically similar and mechanistically related adverse event pairs uncovers a broader spectrum of cross-species associations to clinical outcomes. Stratified analyses by species, drug modality, and exposure control enable better defined searches and more informative answers, which are much needed for human translation. Integrating both target and off-target annotation further enriches mechanistic interpretation, revealing biological pathways and potential safety targets for subsequent validation. To ensure that these advances translate into day-to-day impact, we developed the web app and the multi-agent AI system (ToxAgents), which transform concordance analytics into actionable decision support. The app provides an interactive interface for exploring concordance results, and the ToxAgents layers on structured, role-aligned reasoning for translational safety. The investigative scientist agent interrogates the concordance data, together with the safety-ome of safety-critical targets, to assemble mechanistic rationales and prioritize candidates for further evaluation. In parallel, the regulatory-affairs agent interprets outputs in the context of ICH safety guidelines and organized recommendations for study design. Together, the agents synthesize search results to support human risk assessment, species selection, mechanistic follow-up, and preparation for regulatory interactions. This architecture facilitates broad, standardized access to concordance analytics, promoting wider adoption across scientific and pharmaceutical communities. **Conclusions:** Altogether, this work offers a comprehensive and mechanistically informed concordance framework that directly addresses challenges in preclinical-to-clinical translation. By integrating statistical, pharmacological, and regulatory knowledge and making these insights

accessible through interactive tools, the study provides a transparent foundation for more informed safety assessment, mechanistic exploration, and early development decision-making.

PL 2006 Unique Nonclinical Safety Profile of PF-06835919, A Ketohehexokinase Inhibitor, Highlights A Species-Dependent Mechanism

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Background and Purpose: PF-06835919, a first-in-class ketohehexokinase (KHK) inhibitor, was developed for the treatment of non-alcoholic fatty liver disease (NAFLD). To support its clinical development, nonclinical safety studies were conducted according to regulatory guidelines. In rats, repeat-dose administration of PF-06835919 for up to 6 months produced various findings resembling those produced by peroxisome proliferator-activated receptor (PPAR) activation. Mechanistic studies were therefore conducted to understand the relationship between these findings and PPAR as well as their human relevance. **Methods:** Repeat-dose toxicity studies were conducted in Wistar Han rats (up to 6 months) and beagle dogs (up to 9 months). Investigative studies were conducted *in vitro* or in rats. **Results:** Based on exposures at no-observed-adverse-effect levels (NOAELs), rat was the more sensitive species, and clinical doses were limited to the rat NOAEL (30 mg/kg/day) exposure. Consistent with hepatic microsomal enzyme induction, hepatocyte hypertrophy and higher liver weight were observed in rat toxicity studies, together with secondary hypertrophy/hyperplasia of thyroid follicular cells and vacuolation of cells in the pars distalis of the pituitary gland. The severity/incidence of these findings increased with longer study duration, with preneoplastic eosinophilic foci of cellular alteration in the liver and follicular cell adenoma and diffuse follicular cell hyperplasia/hypertrophy in thyroid observed at higher doses in the 6-month toxicity study. Other findings in the 6-month rat study included increased heart weight, decreased lipid in the mesenteric white fat and increased brown fat (renal hilus) accompanied by changes in cholesterol and triglycerides, increased incidence of decreased/absent corpora lutea in the ovary, and degeneration of seminiferous tubules in the testis. Transmission electron microscopy (TEM) revealed increased endoplasmic reticulum as well as a non-uniform, variable, mild to marked increase in the size and number of peroxisomes in hepatocytes. In 2-, 6-, 16-, and 39-week toxicity studies in dogs, none of these findings, except for lower cholesterol and higher heart weight, were present. In rat liver tissues after 2 weeks of administration of PF-06835919, gene expression levels of CYP 2B1/2B2, 3A1/3A5, 3A2/3A4, UGT 1A1, and UGT 2B1 were significantly upregulated at high doses. In cells expressing a luciferase reporter gene functionally linked to a rat or human PPAR-responsive promoter, PF-06835919 showed no effects on PPAR α or β/δ activities and only weak activity on PPAR γ at 30 μ M, a concentration significantly higher than that required for inhibiting KHK activity or the predicted exposure at the highest planned clinical dose. **Conclusions:** PF-06835919 is not a direct PPAR agonist and does not pose risks in humans via PPAR activation at clinical planned doses. The liver findings and associated thyroid and pituitary gland changes were primarily mediated by test article-related enzyme induction. Induction of UDP-glucuronyl transferases correlated with enhanced thyroid hormone elimination. Hypothyroidism, secondary to the liver enzyme induction, likely caused the increased incidence of decreased/absent corpora lutea. The heart weight increase was an adaptive change to the hemodynamic effects of the test article (heart rate and blood pressure increase). Lipid changes were likely a pharmacological effect of KHK inhibition. Peroxisomes might be upregulated physiologically as an adaptive change associated with metabolic alterations related to the pharmacology of PF-06835919.

PL 2007 The application of New Approach Methods in drug discovery: Three case studies of fit-for purpose model development to improve human risk translation

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Background and Purpose: New approach methodologies (NAMs) have rapidly evolved in recent years. These include *in vitro* systems and assays that provide much needed tools for modern toxicology in drug discovery and development, from mechanistic understanding of specific toxicities to cross-species translation and risk assessment. Implementing NAMs not only reduces animal use but also provides data-driven understanding and prediction of risks in humans at an early stage to help decision making. Here, we present three case studies in which *in vitro* assays were employed for early human risk prediction and assessment. In these studies we sought to understand the human translation of 1) unusual upper GI hyperplasia observed in rodents only following repeat-dose studies, 2) potential for reduced skin toxicity by selectively targeting a mutant form of target protein, 3) local muscle tolerability of different excipients for subcutaneous injection. **Methods:** In case study #1, an air-liquid interface model of primary cells was developed to compare the responses of mouse, rat, dog, and human esophageal epithelial cells to a panel of proprietary compounds being developed for an immunology indication. The cells were grown in Transwell culture and differentiated over the course of 8 days, followed by 7 days of treatment, and then assessed by transcriptomic anal-

ysis and histology. In case study #2, the MatTek EpiDermFT model, starting from an immature and partially differentiated stage, was treated for 10 days with tool molecules alpelisib (PI3K α inhibitor), capivasertib, or MK-2206 (pan-AKT inhibitors), or the AKT1-E17K mutant selective compound VVD-009, and toxicity was assessed by histology. In case study #3, L6 rat skeletal muscle myotubes and human primary skeletal muscle myotubes were differentiated in culture over 72 hours prior to treatment with increasing concentrations of the excipients sulfolbutylether-beta-cyclodextrin (SBE- β -CD) or hydroxypropyl-beta-cyclodextrin (HP- β -CD), and assessed by live cell imaging and ATP measurement of cell viability after 24 hours of treatment. **Results:** Case study #1: Only mouse and rat *in vitro* cultures exhibited a hyperproliferative phenotype as evidenced by increased cell layer thickness and irregularity in response to compound treatment whereas dog cultures did not. This is consistent with the *in vivo* phenotypes observed in the respective 2-4 week tox studies. Rodent cultures also showed increased expression of proliferative markers, indicating that this finding could be due to increased sensitivity to inhibition of this pathway in rodents. Human cultures, similar to that of dogs, did not display a proliferative response. Case study #2: Whereas alpelisib, capivasertib, and MK-2206 all caused a notable thinning of the epidermis and loss of skin barrier integrity, recapitulating their clinical skin toxicity, the AKT-E17K mutant selective inhibitor VVD-009 did not, at doses exceeding the predicted clinical exposures. Case study #3: Whereas a concentration-dependent increase in rat and human myotube death was observed for both SBE- β -CD and HP- β -CD at higher concentrations, SBE- β -CD demonstrated less toxicity at clinically-relevant concentrations. This data is consistent with exploratory studies in rats where SBE- β -CD showed superior local muscle tolerability as compared to HP- β -CD. Based on this data, SBE- β -CD was selected as the excipient for future studies and clinical formulation development for a better injection site safety profile. **Conclusions:** The qualification of NAMs for fit-for purpose use is an ongoing process across the pharmaceutical industry, with needs in many different facets of preclinical de-risking. These studies presented here demonstrate the utility of *in vitro* models for better understanding the clinical translation and potential mechanisms of non-clinical safety findings in animals, or for use where standard animal models have poorly predicted clinical toxicity.

PL 2008 Safety Evaluation of Supraphysiological Tradipitant Dosing under Differential Liver Metabolism

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Background and Purpose: This project aimed to evaluate the safety of Neurokinin-1 receptor antagonist tradipitant in Hesperos, Inc's 4-organ system which is a multi-organ, microphysiological system composed of liver (primary biopsy), cardiac (iPSC-derived), kidney (primary biopsy) and neuronal (iPSC-derived) "organ" modules. **Methods:** Tradipitant (3 μ M, 30 μ M, and 100 μ M) was evaluated over a 14-day period of chronic dosing for toxicity, functional organ testing and biomarker assays in Hesperos, Inc's 4-organ system and compared to vehicle control and positive controls. Each concentration was tested in duplicate per experiment and across an n=4 biological replicates and stratified by low metabolizing liver, medium metabolizing liver, and high metabolizing liver cells. Systems were dosed daily and tested for functional activity at pre-dose, 1-day, 4-day, 7-day, 10-day, and 14-day post initial dose. At each timepoint functional recordings were taken as well as morphology monitoring by phase contrast imaging. For cardiac cells, beat frequency, force, conduction velocity, field potential duration (QT surrogate), and Troponin I were measured and terfenadine, doxorubicin were used as positive controls. For liver cells, a viability assay, toxicity imaging and albumin production were measured and losartan, chlorpromazine were used as positive controls. For kidney cells, kidney injury molecule 1 (KIM-1) release was measured and amphotericin B, gentamicin were used as positive controls. For cortical neurons, spontaneous firing rates were measured and kainic acid, nimodipine were used as positive controls. Medium from systems was also collected and the quantities of tradipitant and metabolites were measured. **Results:** Liver constructs showed the capability to metabolize tradipitant into its respective metabolites (M2, M3, and M4). Tradipitant 100 μ M dose showed potential hepatotoxicity as determined by viability assays and vacuole formation. However, moderate and low metabolizing liver constructs cytotoxicity was not observed at this dose. The moderate liver construct yielded significantly higher levels of albumin when compared to the low metabolizing liver and vehicle control conditions with 3 μ M tradipitant. When dosed with 100 μ M of tradipitant, the moderate liver construct yielded significantly lower levels of albumin when compared to the high metabolizing liver. Chronic exposure of tradipitant had a miniscule effect on cardiac force across all liver constructs and all dose administrations when compared to the vehicle control. However, significant variations between liver constructs were much more apparent. Across all doses, the high metabolizing liver construct showed significant increases in KIM-1 levels throughout the course of compound administration when compared to the vehicle control. The moderate liver construct showed significant increases in KIM-1 levels in only the 30 and 100 μ M doses. The low liver construct only showed significant increases in KIM-1 for the 30 μ M dose. Significant effects on cortical neuron firing rate were only observed in the high metabolic liver systems receiving a 30 μ M dose. Significantly higher levels were observed on day 1 post-dose, however this stabilized and returned to baseline over the course of the study. **Conclusions:** Chronic exposure of tradipitant in Hesperos, Inc's 4-organ system yielded only minor adverse effects in all organ constructs in which it was tested when

compared to the vehicle and positive control. When comparing the safety of the compound across systems containing either high, moderate, or low metabolic activity, significant effects could be observed. This suggests that liver function should be taken into consideration as it may influence physiological outcomes. Importantly, this system was able to demonstrate traditipant had minimal toxicity in the tested organs and is suitable for acute and chronic dosing given the absence of compensatory mechanisms observed.

PL 2009 Development of *in vivo* and *in vitro* Models for De-risking Adrenal Insufficiency

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Background and Purpose: Adrenal glands are common toxicological targets within the endocrine system, and adrenocortical hypertrophy is a relatively common observation, often identified in nonclinical species during drug development studies. In the absence of other indications of a stress response, an *in vivo* ACTH challenge study can be used to demonstrate drug-induced adrenal insufficiency. ACTH challenge studies in rats are typically conducted in male animals to avoid complexities introduced by the estrus cycle of females; however, a female model may be desired in some circumstances. Therefore, we attempted to establish and ACTH challenge model that could be used in both male and female rats. To complement the *in vivo* approach, we also evaluated two *in vitro* adrenocortical models for their potential as screening or mechanistic tools. Merck A is a small molecule drug candidate that caused adrenocortical hypertrophy in rats and monkeys in 1-month tox studies and in a 7-day dose-limiting toxicity study in female rats. The hypertrophy was determined to be an off-target effect. Although there were no degenerative changes and the adrenal findings were not considered adverse, the risk assessment asserted these changes were clinically translatable and likely to require nonclinical and clinical monitoring. The purpose of this research is to develop *in vivo* and *in vitro* models to characterize this toxicity and build our internal capability for de-risking adrenal deficiency. **Methods:** To examine the mechanism of adrenal hypertrophy and inform back-up derisking for Merck A, investigations were planned 1) to confirm/refute hypothesis that the adrenal changes were due to adrenal insufficiency; 2) to assess utility of commercially available adrenal cortical cultures for mechanistic investigation. We optimized dose, time and sampling conditions for the ACTH challenge model in both male and female rats. Adrenal insufficiency was demonstrated for four known steroidogenic inhibitors by measuring plasma corticosterone levels 15 minutes after ACTH or saline injection. Additionally, we developed human HAC15 cells and mouse Y1 adrenal cell culture models to detect perturbations in steroid biosynthesis. We monitored cell viability and distinct steroidogenic intermediates across multi-enzyme pathways for mode of disruption. **Results:** Merck A was evaluated in ACTH challenge model in female rats as previous studies have shown that female rats had higher drug exposure than male rats. A single dose of Merck A inhibited ACTH-stimulated corticosterone release, confirming the working hypothesis of adrenal insufficiency. *In vitro* tests with multiple steroidogenic inhibitors in HAC15 and Y1 cells demonstrated disruption in steroid production, and the responses corresponded with the mechanisms of enzyme inhibition. **Conclusions:** Taken together, we have established the conditions and utility of an *in vivo* ACTH challenge study in male and female Wistar-Han rats. Application of the model confirmed the working hypothesis of adrenal insufficiency for Merck A. In addition, we have developed resource-sparing human and mouse cells models to rapidly detect adrenocortical dysfunction. Further qualification of the *in vitro* platforms presents an opportunity to develop screening approaches and enhance our understanding of structural features associated with adrenal cortical hypertrophy risks.

PL 2010 Size-Dependent Pharmacokinetics of Oral Microplastics: Development of a Physiologically-Based Model with Local Gastrointestinal Binding

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Background and Purpose: Microplastic contamination in food and water poses emerging health concerns, yet a quantitative understanding of their absorption, distribution, and elimination kinetics remains limited. Existing physiologically-based pharmacokinetic (PBPK) models show poor predictive performance for gastrointestinal retention kinetics, with substantial discrepancies between model predictions and experimental measurements. We aimed to develop an improved PBPK model that accurately predicts size-dependent microplastic biodistribution following oral exposure and identifies the mechanistic basis for kinetic differences across particle sizes. **Methods:** We restructured a published PBPK model by incorporating a novel local gastrointestinal tissue binding compartment (AGI_{local}) to capture retention kinetics not explained by systemic absorption. The model was implemented in Julia and validated against experimental biodistribution data for polystyrene particles of four sizes (20nm, 220nm, 1000nm, 6000nm) at multiple timepoints (6h, 12h, 24h, 48h) following 0.1mg oral administration in mice. Parameter estimation was performed using Bayesian inference (NUTS sampler, 500 iterations) and optimization-based methods with weighted likelihood functions (10× GI lumen, 5× GI tissue,

1× systemic compartments). We systematically evaluated parameter requirements across particle sizes and assessed whether constant or time-varying elimination kinetics better describe multi-timepoint data. **Results:** The restructured model with local GI binding dramatically improved predictions across all particle sizes tested. For 20nm particles, prediction error decreased from 91% (original model) to 0.3% (restructured model) for GI lumen retention at 6 hours, with similar improvements for other compartments and sizes. Systematic parameter estimation revealed size-dependent model complexity requirements: smaller particles (20nm) were adequately described by 3 parameters, while larger particles required expanded parameter sets (4-5 parameters for 220nm and 1000nm), including size-specific kidney permeability and tissue binding terms. Kidney clearance rates varied 4-fold across particle sizes (0.079 to 0.020 h⁻¹), demonstrating size-selective renal handling. Critically, parameters optimized using single-timepoint data (6h) failed to predict later timepoints for all particle sizes, with prediction errors escalating from >1% at 6h to >100% at 12h and >900% at 48h. This systematic failure across all particle sizes indicates that time-varying elimination kinetics, rather than constant parameters, are necessary to capture gastrointestinal transit dynamics. Preliminary results suggest fecal elimination rates accelerate over time as particles transit from the stomach to colon, consistent with regional differences in GI motility. **Conclusions:** The integration of local GI binding kinetics into the PBPK models successfully captured experimental microplastic retention patterns that existing models could not predict. The findings have direct implications for human risk assessment, as they allow more accurate estimation of internal exposure and tissue burdens from ingested microplastics. By linking external intake (e.g., diet, water) to predicted tissue concentration, the model supports quantitative risk characterization and margin-of-exposure analysis. The approach also highlights the importance of size-dependent kinetic parameters, since nano- and micro-scale particles differ in retention time and elimination pathways that potentially lead to a higher risk for sensitive populations (e.g., infants and pregnant women). Continued refinement of this model, through empirical fitting of time-varying parameters and validation across particle sizes, is required to enhance the reliability for use in regulatory evaluation and reduce uncertainty in risk assessment.

PL 2011 Microplastics in Human Tissues: Examining Plastic Particulate Accumulative Patterns Across Decades (1980s vs 2020s)

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Background and Purpose: Metabolic (Dysfunction) Associated Steatotic Liver Disease (MASLD) is an emerging liver disease of global concern. Chronic liver disease (CLD; including MASLD) rates are highest in the southwestern United States, including in the state of New Mexico (NM). NM has the highest rate of CLD in the US (36.4/100,000 in 2022), mainly among Hispanic and Native American populations for unknown reasons. We currently have a limited understanding of the role of environmental pollutants in MASLD etiopathogenesis. In particular, plastic pollution is an emerging concern which may underpin hepatic pathophysiology. We hypothesize environmental plastic pollutants can accelerate hepatic metabolic dysfunction in conjunction with established innate risk factors like type II diabetes and obesity. We currently have a limited understanding of NMP routes of bioaccumulation, cellular dysfunction and long-term effects on human health. **Methods:** In the current project, we evaluated NMP presence in decedent human liver tissues for the first time. A total of 32 human liver samples (M=19; F=13) from the years 2016-2024 were obtained from the NM Office of Medical Investigator (OMI) and analyzed for NMP accumulative patterns. Human liver tissues were tested for 12 common environmental plastics (PE, PVC, N66, PET, SBR, PC, PP, PS, ABS, PMMA, PU and N6) using pyrolysis-gas chromatography mass spectrometry (py-GCMS) testing. Additionally, novel imaging techniques (polarization wave microscopy) and fluorescence imaging are used to characterize polymer refractile elements in the hepatic tissues. We also obtained seven archived samples from 1981 for histopathological review and PWM imaging. **Results:** The total measured plastic quantity ranged from 0 µg/gm up to a maximum of 945.5 µg/gm in hepatic tissue, averaging 295.3 µg/gm across 32 liver samples. Next, a detailed histopathological analysis was performed delineating the NMP cellular distribution patterns within the hepatic tissue architecture. We implemented a novel optical imaging technique, polarization wave microscopy (PWM), to identify and measure polarization characteristics of putative, refractile NMP bodies in liver samples for the first time. Additional orthogonal multi-spectral fluorescence and transmission electron microscopy (TEM) imaging approaches confirm the refractile bodies identified using PWM imaging. All human liver samples except one (n=31) showed putative NMPs in the hepatic tissues. Preferential accumulation of NMPs in steatotic hepatic cells is a finding of major concern due to the rapidly increasing incidence of MASLD globally. Also, the hepatic tissues were assessed for key immunological biomarkers of interest - CD68 (Kupfer cells), CD56 (NK cells) and CD3 (Pan T-lymphocyte marker). Using digital pathology-based techniques for quantification, we identified elevated CD68+ macrophages in an NMP burden dependent manner. However, no differences were seen in the CD3 and CD56 immune burdens. Lastly, the seven archived histopathological tissues from 1981 reviewed using H and E and PWM microscopy did not show any refractile NMPs similar to the cases from 2016-2024. This is a novel finding we have observed for the first time reinforcing our main hypothesis of increasing plastic burdens in the past 1-2 decades among humans. **Conclusions:** These findings underscore the urgent need to study biological effects of NMP pollution in human liver tissues. Animal models are needed to understand

the role of chronic NMP pollution as a driver of MASLD pathophysiology and its potential role as an emerging environmental risk factor responsible for chronic liver (and metabolic) dysfunction increasingly observed among humans.

PL 2012 Advancing the method for isolating and quantifying MNPs in human cerebrospinal fluid for Brain Waste Clearance Studies

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Background and Purpose: Micro- and nano-plastics (MNPs) have been detected in human faeces, urine, blood, and various organs, raising concerns about human exposure and potential health implications. However, isolating and quantifying MNPs from complex biological matrices remains a major analytical challenge. Our team has made significant development by developing a novel approach to isolate and quantify MNPs from human tissues using advanced spectroscopic and microscopic techniques. The findings show significant MNPs accumulation in the human brain compared to the liver and kidney, raising concerns about brain waste management and potential toxic effects on the central nervous system (CNS) function. **Methods:** Here, we assess the quantity and physicochemical properties of MNPs in human cerebrospinal fluid (CSF) using pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS). The initial quantitative measurement was conducted on CSF samples (n=14) collected from a brain injury cohort. **Results:** Polymer concentrations ranging between 13.3 and 82.8 µg/ml were detected, with a mean of 22 µg/ml for the summed polymer concentrations. **Conclusions:** To validate our experimental approach, artificial cerebrospinal fluid (aCSF) spiked with known polymer standards will be analyzed to assess matrix interference removal and recovery efficiency. This will help us advance the methodological precision to facilitate the implementation of accurate correlation studies with brain waste clearance and CNS pathologies.

PL 2013 Detection of Microplastics and Other Contaminants in Banked Human Breast Milk Using FTIR

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Background and Purpose: Concerns about the proliferation of plastic products has resulted in a significant body of research. Studies show microplastics (MP), commonly defined as plastic particles and fibers <5 mm, are widely distributed in environmental media, common foods and beverages, wildlife and humans. Once ingested, MP may move easily between compartments. They have been detected in most human organ systems, sputum, semen, blood, and placenta. This study used FTIR to analyze donated human breast milk samples for microplastics and other contaminants. **Methods:** Thirty breast milk samples were analyzed using Fourier Transform Infrared Spectroscopy on a Cary 630 (Agilent San Diego). Spectra were generated and contaminants identified using Open Analysis' Open Specy 1.0 software. Although breast milk is a complex body fluid, purification was not performed. Purification might so the matrix could be preserved. Digestion of organic matter has been shown to damage polymers. Microplastics were found in 46.7% (14 of 30) samples. The most common MPs detected were polypropylene (PP), (polyethylene (PE), and polyvinyl chloride (PVC). Other contaminants found include sodium chloride, weddellite, mab-chminaca, methyl cellulose, pargasite, and pollen. **Results:** Microplastics were found in 14 of 30 samples of breast milk. Sodium Chloride was found in 22 of 30 breast milk samples. The remaining compounds identified occurred far less frequently (weddellite - 4 specimens, mab-chminaca - 1 specimen, methyl cellulose - 3 specimens, pargasite - 2 specimens, and pollen - 1 specimen). **Conclusions:** Microplastics were identified in 14 of 30 samples of donated human breast milk. Donor identities are blinded so demographic data was unavailable. Lacking data such as the total number of pregnancies, duration of pumping session, elapsed time since the most recent birth, maternal age, diet, and occupation prevents more in-depth conclusions. Sodium chloride was found in most samples (22 of 30). Traces of minerals, fibers, pollen, and a synthetic cannabinoid were also detected. Further study should be conducted to elucidate sources of contaminants and their potential to adversely affect nursing infants.

PL 2014 Beyond the Brain: Perfluorooctanoic acid exposure disrupts serotonin signaling in the pancreas and causes gestational diabetes in mice

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Background and Purpose: Gestational diabetes mellitus (GDM) is a form of diabetes that manifests during pregnancy and is characterized by hyperglycemia and glucose intolerance due to pregnancy-driven insulin resistance and inadequate pancreatic beta cell adaptation. GDM has a worldwide prevalence of 1.1-31.5% and is influenced by race, ethnicity, age, body composition, and screening and diagnostic criteria. The

rate of GDM in the US has increased by 30% from 2016 to 2020 and is expected to continue to increase in subsequent years. Women with GDM have an increased risk for pregnancy complications and adverse health conditions following pregnancy, while their fetuses face risks such as premature birth and future type 2 diabetes. Published studies have demonstrated that pancreatic beta cell proliferation is critical for maintaining healthy maternal glucose levels during pregnancy. One mechanism regulating beta cell proliferation is serotonin-dependent activation of the G1 cyclins for the cell cycle by binding of serotonin to the HTR2B receptor. This mechanism is driven by pregnancy-induced activation of the tryptophan hydroxylase 1 gene (*Tph1*) and local islet serotonin production. Previous work from our lab and others has shown that nutritional, genetic, and pharmacologic disruption of serotonin-mediated pancreatic beta cell proliferation causes GDM in a C57BL6/J mouse model. In this study, we sought to determine whether environmental contaminants can also disrupt the serotonin-dependent beta cell proliferation and cause GDM in mice. We chose to study perfluorooctanoic acid (PFOA), a ubiquitous and persistent environmental contaminant, as it is measurable in pregnant women and has been associated with increased risk for GDM in some epidemiologic studies. Preliminary studies from our lab show that gestational exposure to an environmentally relevant dose of PFOA induces glucose intolerance and insulin resistance in pregnant C57BL6/J mice and is accompanied by lower islet serotonin levels and beta cell proliferation rates. This is the first study to identify a potential mechanism by which PFOA induces GDM. Future studies aim to explore how genetic variants may alter serotonin-driven beta cell proliferation and influence susceptibility to GDM. **Methods:** We exposed C57BL6/J female mice to a control or PFOA (8 µg PFOA/kg body weight/day) diet for two weeks prior to mating and throughout gestation (gestational day (GD) 12.5 or 16.5). To assess the metabolic phenotype of these mice, mice underwent glucose tolerance testing (GTT) and insulin tolerance testing (ITT). Another subset of these mice was used for histological analyses, utilizing immunohistochemistry (IHC) staining to measure for islet serotonin abundance, beta cell proliferation, and islet morphology. To validate that the mechanism is dependent on serotonin-dependent activation of the serotonin receptor 2b (HTR2B) signaling, we treated mice with a HTR2B agonist from GD9.5-12.5 or 15.5, the critical window by which *Htr2b* mRNA expression and beta cell proliferation peaks, and assessed beta cell proliferation rates and glucose tolerance, at GD12.5 and 16.5, respectively. **Results:** Gestational PFOA exposure leads to glucose intolerance and insulin resistance in gestational day (GD) 16.5 pregnant mice, but not in non-pregnant or GD12.5 mice, suggesting that this is a pregnancy-specific phenotype that manifests in late gestation. Furthermore, PFOA exposure significantly lowers islet serotonin abundance and beta cell proliferation in GD 12.5 pregnant mice, without significantly altering islet morphology. Treatment with the serotonin receptor 2b (HTR2B) agonist improves beta cell proliferation in GD12.5 PFOA-exposed mice. Ongoing studies seek to assess whether HTR2B agonist treatment improves glucose tolerance in GD16.5 PFOA-exposed mice. **Conclusions:** These results suggest that disrupted serotonin-driven beta cell proliferation is a mechanism causally linked to GDM development in a C57BL6/J mouse model. This is the first study to reveal a potential mechanism by which PFOA induces GDM. Understanding the underlying mechanism can reveal areas for clinical intervention to prevent and treat GDM, improving the health of pregnant women and their offspring.

PL 2015 Fetal rat testis toxicity of a nine-phthalate mixture

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Background and Purpose: Phthalic acid esters (phthalates) are male reproductive toxicants that disrupt fetal testis development, resulting in well-characterized effects in rats, including reduced testicular testosterone production, enlarged seminiferous cords, germ cell clusters, and multinucleated germ cells (MNGs). There is evidence that the human fetal testis is less sensitive to phthalate-induced testosterone reduction than the rat fetal testis, but that induction of MNGs by phthalates is not dependent on testosterone production. We previously showed that the ED₅₀ for dipentyl phthalate-induced MNGs was lower than the ED₅₀ for reduction of testosterone in late gestation, which indicates that fetal testicular germ cells are sensitive to non-androgen-dependent toxic effects of phthalates. Here, we hypothesized that a mixture of nine phthalates would produce a dose-dependent, dose-additive increase in the density of MNGs in the rat, at a lower dose than the dose required to reduce testosterone. **Methods:** We generated a mixture of nine phthalates with well-characterized anti-androgenic effects in fetal rats: benzyl butyl phthalate, di-n-butyl phthalate (DBP), dicyclohexyl phthalate, di-(2-ethylhexyl) phthalate, diheptyl phthalate, dihexyl phthalate, diisobutyl phthalate, diisooheptyl phthalate, and dipentyl phthalate, which each compound being 0.5-log below its ED₅₀ for testosterone reduction in the highest dose group, and covering a 2-log dose range. Timed pregnant dams were treated with the mixture or corn oil vehicle by daily oral gavage from gestational day (GD) 17-21. On GD 21, MNGs were counted in H&E-stained sections, phthalate metabolites were measured in dam serum by GC-MS, and testosterone production was measured by LC-QTOF-MS in media from an ex vivo assay. **Results:** Eight phthalate metabolites showed a log-linear increase in dam serum across the dose range, and additional metabolites were identified in non-targeted analysis. The mixture produced a dose-dependent increase in MNGs with an ED₅₀ of 23.64 mg/kg/d DPeP equivalent (95% CI = 17.63, 23.91); and a dose-dependent decrease in testosterone production with an ED₅₀ of 40.55 (12.47, 147.2) mg/kg/d DPeP equivalent. **Conclusions:** We conclude that phthalates cause germ cell toxicity in a dose-dependent, dose-additive manner, and that this effect is at least as sensitive as reduction of testosterone production.

PL 2016 DDT's Lasting Legacy: Endocrine Activity and Developmental Toxicity of DDT+ and the Emerging Co-Contaminant Tris(4-chlorophenyl)methanol (TCPMOH)

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Background and Purpose: The legacy pesticide dichlorodiphenyltrichloroethane (DDT) is an endocrine disrupting compound associated with reproductive and developmental defects across species. Our collaborators have found >50 DDT-related metabolites and manufacturing byproducts (DDT+) in wildlife near dumping sites, many of which have limited or no toxicity data available. Tris (4-chlorophenyl) methane (TCPM) and tris (4-chlorophenyl) methanol (TCPMOH), also known as TCPMs, are of unknown sources but believed to be DDT byproducts of because of their co-occurrence in the environment and structural similarities to DDT. Here, we investigate the acute and developmental toxicity of the understudied and newly characterized DDT+ with the legacy DDT. We hypothesized that TCPMs would retain similar bioactivity mechanisms to DDT. **Methods:** *IN VITRO STUDIES.* Endocrine disruption was determined by *in vitro* California condor estrogen (ER) activation measured by a luciferase reporter assay. With the Presto Blue assay, we evaluated the cytotoxicity of TCPMs and 12 select DDT+ in HEPG2 cells. LD₅₀ values were calculated by nonlinear regression, and BMD, BMDL, and BMDU were determined using the Hill model at 0.5 BMRF. *IN VIVO STUDIES.* Developmental effects were assessed in zebrafish embryos exposed to DDT+ and TCPMs from 24-120 hpf. Mortality, hatching rate, and morphological deformities were monitored. At 120 hpf, behavioral assays were conducted to monitor larvae movement in response to light and tap stimuli using the Noldus DanioVision Observation Chamber. Total RNA was isolated at 120 hpf, and a clustered heat map was created to compare gene expression changes between DDT+ and TCPM upon exposure. KEGG pathway analysis was conducted to compare responses to acute (96-120hpf) and developmental TCPM and TCPMOH exposure. **STATISTICS.** Cytotoxicity was analyzed by one-way ANOVA and behavioral assays by two-way ANOVA followed by Tukey's multiple comparison test. Developmental outcomes and differences in group frequencies were compared by Fisher's exact test ($\alpha = 0.05$). **Results:** DDT+ activated condor and human ERs, with condor ERs showing greater sensitivity. TCPMOH was most cytotoxic of all tested DDT+, significantly reducing cell viability from 12.5 μ M ($p < 0.05$) and yielding an LD₅₀ of 32.52 μ M. Benchmark dose modeling produced a BMD of 33.959 μ M, with confidence limits ranging from 31.685 μ M (BMDL) to 36.401 μ M (BMDU). Developmental exposures to TCPMOH in zebrafish embryos increased mortality, impaired growth, and induced teratogenic defects: pericardial edema, swim bladder deflation and craniofacial deformities were most pronounced at 1 μ M at 5 dpf. Behavioral assays showed TCPMOH exposure during zebrafish development altered locomotor activity, causing hyperactivity manifested as increased total distance traveled, mean velocity, and time in movement in the Light/Dark assay. Heatmap analysis shows the para-substituted compounds *p,p'*-DDD, *p,p'*-DDT and *p,p'*-DDE cluster closely together, whereas TCPM and TCPMOH form a separate branch. KEGG pathway analysis revealed developmental transcriptomic responses to TCPM and TCPMOH were correlated ($R^2 = 0.8$), with downregulated steroid hormone biosynthesis, circadian rhythm, and retinol metabolism. **Conclusions:** DDT+ presented endocrine disrupting potential through ER receptor activation. Among the tested compounds, TCPMOH showed the greatest acute toxicity and induced significant developmental effects, including morphological abnormalities and altered behavioral patterns. *P,p'* isoforms seem to act through similar modes of action, as they showed comparable estrogenic activity, cytotoxicity, and transcriptomic patterns. Ongoing analyses with additional DDT+ are being conducted to further characterize and compare their induced developmental outcomes. Overall, this study characterizes the developmental toxicity of the emerging DDT+ and highlights the need to further investigate their ecological and health impacts, especially TCPMs which may be particularly toxic but understudied.

PL 2017 Co-Exposure to Mono(2-ethylhexyl) Phthalate and High Temperature Inhibits Mouse Antral Follicle Growth and Estradiol Synthesis *In Vitro*

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Background and Purpose: Di(2-ethylhexyl) phthalate (DEHP) is a plasticizer found in common consumer goods, such as food and beverage packaging, floor and ceiling tiles, automobile and furniture upholstery, and medical devices. DEHP is rapidly metabolized upon ingestion to mono(2-ethylhexyl) phthalate (MEHP), which is bioactive and known to exert endocrine-disrupting toxicities. Due to the extensive use of DEHP in consumer goods, humans are ubiquitously exposed and therefore vulnerable to endocrine and reproductive dysfunction by MEHP. Studies have shown that MEHP exposure disrupts the essential ovarian processes of antral follicle growth and steroidogenesis, both critical for ovarian function and female fertility. Simultaneously, humans are ubiquitously exposed to non-chemical stressors like rising global temperatures, and studies have shown that exposure to elevated temperatures can also impair female reproductive health. Given the unavoidable exposure to both increasing heat and DEHP, this study used an *in vitro* follicle culture system to test the hypothesis that exposure to high temperature will exacerbate

the negative effects of MEHP exposure on antral follicle function. **Methods:** Antral follicles were isolated from 4-5-week-old CD-1 mice and cultured for 24-96hr in media containing follicle-stimulating hormone (FSH) at a control temperature (CT; 37°C) or high temperature (HT; 42°C). Follicles were treated with vehicle control (dimethyl sulfoxide; DMSO) or MEHP (0.2-20 μ g/ml) in both temperature groups. Separate incubators were used for the CT and HT treatment groups, in which all HT treatments occurred for 8hr per 24hr period to model daytime, occupational exposure to high temperatures. Follicular growth was measured every 24hr. Follicles and media were collected at 24, 48, 72, and 96hr for gene expression analyses and sex steroid hormone measurements, respectively ($n = 3-7$ with 5-10 follicles/group/replicate; $p < 0.05$). **Results:** At 24hr, mRNA levels of *Ccnd2*, *Hsd17b1*, and *Cyp19a1* were decreased by HT+MEHP while mRNA levels of *Cdkn1a* were increased by HT+MEHP compared to CT+DMSO. Additionally, mRNA levels of *Fshr* at 24hr were increased by HT+DMSO and HT+MEHP compared to CT+DMSO. At 72hr, follicle growth, mRNA levels of *Ccnb1* and *Cyp11a1*, and estradiol levels were decreased by HT+MEHP compared to CT+DMSO. Additionally, mRNA levels of *Cyp19a1* and estrone levels at 72hr were decreased by HT+DMSO and HT+MEHP compared to CT+DMSO. At 96hr, follicle growth was decreased by HT+MEHP and both estrone and estradiol levels were decreased by CT+MEHP, HT+DMSO, and HT+MEHP compared to CT+DMSO. **Conclusions:** These results suggest that HT+MEHP co-exposure dysregulates cell cycle gene expression and decreases steroidogenic gene expression at earlier time-points leading to inhibited antral follicle growth and estradiol synthesis over time. The early increase in *Fshr* gene expression by HT+MEHP co-exposure may indicate a compensatory mechanism as a result of the impaired cell cycle progression and steroidogenesis. Further, several of the defects in cell cycle regulation, follicle growth, and steroidogenesis occur by HT+MEHP co-exposure but not by individual exposures to HT or MEHP alone. These findings demonstrate that HT and MEHP co-exposure may exert additive/synergistic effects. Thus, exposure to ubiquitous chemical and non-chemical stressors like MEHP and HT disrupt critical ovarian processes and may potentiate ovarian dysfunction when combined. Supported by R01ES033767, UL1TR001998, and P30ES026529.

PL 2018 Acute Exposure of Imidacloprid Affects Uterine Homeostasis in Mice

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Background and Purpose: Imidacloprid (IMI) is the most widely used neonicotinoid insecticide, leading to ubiquitous environmental and human exposure. IMI exerts its insecticidal action by binding to and activating nicotinic acetylcholine receptors (nAChRs). Although mammalian nAChRs exhibit lower binding affinity, IMI exposure has been associated with neurological, immune, metabolic, and reproductive dysfunction. However, the potential uterine effects of IMI remain largely unexplored. This study aimed to determine whether low-dose, subacute IMI exposure disrupts uterine homeostasis and early pregnancy-related remodeling. **Methods:** Adult non-pregnant CD1 female mice ($n = 15$ /group) were exposed to IMI via drinking water for 30 days at 0 (vehicle), 0.2, 2, 20, 200, or 2000 μ g/L, representing environmentally relevant to high-end exposure levels. Uterine tissues were collected and processed for histological and molecular analyses. Hematoxylin and eosin (H&E), KI67, F4/80, and Picrosirius Red staining were used to assess cellular proliferation, macrophage infiltration, and collagen deposition, respectively. qPCR quantified expression of genes related to oxidative stress, inflammation, and fibrosis. To further understand the impact of IMI on pregnancy-related processes, we examined the differentiation of uterine stromal cells into decidual cells—a step critical for the establishment of pregnancy. Mouse endometrial stromal cells (MESC) were isolated from day-4 pregnant mice, cultured, and treated with IMI. *Igfbp4* protein expression was evaluated by immunocytochemistry as a marker of stromal differentiation during decidualization. **Results:** Low-dose IMI exposure (0.2 μ g/L) appears to increase luminal epithelial proliferation and myometrial thickness based on H&E staining, with KI67 analysis underway to verify proliferative activity. Enhanced macrophage recruitment was observed through F4/80 immunoreactivity. Picrosirius Red staining showed early collagen deposition within the stroma, suggesting activation of fibrotic remodeling. qPCR analysis showed upregulation of inflammatory and oxidative stress-related transcripts, while *Igfbp4* expression was possibly reduced *in vitro*, indicating potential impairment of stromal cell differentiation processes. **Conclusions:** These findings demonstrate that environmentally relevant IMI exposures may alter uterine remodeling pathways, promoting glandular hyperplasia, inflammation, and early fibrotic activity. This work identifies previously uncharacterized effects of low-dose neonicotinoid exposure on uterine remodeling and early pregnancy-related changes, underscoring IMI as a potential reproductive toxicant of concern. Supported by NIH R21 ES036520, NIH R25 ES025059

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Background and Purpose: Neonicotinoids are among the most widely used insecticides globally. Epidemiological studies have associated gestational exposures to neonicotinoids with fetal growth restriction and developmental abnormalities. Although designed to target insect nicotinic acetylcholine receptors (nAChRs), several neonicotinoid metabolites have greater binding affinity to mammalian nAChR subunits. nAChRs are also present in the human placenta, raising concern for pregnancy-specific toxicity. This study investigated the placental transfer and functional impact of neonicotinoid parent compounds and their metabolites. **Methods:** To evaluate pregnancy kinetics of neonicotinoids, pregnant C57BL/6J mice received a single oral dose of a neonicotinoid mixture (10 mg/kg-bw; acetamiprid (ACE), imidacloprid (IMI), clothianidin (CLO), and dinotefuran (DINO)). Plasma, maternal liver and brain, placenta, and fetal liver and brain ($n = 3/\text{timepoint}$) were harvested at 0, 3, 6, 12, 24, 48, and 72 h after exposure for mass spectrometry detection. Concentrations of parent compounds and their biotransformation products (desmethyl-N-acetamiprid (D-ACE), desmethyl-clothianidin (D-CLO), dinotefuran-urea (DU), 5-hydroxy-imidacloprid (5-OH-IMI)) were quantified and toxicokinetic parameters ($t_{\max}$, $C_{\max}$, CL_{plasma}) were assessed. To examine the functional impact of neonicotinoids, human placental extravillous trophoblasts (EVTs; HTR8/SVneo cell line) were exposed to ACE, IMI, CLO, DINO, D-ACE, D-CLO, DU, imidacloprid-olefin (IMI-O), desnitro-imidacloprid (DNI), and 3-hydroxy-imidacloprid (3-OH-IMI) individually or in mixtures (4-parent or 4-metabolite mixtures) across environmentally relevant concentrations (0.002, 0.02, 0.2, or 2 μM). Proliferation (5 d) and migration (24 h) were assessed using live-cell imaging and scratch wound assays, respectively. In addition, EVT invasion was assessed over 96 h after 2 μM exposures to neonicotinoid parent and metabolite compounds using a novel 3-D microphysiological platform, where collagen bead microtissues coated with GFP-tagged EVTs are embedded in a collagen-Matrigel mixture. **Results:** All parent neonicotinoids and their metabolites were detected in plasma and maternal, placental, and fetal tissues within 3 h post-exposure with peak concentrations ($t_{\max}$) observed at 3 h. Notably, concentrations of D-ACE and 5-OH-IMI were higher in fetal vs. maternal liver at most timepoints. Plasma clearance (CL_{plasma}) occurred between 12-24 h for most compounds. Maximum concentration ($C_{\max}$) for all compounds ranged between 0.16 and 508.93 ng/mL, with D-ACE reaching the highest concentration. CLO exhibited the highest placental-to-plasma ratio of ~ 0.144 . *In vitro*, EVT proliferation was significantly reduced after 5-day exposures to IMI-O (0.002 μM) and 3-OH-IMI (0.2 and 2 μM). Migration measured at 18 h decreased with D-ACE (0.002 and 0.02 μM), DNI (0.02 μM), 3-OH-IMI (0.002 and 0.2 μM), and DU (2 μM). Parent compounds did not alter proliferation or migration at any concentration individually or in a mixture. In the 3-D placental microtissue model, exposure to 3-OH-IMI for 96 h increased EVT invasion at all doses tested. **Conclusions:** This study provides the first integrated evidence that neonicotinoid metabolites pass through placenta and rapidly reach fetal tissues, including the brain, within hours of maternal exposure. Moreover, they selectively impair key placental functions in a compound-specific manner. Our findings highlight the need to consider metabolite-specific toxicity and placental-fetal transfer in environmental risk assessments.

PL 2020 **Advancing Dermal Toxicity Assessment for Botanicals: *in silico* approaches**

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Background and Purpose: Botanicals, including derivatives from plants, fungi, and algae, have long been used in cosmetics and personal care products, with growing consumer demand for natural ingredients. However, their complex and variable chemical compositions pose challenges for traditional toxicology testing. Because skin contact is a primary route of exposure, assessing dermal toxicity is essential to identify potential risks including skin irritation, sensitization, or phototoxicity. In this study, we focused on endpoints such as sensitization and evaluated whether *in silico* tools could predict this endpoint for botanical constituents and determine if these predictions fall within their applicability domain. **Methods:** We selected 86 chemical constituents from 10 botanicals based on their potential to induce dermal toxicity, including skin irritation, sensitization, and phototoxicity. The set of botanical materials included poison ivy ethanol extract, which is positive for sensitization; bergamot essential oil, positive for phototoxicity; and cypress spurge ethanol extract, positive for irritation, as well as botanical extracts not expected to cause adverse effects. Two predictive platforms were used: the OECD (Q)SAR Toolbox, which groups chemicals and fills data gaps using read-across and structure-activity relationships, and Derek Nexus which predicts sensitization, irritation, and phototoxicity by applying structural alerts from historical *in vivo* data, fragment-based rules for negative outcomes, and k-Nearest Neighbors modeling for potency estimation. Each constituent was assessed for domain applicability and predicted outcome for the dermal endpoints. A negative prediction outside the applicability domain was considered inconclusive.

Results: Among the 86 botanical constituents analyzed for skin sensitization, 83.7% were predicted as sensitizers by Derek Nexus and 70.9% by the OECD Toolbox, while 5.8% and 17.4% of the respective predictions were inconclusive. The two models showed a 66% concordance rate. Strong concordance was observed for constituents derived from hops, thyme, and cypress spurge, whereas notable divergence was found among models for poison ivy constituents. Sensitizing compounds were identified in all the botanicals analyzed. **Conclusions:** *In silico* tools can support dermal safety assessment of botanicals on a constituent-by-constituent basis, provided chemical characterization is available. For the sensitization endpoint, although both models are included as Defined Approaches in OECD Guideline 497, their predictions were not as concordant as expected, suggesting that *in silico* sensitization tools may not yield consistent outcomes for some natural complex substances components. While their predictive power is constrained by the complexity and variability of botanical compositions, the results can complement other non-animal methods and contribute to hazard identification and weight-of-evidence approaches for regulatory safety evaluations. Integrating computational approaches into dermal safety assessment frameworks will accelerate evaluations and establish additional scientifically robust foundations for botanical toxicity screening. These findings highlight both the utility and the current limitations of applying *in silico* methods to complex botanical mixtures.

PL 2021 **Understanding the Toxicity of Plant-Derived Prenylated Phenolics using QSAR Modelling and Molecular Dynamics Simulations**

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Background and Purpose: Prenylated phenolics are plant-derived compounds with promising antimicrobial activity. Although *in vitro* cytotoxicity has been widely reported, structure-activity relationships (SARs) remain poorly understood because of variability in experimental conditions. This study aimed to establish a consistent cytotoxicity dataset suitable for quantitative structure-activity relationship (QSAR) modelling and to identify molecular features influencing cytotoxicity in prenylated phenolics. **Methods:** A structurally diverse set of 41 prenylated phenolics was evaluated for cytotoxicity in human osteosarcoma (U2OS) cells using the resazurin (Alamar Blue) and CFDA-AM viability assays after 48 hours of exposure. The octanol-water partition coefficient ($\log K_{ow}$) was estimated using EpiWeb (v4.1) to predict general cytotoxicity as described by Kramer et al. (Toxicology *in Vitro*, 2009, 23, 1372-1379). Molecular structures were energy-minimized, and descriptors were calculated *in-silico* using Molecular Operating Environment (MOE v.2024.06). The dataset was divided into a training and test subsets. QSAR models were generated using genetic algorithm-multiple linear regression (GA-MLR), and model performance was assessed based on fitting performance and external predictivity. For candidate compounds, steered molecular dynamics (MD) simulations were performed to investigate phospholipid membrane interactions and assess insertion behavior, providing mechanistic context for the observed cytotoxicity. **Results:** Cytotoxicity of prenylated phenolics, as measured by the resazurin assay, ranged from $IC_{50} = 3.0 \mu\text{M}$ (Kurarione) to $IC_{50} = 74.1 \mu\text{M}$ (α -Isowighteone). A weak but statistically significant correlation was observed between measured and predicted IC_{50} values (Pearson's $r = 0.345$, $p = 0.027$), indicating limited predictive power of the general cytotoxicity model. These findings emphasize the need for more robust, compound class-specific QSAR approaches for prenylated phenolics. Development of such models is ongoing and will be reported at the conference. Preliminary data analysis showed a significant positive correlation (Pearson's $r = 0.50$, $p < 0.05$) between cytotoxicity (pIC_{50} , resazurin assay) and the fractional negative surface area (FCASA-), as well as a weak positive trend (Pearson's $r = 0.26$, $p < 0.10$) with hydrophobicity ($\log P(o/w)$). MD simulations showed more favorable and deeper membrane insertion for prenylated phenolics with increased hydrophobicity. **Conclusions:** This study established a robust cytotoxicity dataset for a structurally diverse set of prenylated phenolics. The limited predictive accuracy of general cytotoxicity QSAR models underscores the need for class-specific modelling approaches. Preliminary insights indicate that both increased fractional negative surface areas and hydrophobicity contribute to toxicity. Ongoing work focusses on developing class-specific QSAR models to support the rational design of prenylated phenolics with optimized antimicrobial efficacy and reduced mammalian toxicity.

PL 2022 **Integration of computational models to predict botanical phytochemical constituent clearance routes by the Extended Clearance Classification System (ECCS)**

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Background and Purpose: The Extended Clearance Classification System (ECCS) is a framework that predicts a chemical's predominant rate-determining clearance route: metabolism, hepatic uptake, or renal clearance. The ECCS prediction is based

upon molecular weight, ionization state, and membrane permeability, which could be predicted by quantitative structure-activity relationship (QSAR) models. The ECCS also indicates potential chemical interactions via drug-metabolizing enzymes and transporters. This study used the ECCS to evaluate phytochemical constituents and predicted drug-metabolizing enzyme and transporter pathways to understand botanical clearance in humans. **Methods:** First, 82 phytochemical constituents were classified into six ECCS classes based on QSAR-predicted properties. Next, constituents in classes 1A and 2 were further explored as potential substrates for 18 drug-metabolizing enzymes followed by predictions for hepatic clearance, while constituents in classes 3 and 4 leveraged predictions for glomerular filtration and renal transporters. Finally, potential interactions between phytochemical constituents and drugs were discussed. **Results:** Results showed that more than half of the phytochemical constituents were in ECCS class 2, whose Phase I metabolism were predicted to be predominantly mediated by CYP3A4, CYP2D6, and CYP1A2. Additionally, over 20% of the phytochemical constituents fell into ECCS class 4, which were predicted to be predominantly cleared in unchanged forms by glomerular filtration and active renal secretion by OAT1/3 or OCT2. Classes 1A and 2 compounds exhibit high interaction potential via CYPs, while classes 3 and 4 compounds have relatively low potential for renal uptake transporter mediated interactions. **Conclusions:** This study represents a data-driven framework for exploring and contextualizing botanical constituent information to inform safety evaluations. Additionally, it introduces the term “botanickinetics” as a more generalizable term for the ADME kinetics of complex botanical exposures.

PL 2023 Roots to Neurons: Evaluating Developmental Neurotoxicity (DNT) Potential of Botanicals Using an *In Vitro* Testing Battery

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Background and Purpose: The usage of botanicals is widespread and growing in the United States. Most botanicals have not been evaluated for developmental neurotoxic (DNT) potential, and some have been shown to adversely affect the central nervous system, raising concern and calling for evaluation. International efforts have proposed new approach methodologies (NAMs) for DNT testing resulting in the DNT *In Vitro* battery (DNT IVB) which consists of multiple assays designed to detect changes in key neurodevelopmental processes (KNDPs) of proliferation, apoptosis, cell migration, neuronal differentiation, neurite outgrowth, neuronal maturation and synaptogenesis, oligodendrocyte differentiation, and network formation using human and rat (primary cortical) cells. The objective of this project was to screen botanicals in the DNT IVB and evaluate the resulting data using Pareto frontier analysis of potency, selectivity, and activity confidence to comparatively assess potential DNT hazard. **Methods:** Eight botanicals selected based on signals of neurotoxicity in the literature (Aconite, Bupleurum, Cassava, Guarana, Kratom, Oleander, Wormwood, and Yohimbe) were assessed for DNT potential using a DNT Battery comprised of 2D and 3D *in vitro* assays to assess the impact on neurodevelopmental processes and a zebrafish assay to measure neurobehavior. Multiple samples for each botanical, except for Oleander and Cassava, were evaluated in the battery, which varied by preparation (i.e., powders vs extracts) and plant parts (i.e., leaf vs whole plant). **Results:** Oleander exhibited the greatest potential for DNT effects compared to the other botanicals as it displayed the highest selectivity and potency in multiple DNT assays. Fu Zi (Zhi), a popular Chinese herbal medicine and a processed root of Aconite, was the only botanical sample that showed no activity in any of the assays, suggesting the least potential for DNT effects. Our findings also show that potency and activity of the botanicals can differ not only by ingredient but also by preparation of the same ingredient and by lot number (e.g., Kratom samples differed across preparations and lot numbers). Wormwood samples were the most similar in terms of potency and activity. Additional observations include differences between test systems and test methods that resulted in diverse outcomes from assays for the same neurodevelopmental process (e.g., migration). For example, out of the 3 DNT assays that measure neural cell migration, ‘Guarana Extract’ was active only in the NPC2b assay that uses neurons and was inactive in the NPC2a assay that uses radial glial cells and the NPC2c assay that uses oligodendrocytes. Out of the 22 tested botanical-derived samples, only five were active in the zebrafish neurobehavioral assay, suggesting limited toxic potential of these botanicals on neurobehavior. **Conclusions:** In summary, exposure to botanical extracts affects neurodevelopmental activity, although consistent results across samples of the same ingredient were not observed. These inconsistencies are likely due to the variations in botanical processing and preparation which affects their chemical composition and potency. The diverse activity phenotypes of botanicals and botanical-derived compounds further highlight the complexity of hazard characterization, including DNT evaluation.

PL 2024 Emerging Mycotoxins and Fungal Contaminants in Seized Cannabis in Arizona and California, 2023-2024

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Background and Purpose: The contamination level of cannabis in the illicit market - including unlicensed production (i.e., black market cannabis) within a legalized state and products that failed compliance testing in the gray market - is largely unknown. **Methods:** The Maricopa County Sheriff's Office and the U.S. Postal Inspection Service collected 118 illicit cannabis flower samples between 2023 and 2024. Previous analysis identified 7 *Aspergillus* and *Fusarium* mycotoxins and metabolites (Stephens et al., 2023, Environmental Health Perspectives 133). In this follow-up study, we evaluated 8 additional emerging mycotoxins in a subset of samples using liquid chromatography-mass spectrometry. Twenty-three samples' fungal communities were sequenced through three separate targeted amplicon sequencing assays using NovaSeq1k p1 600 cycle kit run with 250bp forward and reverse reads. A primer set amplifying the transcription elongation factor 1α (EF1α), Internal Transcribed Spacer-1 (ITS-1), and ITS-2, before sequencing, was performed to speciate *Aspergillus* and *Fusarium* spp. **Results:** Three out of the eight tested emerging mycotoxins - alternariol, enniatin A, and beauvericin - were detected in 11 samples. Using targeted amplicon sequencing of the EF1α gene, we identified *F. cateniforme*, *F. languescens*, *F. cuspidatum*, *F. clavus*, *F. annulatum*, and unknown *Fusarium* species complexes. In further analysis, using the ITS-1 and ITS-2 sequencing data, more species of *Aspergillus* and *Fusarium* were identified. Six *Fusarium* species, including *F. chlamydosporum*, *F. oxysporum*, *F. proliferatum*, *F. solani*, *F. trinctum*, and *F. venenatum*, detected by ITS-1 and/or ITS-2 all came from separate species complexes. All but one *Fusarium* species identified (*F. solani*) are known producers of mycotoxins. The *Aspergillus* species detected in samples came from three general subgenera: *Circumdati*, *Aspergillus*, and *Nidulantes*. The six identified *Aspergillus* species are *A. flavus*, *A. insuetus*, *A. ochraceus*, *A. piperis*, *A. ruber*, *A. sclerotiorum*, and *A. versicolor*. Three of the six species of *Aspergillus* detected have not been recognized as producers of mycotoxins (*A. insuetus*, *A. piperis* and *A. ruber*). *A. versicolor* has been reported to produce sterigmatocystin, and was identified in two out of three sterigmatocystin-positive samples by both ITS-1 and ITS-2. There were eight sterigmatocystin negative samples that contained *A. versicolor*, as well. Overall, species of *Aspergillus* and *Fusarium* were identified in both samples that were mycotoxin positive and negative. **Conclusions:** Emerging mycotoxins and toxicogenic *Fusarium* and *Aspergillus* spp. pose a health risk to cannabis consumers. Seized illegal cannabis products contain *Fusarium* and *Aspergillus* spp. in both samples that were mycotoxin positive and negative; suggesting the possibility of production of mycotoxins in samples.

PL 2025 Case studies: Assuring consumer safety of botanical extracts, as cosmetic ingredients, through conscious safety design, new approach methodologies, and next-generation risk assessment (NGRA)

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Background and Purpose: Due to increased consumer demand for naturally sourced cosmetic ingredients and bans on animal testing, new non-animal safety assessment methods have become essential. This research presents the integration of New Approach Methodologies (NAMs) and Conscious Safety Design (CSD) concept in assessing consumer safety of botanical extracts to be used as cosmetic ingredients. **Methods:** CSD is a holistic approach that integrates safety considerations into the early design process of botanical extract preparations. This approach emphasizes the importance of early collaboration among toxicologists, analytical chemists, applied research scientists, engineers, and quality/regulatory staff among others to create safer botanical extracts that are not only functional for their intended biological activities but also safe for all end-users when formulated into cosmetic products. The four elements of CSD and NAM/NGRA are: (1) extraction: water, solvent, supercritical fluid, or electrokinetic (source identification, biological activities, effective concentration, substances of toxicological concern), (2) NAM and toxicology gap analysis: phytochemical/compositional analysis, literature review, use history, *in silico*, in chemico and *in vitro* assays, read-across, (3) next-generation risk assessment (NGRA) and validation: in-use simulated exposure assessment, qualitative and quantitative risk assessment, panel testing (effectiveness & safety), and (4) postmarketing surveillance. **Results:** Our case studies demonstrate the effectiveness of CSD and NAM/NGRA approaches in assuring the consumer safety of botanical extracts being used as cosmetic ingredients. The emphasis was given on in-depth review in historic botanical use, characterizing product categories and exposure, conducting *in vitro* irritation, sensitization, phototoxicity, mutagenicity tests, and confirming compatibility through human volunteer human patch tests on over 3,000 volunteers.

across 20 ingredients and more. **Conclusions:** CSD and NAM/NGRA approaches are effective animal alternative methods in assuring the consumer safety of botanical extracts being used as cosmetic ingredients.

PL 2026 Profiling Human Hepatic Biotransformation Enzyme Gene Expression According to PFAS Exposure

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Background and Purpose: Exposure to per- and polyfluoroalkyl substances (PFAS) has been associated with adverse health outcomes, including cardiometabolic disease, decreased immune function, and malignancy. The widespread exposure of humans to PFAS along with the high utilization of pharmaceuticals to treat these associated diseases raises concern about potential pharmacokinetic and pharmacodynamic interactions. PFAS and pharmaceuticals share disposition pathways including binding to serum albumin, cell membrane transport in key tissues via drug transporters, enterohepatic recirculation, along with renal reabsorption and excretion. As PFAS bioaccumulate in livers and kidneys, they activate transcription factors and nuclear receptors that regulate the expression of drug metabolizing enzymes. Therefore, the purpose of this study was to evaluate associations between PFAS concentration in banked human liver specimens and expression of mRNAs encoding drug-metabolizing enzymes. **Methods:** Human liver biospecimens were obtained from 34 cadaver donors through the National Disease Research Interchange. 54 PFAS were measured in the liver biospecimens using targeted liquid chromatography high-resolution mass spectrometry (LC-HRMS). Individual PFAS that were detected in more than 60% of the samples were included in the subsequent statistical analysis. For each PFAS, samples were ranked by concentration and divided into tertiles for comparison of mRNA expression levels. SYBR Green-based RT qPCR was conducted using 96-well plates formatted for the Human Drug Metabolizing Enzyme RT2 Profiler PCR Array System to measure relative mRNA abundance of 150 phase-I and -II biotransformation enzymes and 5 housekeeping genes. Target gene expression was normalized to housekeeping genes, and fold changes were calculated as $2^{-(\Delta\Delta Ct)}$. All data were analyzed using descriptive statistics. Differences in gene expression changes according to PFAS concentration were assessed using Welch's T-test. **Results:** PFAS were detected in all 34 individuals, with 17 compounds, including linear and branched isomers, found in ≥ 3 livers and 6 detected in 60% of specimens. The six most frequently detected PFAS were perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluoroundecanoic acid (PFUDA), linear-perfluorohexanesulfonic acid (L-PFHxS), linear-PFOS (L-PFOS), and branched PFOS (Br-PFOS). The gene expression of phase I metabolizing enzymes: Alcohol dehydrogenase (ADH) 1A, 1B, 1C, 4 and 6; Cytochrome P450 (CYP) 1A2, 2E1, 3A4, 3A43 and 7A1; and Flavin-containing Monooxygenase 3 were elevated at high PFOA, PFNA, L-PFHxS, PFUDA, L-PFOS and Br-PFOS concentrations. Similarly, phase II enzymes: Epoxide Hydrolase 1, Glycine-N-Acyltransferase, Glutathione S-transferase alpha 1, Sulfotransferase (SULT) 1A1, 1A2, 1E1, UDP-glucuronosyltransferase (UGT) 1A9, UGT2B4, 2B7, 2B10, 2B17, 2B28, and Thiosulfate Sulfurtransferase were enriched compared to samples in the lowest 33rd percentile. The phase I enzyme CYP21A2 and phase II enzyme Indolethylamine N-methyltransferase were lower at high PFAS concentrations. **Conclusions:** This study revealed novel associations between hepatic PFAS levels and altered expression of genes encoding Phase-I and -II biotransformation enzymes, suggesting potential effects of the ADME of pharmaceuticals and endogenous compounds. Supported by T32ES007148 and P30ES005022.

PL 2027 Per- and Polyfluoroalkyl Substances (PFAS) Antagonize Retinoic Acid Receptor (RAR) and Retinoid X Receptor (RXR) Activity

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Background and Purpose: Defects of the head and face account for approximately one-third of all congenital abnormalities worldwide and are a significant threat to neonatal and infant mortality. Prenatal exposure to per- and polyfluoroalkyl substances (PFAS) is associated with significant birth defects in humans and laboratory animals, including craniofacial abnormalities impacting eye and philtrum development. In chordates, craniofacial development is highly dependent on retinoic acid homeostasis and signaling, which regulates the expression of over 500 target genes through agonism of the nuclear retinoic acid receptor (RAR; $\alpha/\beta/\gamma$) and retinoid X receptor (RXR; $\alpha/\beta/\gamma$). Activation of the RAR/RXR heterodimer is driven by RAR agonism, with binding to RXR yielding synergistic effects on transcription. Throughout pregnancy, systemic concentrations of the predominant RAR agonist, all-trans-retinoic acid (atRA) are tightly maintained at approximately 5 nM. This homeostasis is achieved through an autoregulatory feedback loop in which excess atRA drives the transcription of genes encoding members of the cytochrome P450 (CYP) 26 and 3A families, which actively metabolize retinoic acid. PFAS share many structural similarities with atRA, affording them an increased capacity for ligand recognition in other biological pathways mediated by retinoid gradients. We have previously demonstrated the ability for PFAS to inhibit the homeostatic oxidative activities of CYP26A1 and

fetal/neonatal CYP3A7. Additionally, we found that perfluorodecanoic acid (PFDA) significantly downregulates transcription of retinoid target genes in female primary human hepatocytes. We therefore hypothesize that PFAS antagonize RAR and RXR activity, thereby contributing to the craniofacial defects associated with prenatal exposure. **Methods:** Twelve PFAS (FBSA, PFBS, PFPeA, GenX, FHxSA, PFHxS, 4:2 FTS, PFOA, PFOS, PFNA, PFDA, and PFUnDA) were tested at 10 and 100 μ M for their capacity to antagonize RAR $\alpha/\beta/\gamma$ and RXR $\alpha/\beta/\gamma$ activity based on their widespread use and/or presence in fetal or maternal tissues. Screenings were conducted using the INDIGO Biosciences cell-based luciferase reporter assay for each isoform (State College, PA). Briefly, incubations were performed in the presence of atRA or 9-cis-RA agonist at the EC₅₀₋₈₀ for each respective receptor. Reference compounds (RAR α , BMS 195614; RAR β/γ , CD 2665; and RXR $\alpha/\beta/\gamma$, UVI 3003) were included as positive controls for antagonism. Following a 22-24 h incubation period (37°C, 5% CO₂), media was removed, and luciferase detection reagent was added. After 5 min, luminescence was quantified with a microplate reader (TECAN; Männedorf, Switzerland) at 500 mSec per well. The log₂(FoldChanges) of each treatment were quantified against the vehicle control, and statistical significance was calculated using a two-way ANOVA with Dunnett's post hoc test for multiple comparisons. Experiments were performed in triplicate. **Results:** Our results indicate that FBSA, PFBS, PFPeA, GenX, FHxSA, 4:2 FTS, PFNA, PFDA, and PFUnDA significantly antagonized RAR and RXR activity ($p_{adj} < 0.05$). An inverted U-shaped relationship was observed between carbon chain length and receptor activity, in which the shorter ($\leq C6$) and longer PFAS ($\geq C9$) were more potent antagonists compared to mid-length PFAS (e.g., PFOA and PFOS (C8)). The 11-carbon PFUnDA demonstrated the highest potency for receptor antagonism across all six isoforms. PFUnDA (100 μ M) significantly antagonized RAR activity with log₂(FCs) of -4.62 (α), -1.35 (β), and -4.52 (γ) ($p_{adj} < 0.001$). PFUnDA (100 μ M) also inhibited RXR activity with log₂(FCs) of -0.52 (α), -1.39 (β), and -2.31 (γ) ($p_{adj} < 0.05$). In addition to PFUnDA, FHxSA and PFDA both significantly antagonized all six RAR and RXR isoforms ($p_{adj} < 0.01$). **Conclusions:** Nine of the twelve PFAS tested (FBSA, PFBS, PFPeA, GenX, FHxSA, 4:2 FTS, PFNA, PFDA, and PFUnDA) significantly antagonized retinoid receptor activity *in vitro*. Interference with retinoid signaling during human development leads to severe congenital defects and malformations of the head and face. Biomarkers of PFAS teratogenicity largely overlap with those implicated in altered retinoid signaling during development. We therefore offer our findings as further mechanistic evidence for PFAS teratogenicity through altered retinoid signaling via RAR and RXR antagonism.

PL 2028 Identification of PFAS-binding proteins by a toxicoproteomic tool

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Background and Purpose: Although transcriptomic and proteomic approaches are informative in toxicological research, they often fail to pinpoint the precise molecular targets of chemicals. To address this gap, we developed a proteome-wide binding assay and a computational pipeline to identify proteins binding to per- and polyfluoroalkyl substances (PFAS). **Methods:** Perfluorooctanoic acid (PFOA) and perfluorononanoic acid (PFNA) were covalently attached to carboxyl-functionalized ferrite-glycidyl methacrylate (FG) beads using click chemistry. The PFAS-conjugated beads were incubated with whole-cell extracts derived from mouse P19 teratoma cells and Hepa1c1c7 liver cells. After several washing steps, bound proteins were first visualized by gel electrophoresis and silver staining, and subsequently identified by liquid chromatography-mass spectrometry (LC-MS). Differential Enrichment analysis of Proteomics data (DEP) software was used to identify differentially bound proteins to PFAS-conjugated beads compared to control beads and to predict molecular pathways affected by PFAS exposure. **Results:** PFOA-conjugated beads differentially captured 474 proteins from P19 cell extracts and 378 proteins from Hepa1c1c7 cell extracts compared with control beads. PFNA-conjugated beads bound 470 proteins in P19 cells and 448 proteins in Hepa1c1c7 cells. Most bound proteins overlapped between the two cell types (306 for PFOA and 344 for PFNA) indicating that PFAS targets were not restricted to specific cell types. Functional enrichment analysis suggested that the bound proteins participate in fundamental cellular processes, including DNA, RNA, protein, and macromolecule metabolism; RNA processing; and the organization of chromosomes, organelles, and protein complexes. Proteins involved in xenobiotic responses and cellular stress were also detected. **Conclusions:** Toxicoproteomic tools enable rapid and systematic identification of chemical targets and offer a more direct route to identifying molecular initiating events. In our assays, each PFAS captured hundreds of proteins with substantial overlap between the two cell extracts, indicating that PFAS interact with large protein complexes critical for essential cellular functions.

PL 2029 Individual chemical concentration-responses predict neurobehavioral effects of a human-relevant PFAS mixture in larval zebrafish

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Background and Purpose: Humans are ubiquitously and simultaneously exposed to per- and polyfluoroalkyl substances (PFAS), a class of widespread environmental contaminants comprised of >14,000 unique structures. Some PFAS transfer through the placenta and bioaccumulate in the brain, indicating the need to investigate the potential of these compounds to cause developmental neurotoxicity (DNT). Epidemiological studies report conflicting findings on the link between early life PFAS exposure and DNT in humans, which may be attributed to the limited focus on co-occurring chemical mixtures. Here, we aimed to assess exposure of six prominent PFAS detected in the blood of Scandinavian populations and their mixture to elucidate effects on neurodevelopment in an early life-stage zebrafish behavior assay. Furthermore, we applied mixture modeling approaches to examine whether mixture effects were predictable from individual chemical concentration-response profiles, revealing a novel application of behavior-based New Approach Methods (NAMs) in chemical mixture risk assessment. **Methods:** Zebrafish were developmentally exposed to single PFAS (perfluorooctanesulfonic acid (PFOS), perfluorohexanesulfonic acid (PFHxS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), or perfluoroundecanoic acid (PFUnDA)), a mixture in ratios found in the blood at 10-100x the blood level, or the solvent control from 1-5 days post fertilization (dpf). At 5 dpf, larvae were subjected to the Visual and Acoustic Motor Response (VAMR) NAM. To derive concentration-response curves, motor activity was modeled using Generalized Additive Mixed Effects Models for visual motor response data and Linear Mixed Effects Models for startle response data. Frechet distances were calculated between chemical concentrations and control modeled responses. The winning model was selected out of 10 curve-fitting models by the tcpl2 v3.0 pipeline. To predict mixture effects on different endpoints, Concentration Addition (CA) and Independent Action (IA) models were computed and predictions were compared to observed mixture responses. **Results:** Larvae exposed to PFOS, PFUnDA, PFOA, PFNA, or 10-100x the human blood level mixture displayed heightened startle in response to high intensity acoustic stimuli. Exposure to PFOS, PFHxS, PFUnDA, or the mixture at 56-100x the human blood level caused dark-phase hyperactivity. Best fit concentration-response curves for single compounds active in the acoustic startle and dark phase endpoints demonstrated concentration-dependent relationships in the same direction as the observed mixture effect, thereby allowing their inclusion for mixture toxicity predictions. CA and IA predicted mixture curves for these endpoints revealed similar fits with the observed mixture curves, indicating model predictions could accurately capture experimental mixture effects. AC50 values derived from CA and IA mixture predictions were within $\pm 0.8 \mu\text{M}$ of the observed mixture AC50 values, suggesting active compounds within an endpoint obey rules of additivity and single component response curves can be used to predict mixture effects. **Conclusions:** This work demonstrates the applicability of additive mixture predictions for rapid, behavior-based assessment of co-occurring chemical mixtures. By examining a PFAS mixture comprised of compounds found in human blood at a fixed ratio, we extend the implementation of zebrafish embryo NAMs for DNT testing to a real-life exposure scenario. Analytical chemistry is ongoing to link behavioral effects to zebrafish tissue doses, ultimately allowing for extrapolation to human blood concentrations.

PL 2030 PFAS compounds potentiate DNA damage in HEK293T cells and zebrafish embryos at parts per billion exposure levels and below.

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Background and Purpose: Per- or Polyfluoroalkyl substances (PFAS) are persistent environmental contaminants, some of which have been shown to cause injury and disease, such as immune deficiencies, cancer, and metabolic disease in humans. For this study, we selected three PFAS compounds with differing carbon chain lengths: perfluorooctanoic acid (PFOA, 8C), perfluoropentanoic acid (PFPeA, 5C), and perfluorobutane sulfonic acid (PFBS, 4C), to determine if chain length corresponds with genotoxicity *in vitro* and *in vivo*. **Methods:** To determine genotoxicity, we separately tested each chemical against vehicle control at dose levels ranging from 0.02 to 20 parts per billion in HEK293T cells for 24 hours and in zebrafish embryos from 4 hours post-fertilization to five days using single-cell electrophoresis (i.e., comet). Each compound was tested at least twice under neutral and alkaline electrophoresis conditions using HEK293T cells and zebrafish embryos. The webtool, CometScorer 2.0, was used to compute Olive Tail Moments (OTM) that describe the length, width, and brightness of comet features, with higher OTMs indicating increased damage. **Results:** Our data show increased genotoxicity, *in vitro* and *in vivo*, for all chemicals tested and under alkaline or neutral electrophoresis conditions. The lowest observable effect levels were 0.02 and 0.2 ppb, depending on the exposure paradigm and host system. **Conclusions:** Our results indicated that the PFAS compounds tested induce DNA damage at environmentally relevant levels. OTMs between neutral and alkaline comet conditions were different in effect amplitude, with alkaline comet showing higher OTMs. We interpret these data as the PFAS potentiating

DNA damage that is realized in alkaline comet but not neutral comet conditions. Furthermore, DNA damage in zebrafish showed similar relationships to the *in vitro* model, supporting our hypothesis that PFAS potentiate DNA damage independent of the host cell. Finally, we are seeing effects at parts per trillion (ppt) concentrations. The EPA has set the maximum contamination level for PFOA at 4 ppt; based on our data, we conclude that the EPA MCL is appropriately low.

PL 2031 PFAS Exposure Attenuates Tryptophan Metabolism through the Kynurenine Pathway in the Human Placenta

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Background and Purpose: The placenta is an essential organ that forms the interface between the maternal and fetal bloodstreams. The placenta performs numerous functions essential for successful pregnancy, including transfer of nutrients, removal of waste, and protection from infections and maternal-immune mediated rejection. Impaired placental function is associated with pregnancy complications that adversely impact maternal and fetal health and survival. A key pathway in the placenta is tryptophan (TRP) catabolism through the Kynurenine Pathway (KP) which is active from mid to late gestation in human pregnancy. Indoleamine dioxygenase (IDO1) is the rate limiting enzyme that initiates TRP catabolism in the KP. Studies in pregnant mice revealed that the KP is essential for promoting a tolerogenic maternal-fetal environment that allows the survival of the semi-allogenic fetus. Deficiency in this pathway, when the ratio of available TRP is high relative to KYN levels, is associated with growth restriction, preeclampsia, and miscarriage in human pregnancy. Published studies using animal models show that placental TRP catabolism is vulnerable to environmental toxicant exposures, but to the best of our knowledge, no study has been performed in humans. Per or Polyfluoroalkyl Substances (PFAS) are widespread toxicants that are extremely stable in the environment and have been detected in human pregnancies in maternal blood and placenta samples. Previous murine studies have found that PFOA exposure alters several amino acid metabolic pathways, including TRP metabolism in the brain and liver. Additionally, PFAS exposure is associated with liver inflammation and reduced Tryptophan 2,3-dioxygenase (TDO), the enzyme responsible for TRP metabolism in the liver in humans. The current project investigates TRP catabolism in the human placenta. **Methods:** The cohort in this investigation is part of an ongoing, prospective longitudinal study Understanding Pregnancy Signals and Infant Development (UPSIDE) that has prospectively followed a diverse sample of dyads from the first trimester of pregnancy into childhood. During trimester 2, pregnant women consented to a blood draw, from which serum was separated for PFAS assessment (n=286). PFAS analytes were measured in serum samples using LC/MS analysis by the Wadsworth Human Health Exposure Analysis Resource (HHEAR) laboratory. Placental samples were also collected at the time of birth, aliquoted into 50ml tubes, and stored at -80C. Metabolomics assessments for amino acids were completed on a subset of placental samples (n=40) using LC/MS analysis by the University of Pennsylvania's Metabolomics Core Facility. This study focused on TRP, Kynurenine (KYN) and the KP metabolites Kynurenic Acid (KA) and 3-Hydroxy-Kynurenine (3-HK). Samples were shipped to University of Pennsylvania's Metabolomics Core for LC/MS analysis. Prior to analysis, PFAS values below LOD were imputed with the LOD/sqrt2, a common statistical method for linear regression analyses. Analytic methods planned for this investigation include Pearson's Correlations, linear models, and Bayesian Kernel Regression Model (BKRM). **Results:** Serum samples from all 40 women had detectable levels of PFOS, PFOA, PFNA, PFHxS, and PFDA levels. There were slightly lower rates of detection for PFPEA (75%) and PFUNDA (65%). Preliminary analysis of linear models suggests that levels of PFPEA (est= -0.59, se= 0.34, p= 0.09) and PFUNDA (est= -2.9, se= 1.3, p= 0.03) have an inverse relationship with TRP levels in the placenta. PFPEA levels have a positive relationship with KYN (est= 1.3, se= 0.37, p= 0.001) and with the KYN/TRP ratio (est= 0.34, se= 0.12, p= 0.007). **Conclusions:** These preliminary results suggest that PFAS analytes, especially PFPEA, are associated with indicators of increased KP activity. This is a unique and significant finding considering no other studies have investigated the influence of PFAS on placental KP activity. Analyses are ongoing and will include additional statistical models and assessments of IDO protein levels in the human placenta, a key mediator of KP activity.

PL 2032 Extracting Medical Devices: A Solution for Multilaminates, Soft Polymers, Superabsorbers and Various Adhesives

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Background and Purpose: The diversity of materials used in producing medical devices often results in difficulties when testing for biocompatibility. Soft polymers, hydrogels, various adhesives, multilaminate structures and superabsorbent materials typically degrade or become otherwise compromised when performing traditional extractions as per ISO 10993, resulting in non-compliant extracts which are both physically unsuitable for biocompatibility testing and not clinically representative of the patient chemical exposure profile. The quantity of available material is overestimated, and this impacts the accuracy and appropriateness of the Toxicological Risk Assessment. Furthermore, further modification (i.e.: filtration) is often required before the extract can be used within biocompatibility test systems,

an approach which is currently not accepted by certain regulators (e.g.: the FDA). **Methods:** Extraction of wound dressings was performed in compliance with the requirements of ISO 10993-12 using two different extraction vessels: traditional (polybag) and novel (fixture). Following visual observation, the chemical profiles of extracts generated in both vessels were compared using gravimetric analysis (NVR), GCMS, QTOF-LCMS, ICP-MS and GPC. To verify the analytical evaluation, a few devices were confirmed through traditional *in vivo* systemic toxicity endpoint testing (acute and repeated dose) to verify any incompatibility with the use of the fixture; with the *in vivo* work, the pH and osmolality of the fixture extracts were also measured. **Results:** Generating extracts from wound dressing medical devices was feasible using the novel fixture extraction method. Comparing fixture extraction to extraction using a traditional (polybag) extraction vessel, device degradation was significantly minimized and the physical destruction of test articles was eliminated. NVR highlighted a significant reduction in the quantities of degradation particulates, and analytical chemistry (GCMS and LCMS) indicated no unaccountable difference in the chemistries present within the extracts generated using both the traditional and novel extraction method. Furthermore, GPC demonstrated a small reduction in the average molecular weight of extracted polymers, and it was demonstrated that the pH and osmolality of fixture extracts was not significantly changed from that of the extraction solvent. ICP-MS was determined to be incompatible with fixture extraction. Finally, systemically injecting the polybag extract resulted in equivocal results and failing to meet the test criteria in the acute and repeated dose systemic toxicity studies, respectively, whereas exposure to the fixture extracts following the same dosing protocols showed no evidence of systemic toxicity. **Conclusions:** Using a commercially available and standardized fixture for extracting medical devices (i.e.: wound dressings) composed of materials such as soft polymers, hydrogels and adhesives, multilaminates, and superabsorbers allows for the generation of extracts in compliance with ISO 10993 which are both (i) representative of an exaggerated patient chemical exposure profile and (ii) suitable for using in biocompatibility testing.

PL 2033 Approaches for the Biocompatibility and Toxicological Assessment of Absorbable-Degradable Medical Devices with Extraction Vehicle Solvent Incompatibility

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Background and Purpose: Medical devices which have absorbable/degradable properties or are incompatible with test systems (biological and analytical), pose unique challenges for assessing their safety per the traditional ISO 10993 testing paradigm. Adaptations to these methods are required, in particular test article/dose preparation and how chemical characterization and the associated toxicological risk assessment can be performed. Non-solid materials, and those intended to change form during clinical use, such as hydrogels, collagens, wound rinse solutions, bone putties and degradable polymer sutures and implants. Certain materials can be incompatible with various extraction parameters within ISO 10993-12 leading to adaptations in approach. For many medical devices, a traditional approach to determining extractables includes an exhaustive extraction, and when a device is intended to change its physical and chemical properties during clinical use (eg, bioabsorbable/degradable devices), determining exhaustivity due to these intended properties may not be feasible. These challenging material types require a customized approach to best determine their complete safety profiles and efforts are underway by ISO TC 194 and ISO TC 150 working groups to create guidance on considerations and a potentially predictive pathway. These considerations are presented from a historical evaluation of bioabsorbable/degradable devices with the purpose of providing solutions, but also solicit feedback from industry for a collective approach. **Methods:** A historical review of ISO 10993 testing approaches resulting in a successful evaluation of safety for absorbable/degradable devices was performed and determined: 1) common challenges for sample preparation for absorbable/degradable material types, 2) sample preparation adjustments were necessary, 3) the particular biological and analytical chemistry methods they are incompatible with, 4) solutions for ensuring applicable biological endpoint(s) could be addressed, and 5) potential drawbacks to approaches used. **Results:** While the majority of material types and individual methods had solutions to any incompatibilities which were found to be suitable, it was determined that a robust pre-emptive mitigation plan was missing from a robust biological evaluation plan in some instances. When biological testing was performed via an extraction, sample preparation was found to require the greatest level of adaptation due to the variety to dose methods required across the applicable ISO 10993 series. Direct testing methods were best optimized for cytotoxicity, irritation, hemocompatibility and systemic toxicity while the rabbit material mediated pyrogen test was one of the most challenging. The strategic use of comparative controls allowed for a real-world clinical impact assessment of results which may have otherwise shown a "positive" result. For extractables chemical characterization testing and particular genotoxicity vehicles, the greater challenge of material compatibility with extraction solvent compared to most biological extractions vehicles lead to an expansion of incompatible materials including soft plastics. An Analytical Evaluation Threshold (AET) could not be met for certain cases and utilizing biological testing was required to support required endpoints (systemic toxicity and genotoxicity) which may have otherwise been addressed by chemical characterization. The findings, in particular for ISO 10993-12, have led to a proposal for an ISO Technical Specification document which uses known data to predict the results of extraction based on material type. **Conclusions:** The challenge with any approach that may

operate tangentially to a standard or guidance, is simultaneously ensuring both testing feasibility and regulatory acceptance. This historical review presents options and pathways which have been shown to generate optimal data using risk-based and historical perspective for modifications of ISO 10993 methodology, while promoting and helping predict the success of future medical devices with intended physical and chemical property changes during clinical use. Medical device manufacturers and the lab community can coordinate efforts to create a least burdensome (time, cost, and effort) pathway for the evaluation of absorbable/biodegradable materials and those with extraction vehicle incompatibility for testing.

PL 2034 Target Selection for Leachables Testing Using Historical Data of Pharmaceutical Container Closure Systems

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Background and Purpose: Extractables and leachables (E&L) testing is widely used to identify and quantify impurities originated from the polymeric materials used in pharmaceutical manufacturing, packaging, and medical devices. Leachables testing provides a direct measurement of chemical migration under actual or accelerated conditions and serves as an important step in the overall E&L assessment process verifying patient safety and affording opportunities for risk mitigation. While regulatory guidelines such as draft ICH Q3E, USP <1664>, and ISO 10993-18 provide direction on when and how to conduct leachable testing, these guidelines do not explicitly define the scientific rationale or criteria for selecting leachable targets. **Methods:** Leachable target selection is guided by the extractables profile of the test article and is based on four main considerations: 1) the known toxicity or safety threshold of the compound; 2) its prevalence in extraction studies of similar materials; 3) its representativeness of specific material classes with known leachables profiles; and 4) the physiochemical properties and potential leachability under relevant storage/contact conditions. Ideally, leachable targets should be both targetable and quantifiable using an established analytical assay. Following these criteria, 27 leachable targets were selected and monitored in annual stability samples from multiple drug substance and drug product containers. Additionally, non-targeted analysis (NTA) was also conducted to identify any unanticipated leachables. **Results:** The four selection factors ensure the leachable target is toxicologically relevant and representative of compounds with a high likelihood of migrating from container materials under real-world use conditions. Among the 27 targets under annual monitoring, 11 were detected in the leachables testing of 25 drug products and 10 drug substances over a 5-year period. Two compounds, caprolactam and polyethylene glycols (PEGs) (molecular weight between 400 to 600 dalton), were consistently detected above their analytical assay limits of quantification. Caprolactam was reported in the leachable samples from the drug substance bags (detection frequency: 75%; n=24) with a maximum patient exposure of up to 36.4 µg/dose. PEGs were reported in the leachable samples from the vial-stopper systems (100%; n=33) with a maximum patient exposure of up to 875.7 µg/dose. Notably, bisphenol A (BPA), a compound identified in the extraction study of polycarbonate carboys (n=7), was not detected in any drug substance samples analyzed. With a few exceptions, no new leachables were detected in any drug products or drug substances using NTA with highly sensitive analytical assays capable of detection at ppm-ppb levels. **Conclusions:** This study demonstrates a systematic approach to leachable target selection which integrates extractable data interpretation, compound safety assessment, and analytical feasibility. Historical leachable data from pharmaceutical container systems indicates that the proposed approach effectively identifies representative leachable compounds with minimal unexpected findings from NTA general screening. Moreover, all leachables were reported at levels well-below their respective permitted daily exposure (PDE) values at all time points, providing empirical evidence that the current quality management system is effectively ensuring patient safety with respect to potential leachables exposure.

PL 2035 A Novel Alternative to Headspace Sampling for the Detection of Volatile Organic Compounds Utilizing Passive Headspace Concentration

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Background and Purpose: The current method used for analysis of volatile organic compounds (VOCs) from medical devices per ISO 10993-18:2020 is static headspace sampling, where a medical device is extracted with a polar aqueous solvent in a clean vessel for a set duration of time at a specific temperature using continuous mechanical agitation. The resulting liquid extract is then analyzed via headspace gas chromatography-mass spectrometry (HS GC-MS) to identify and quantify the compounds present. This information is then used in a toxicological risk assessment (TRA) to evaluate potential patient safety risks arising from exposure to these compounds. Recent deficiency questions from the U.S. Food and Drug Administration (FDA) are challenging whether VOCs are substantially lost during transfer of the polar aliquot to a vessel. The proposed FDA approach to address these deficiencies is to perform headspace analysis on the entire device. However, most medical devices are not compatible with the headspace vial configuration used for HS GC-MS analysis, as the

maximum available volume is limited to 20 mL with an inner neck diameter of only 11 mm. To address the FDA's request, a new semi-quantitative method was developed for medical devices: Passive Headspace Concentration for Medical Devices (PHC-MD). This approach was based on ASTM E1419-19 and ASTM E1618-19, standards originally created for fire debris analysis. It uses passive headspace concentration (PHC) to extract VOCs from an entire medical device without the need for a polar solvent. PHC has historically been used as a qualitative method for the identification of ignitable liquid residues in fire debris. In this study, we adapted, developed, and validated a PHC method for VOCs in medical devices that can provide quantitative amounts of VOCs which can then be evaluated in a TRA to understand the exposure risk to a patient. **Methods:** Residual Solvents Class 1 mix and Class 2 Mix A at various concentrations were spiked into gas-tight glass vials. PHC extraction was performed by hanging a small piece of activated charcoal into the headspace of the vial. The vials were sealed and heated for 24 hours at 100°C to drive volatiles into the headspace of the container, allowing interaction with the activated charcoal, which acted as sink for volatile organic compounds. The activated charcoal was removed from the container upon cooling and desorbed using dichloromethane. Sample analysis was performed on an Agilent Technologies 8890 gas chromatograph equipped with an Agilent 5977B mass selective detector. The resulting data was compared to traditionally conducted HS GC-MS data then evaluated using ISO 10993-17:2023 toxicology methods. **Results:** Method accuracy, precision, specificity, limit of quantitation (LOQ), and linearity were established to understand the robustness of this method for use in medical devices. When the results from the PHC-MD method were compared to historical data from traditional HS GC-MS analysis, we found that the PHC-MD method identified more compounds. This shows that the new method is both more sensitive as it is able to detect lower levels of volatiles, and more conservative than the previous approach. Permitted daily exposure limits for several VOCs specified in USP <467> (Residual Solvents) were evaluated as a toxicologically relevant framework for determination of suitable reporting limits for VOCs to ensure that the results were meaningful for toxicological risk assessments. **Conclusions:** Establishing a passive headspace concentration method for the detection of volatiles from an entire final, finished medical device is critical to address repeated FDA deficiencies surrounding the analysis of VOCs. It is also important for the accurate toxicological risk assessment to ensure patient safety has been adequately evaluated. This new method can be used on the entire medical device without the use of a polar solvent, expanding its applicability to medical devices that are incompatible with standard HS-GC vial configurations.

PL 2036 CN Bio Microphysiological System Platform to Assess Mechanisms of Hepatotoxicity

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Background and Purpose: While advancements have been made in 2D hepatocyte culture, there have remained gaps in the ability to predict human drug-induced liver injury (DILI). Complex *in vitro* models, such as microphysiological systems (MPS), which integrate 3D architecture and media microfluidics have aimed at improving the ability to predict DILI. However, their widespread use in pharmaceutical research has remained limited. We optimized a human liver MPS with hepatocytes, Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs), and stellate cells (SCs) at physiologically relevant ratios. We then evaluated whether the liver MPS could reliably distinguish between cholestatic and mixed DILI mechanisms by assessing functional markers, bile acid secretion, and gene expression profiles following administration of known mixed and cholestatic DILI compounds and controls. **Methods:** To recapitulate native human liver composition and function, we optimized cellular input of hepatocytes, KCs, LSECs, and SCs at relative proportions representative of native human liver. Cells were seeded at a density of 600,000 total cells per well in Williams E media in the CN Bio PhysioMimix® platform. Media was exchanged every other day for 22 days. To determine whether the model could distinguish cholestatic DILI from mixed DILI, 10 positive and negative controls were evaluated, with compound administration initiated on Day 8 at 1x, 5x, 15x and 50x Cmax. Samples were collected at 24 hours, 1 week, and 2 weeks post-administration. Non-terminal readouts included albumin, LDH, and bile acids as markers of liver health and function. At Day 22, expression of 21 genes involved in bile acid transport, metabolism, and homeostasis was profiled. **Results:** Administration of mixed and cholestatic DILI compounds resulted in significant, dose- and time-dependent cell damage, as indicated by decreased albumin production and increased LDH at all time points. Mixed DILI compounds had a greater impact on albumin output and overall toxicity, particularly at higher concentrations (15x and 50x Cmax), while cholestatic DILI compounds caused more pronounced downregulation of genes involved in bile acid metabolism (CYP7A1, CYP8B1) and transport (NTCP, BSEP). Of the five bile acids (TCDCa, CA, TCA, GCA, GCDCA) profiled, significant reductions in GCA and GCDCA over two weeks were observed. **Conclusions:** Collectively, these findings demonstrate that mixed and cholestatic DILI compounds disrupt hepatobiliary homeostasis, impair liver cell viability, and alter key regulators of bile acid regulation. Successful integration of MPS into pharmaceutical research relies on careful characterization, definition of relevant context(s) of use (COU), and COU qualification. Current data demonstrate the feasibility of utilizing the liver MPS to assess mechanisms of hepatotoxicity, including cholestatic DILI, *in vitro*.

PL 2037 Role of Hepatocyte-Specific Egr1 in Liver Injury and Regeneration After Acetaminophen Overdose

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Background and Purpose: Acetaminophen (APAP) overdose is the leading cause of acute liver failure (ALF) in Western countries. Current treatment options for APAP-induced ALF are limited, including N-acetylcysteine effective only when administered within the first 24 hours after overdose and liver transplantation, which presents significant challenges. Studies from our laboratory and others have shown that timely liver regeneration is critical for patient survival. However, the mechanisms of liver regeneration after APAP overdose and the transcriptional factors that drive the regenerative response are not entirely clear. Early Growth Response 1 (Egr1) is an early response transcription factor rapidly activated after liver injury. Previous studies have shown that Egr1 protects after APAP overdose at early time points during the injury phase, improving mitochondrial respiratory function and fatty acid β -oxidation activity. While Egr1 deficiency exacerbates liver injury, its role during regeneration remains unclear. Studies from our lab performed using the incremental dose model, which includes a moderate/regenerative dose and a severe/nonregenerative dose, showed a steady decline in Egr1 expression in both doses during the first 24 hours but a significant increase only in the moderate/regenerative dose at 48 hours, coinciding with hepatocyte proliferation. Based on these studies, we hypothesize that increased Egr1 during APAP-induced liver injury regulates liver regeneration.

Methods: Two-month-old male Egr1 floxed mice were injected with AAV8-TBG-iCre to generate hepatocyte-specific Egr1 knockout (hEgr1 KO) mice. Wild-type control mice were injected with AAV8-TBG-GFP. One week after AAV administration, mice were fasted overnight and then administered a 300 mg/kg overdose of APAP. Liver and blood samples were collected at multiple time points over 72 hours. **Results:** hEgr1 KO mice exhibit a significantly greater liver injury compared to controls at 6 and 24h after APAP administration, as determined by elevated ALT activity in serum and hepatic necrosis. CYP2E1 protein level shows no difference at baseline. The absence of Egr1 was associated with low GSH levels and high JNK and ERK phosphorylation. Importantly, despite significantly greater initial injury, hEgr1 KO mice showed recovery similar to that of controls. Both groups display similar levels of proliferation at 48h and 72h after APAP overdose, exhibiting no significant difference in Ki67-positive cells by IHC. hEgr1 KO mice show a reduction of Cyclin D1 protein at 24h compared to control, but no difference was observed at 48 and 72h after APAP treatment. This was accompanied by increased protein levels of CDK4 and phospho-Rb in hEgr1 KO mice. hEgr1 KO mice had increased AKT phosphorylation at 72h after APAP overdose. The expression of cytokines *Il6*, *Il1b*, and *Il10* is increased at 6 and 24h after APAP overdose in hEgr1 KO mice, associated with higher injury levels. *Ifng* shows no significant difference between the mouse groups. Key mediators of liver regeneration, *Tnfa* and *Tgfb1*, had increased expression in hEgr1 KO mice at 48h, but *Il6* mRNA levels were lower. Nuclear receptors, other liver regeneration mediators, *Ppara*, *Car*, and *Fxr* showed higher expression in control mice at 24h after APAP overdose than in hEgr1 KO mice, with *Ppara* alone showing high expression in control mice at 48h. **Conclusions:** Taken together, these data indicate that hepatocyte-specific Egr1 protects from initial liver injury after APAP overdose, but its loss during the regenerative phase does not affect liver regeneration. In fact, rapid recovery of hEgr1 KO mice despite significantly higher injury suggests that inhibition of Egr1 during the regenerative phase may improve liver regeneration by stimulating pro-regenerative cytokines (TNF α) and inhibiting anti-regenerative cytokines (IL-6). Supported by NIH R01DK98414

PL 2038 MRP4 Modulates Hepatic Response to Phenobarbital by Regulating Proliferative and Metabolic Pathways

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Background and Purpose: Membrane transporters are essential for hepatic homeostasis by mediating the movement of metabolites and xenobiotics. Among these, Multidrug Resistance Associated Protein 4 (Mrp4/ABCC4) plays a role in hepatic defense and adaptation to chemical stress. Our laboratory previously showed that hepatic Mrp4 expression increases after acetaminophen intoxication and colocalizes with zones of hepatocyte proliferation. Notably, Mrp4 deficiency did not reduce hepatocellular proliferation following partial hepatectomy (PH). Rather, knockout mice developed significant hepatic steatosis post-PH. To further delineate the role of Mrp4 in hepatocyte growth and metabolic remodeling, we used a phenobarbital-induced hepatomegaly model, a classical mitogenic stimulus that promotes hepatocellular proliferation and metabolic adaptation. **Methods:** Adult wild-type and Mrp4^{-/-} mice received daily intraperitoneal doses of phenobarbital (50 or 100 mg/kg) for 10 days. Liver-to-body weight ratios and serum ALT levels were measured to assess hepatic growth and injury. Histological evaluation was performed on formalin-fixed, paraffin-embedded liver sections using H&E staining to assess hepatocellular hypertrophy and mitotic figures. Hepatocyte proliferation was quantified by Ki67 immunohistochemistry. Hepatic triglyceride content was measured in frozen liver tissue using a colorimetric triglyceride assay following lipid extraction, and neutral lipid accumulation was visualized via Oil Red O staining. Gene and protein expression of markers involved in cellular proliferation, xenobiotic metabolism, and lipid regulation were quantified using qPCR and immunoblotting, respectively. **Results:** Mrp4^{-/-}

mice exhibited significantly higher liver-to-body weight ratios and elevated ALT levels compared to wild-type counterparts, indicating amplified hepatic enlargement and injury. Histology revealed hepatocellular hypertrophy with increased mitotic figures in phenobarbital-treated Mrp4^{-/-} livers. An increase in Ki67-positive hepatocytes was observed in Mrp4^{-/-} mice treated with 100 mg/kg phenobarbital, although this change was not statistically significant compared with wild-type mice treated with the same dose. Gene and protein analyses showed upregulation of proliferative markers (Pcna, Cdk1/2, Cyclin B1, c-Myc) and xenobiotic metabolism-related genes (Cyp3a11, Cyp2b10, Cyp2c29, Ugt1a1, Sult1a1, Nqo1, Ahr), with concurrent suppression of Cyp7a1, Cyp2e1, and Gstp1, consistent with selective reprogramming of hepatic detoxification pathways in phenobarbital-treated Mrp4^{-/-} animals. Mechanistically, increased phosphorylation of cAMP response element-binding protein (CREB) and decreased Salt-inducible kinase 2 (SIK2) levels indicate activation of CREB-mediated transcription. Lipid-regulatory genes (Lpl, Fabp4, Ppar α , Dgat1, Acot2) were elevated, whereas Fasn, Acc, and Ppara were downregulated, in line with early lipid accumulation confirmed by Oil Red O staining. Consistently, liver triglyceride levels were significantly higher in Mrp4^{-/-} mice compared to wild-type animals. **Conclusions:** Loss of Mrp4 markedly amplifies phenobarbital-induced hepatic proliferation and metabolic remodeling. This phenotype involves enhanced xenobiotic enzyme expression, activation of CREB signaling, and dysregulation of lipid metabolism, revealing a previously unrecognized role for Mrp4 in maintaining hepatic metabolic balance under mitogenic stress.

PL 2039 Silencing hepatic CAPN4 protects against diet-induced MASH in mice

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Background and Purpose: Metabolic dysfunction-associated steatohepatitis (MASH) is a severe liver disease characterized by hepatocellular inflammation and fibrosis that affects a significant portion of the population. Plasma proteomic profiling reveals a distinctive pattern of protein degradation in MASH compared to healthy liver, with computational analysis implicating elevated calpain-mediated proteolysis. Calpains (particularly Calpain 1/2 isoforms) are Ca²⁺-dependent proteases activated under cellular stress that regulate tissue remodeling and inflammation, yet their specific role in MASH pathogenesis remains poorly understood. This study investigated whether hepatic Calpain 1/2 activity is necessary for MASH development. **Methods:** C57BL/6J mice received rAAV8 vectors encoding shRNA targeting Capn4 or scrambled control sequences under hepatocyte-specific (albumin) promoter control. After 4 weeks of vector integration, mice were fed either a low-fat control diet (13% saturated fat) or a Western-style high-fat, high-fructose diet (42% saturated fat) for 12 weeks. Hepatic Capn4 expression was quantified by qPCR. Analysis included plasma biochemistry, histopathological assessment of liver injury and steatosis, and quantitative PCR analysis of inflammatory gene expression to determine the contribution of calpain activity to MASH pathogenesis. **Results:** rAAV8-mediated knockdown achieved robust suppression of hepatic Capn4 expression. As expected, Western diet induced obesity, insulin resistance, and severe SteatoHepatitis. Despite minimal systemic differences in obesity and adiposity metrics, Capn4 knockdown substantially attenuated liver injury and fibrosis as well as suppressed key inflammatory genes associated with MASH progression. **Conclusions:** This study reveals that Capn4-mediated hepatic calpain activity is a critical driver of MASH development, specifically affecting disease initiation rather than advanced fibrosis progression. Blocking hepatic calpain proteolysis disrupts early pathogenic mechanisms while having minimal impact on systemic metabolic parameters. These findings establish Capn4 targeting as a potential therapeutic strategy for early intervention in MASH and highlight the critical role of hepatic Calpain 1/2 in disease development.

PL 2040 The Impact of Cyp2e1 KO and Sex on Response to DDC Diet

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Background and Purpose: Cytochrome P450 2E1 (CYP2E1) is a mammalian monooxygenase that is expressed at high levels in the liver. Within hepatocytes, CYP2E1 localizes to both the endoplasmic reticulum and the mitochondria. CYP2E1 metabolizes small hydrophobic compounds and often generates toxic metabolites. Although the enzyme is well studied for its role in xenobiotic toxicity such as metabolism of benzene or acetaminophen, its role in endogenous metabolism is less well understood. CYP2E1 is also highly conserved among mammals, and no clearly deleterious polymorphisms have been reported. Our previous work identified pathological changes in the bile duct region of CYP2E1 knockout (KO) mice, with more severe changes observed in females. To determine how this baseline biliary pathology affects liver function, we fed twelve-week-old mice a diet containing 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), a toxic porphyrinogenic compound. DDC prevents incorporation of iron into protoporphyrin IX, leading to its accumulation in hepatocytes, the formation of bile duct plugs, and pathology similar to primary sclerosing cholangitis. We hypothesized that female KO mice would experience worse outcomes compared to KO males and female wild-type (WT) controls, as measured by fibrosis

and increases in serum ALP, ALT, AST, and GGT. **Methods:** Two cohorts of male and female CYP2E1 KO mice were placed on a diet containing 0.1 percent DDC for two weeks beginning at twelve weeks of age. Histology was assessed by hematoxylin and eosin staining and picrosirius red staining for fibrosis. For serum biochemistry, approximately 500 microliters of blood were collected from each animal, allowed to clot for thirty to sixty minutes, and centrifuged at 10000 times g at 4 degrees Celsius. One hundred microliters of serum were submitted to our Department of Laboratory Animal Resources for a comprehensive diagnostic chemistry panel, which included ALT, total bilirubin, albumin, ALP, amylase, BUN, calcium, phosphate, creatinine, glucose, sodium, potassium, total protein, and globulin. **Results:** All groups displayed significant (p<0.05) elevations in total bilirubin, ALP, amylase, BUN, calcium, phosphate, sodium, and potassium compared to normal ranges. All males on the DDC diet (12 of 12) showed elevated ALT regardless of genotype. In contrast, among females, four of six animals in both KO and WT groups did not show ALT elevation. Other serum measures remained within the normal range across groups. Histological analysis with hematoxylin and eosin and picrosirius red staining revealed characteristic onion ring fibrosis in portal tracts and the presence of bile duct plugs in all animals. Quantification of fibrosis severity and statistical comparison is ongoing. To further define the effects of DDC, ongoing work will assess total bile acids, proliferation markers, and levels of Sox9 and CK19. **Conclusions:** CYP2E1 knockout did not alter the overall pathology induced by the DDC diet, despite baseline biliary differences in KO animals. An unexpected observation was that female mice of both genotypes showed no ALT elevation, suggesting that their hepatocytes may be protected from DDC-induced injury. These findings underscore a sex specific response to DDC injury that warrants further investigation, particularly given the strong male predominance (2-3x higher in males) observed in human PSC.

PL 2041 Real-Time Hepatic Surveillance in Residents Following the East Palestine, OH Chemical Disaster

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Background and Purpose: The February 2023 train derailment in East Palestine, Ohio, released vinyl chloride and other hepatotoxic chemicals, creating urgent need for health surveillance. This study represents the first systematic liver function assessment in residents potentially exposed to these environmental toxicants, establishing critical baseline data for long-term health monitoring. **Methods:** Adults ≥ 18 years within 8 miles of the derailment site have been recruited since June 2024. Participants provided informed consent for blood sampling and complete questionnaires on demographics, alcohol consumption, medical history, and health status. Liver function was assessed via serum ALT and AST levels. FibroScan examinations were performed to evaluate liver stiffness and hepatic steatosis. Body mass index (BMI) was calculated from measured anthropometric data. **Results:** To date, 88 participants enrolled (mean age 65.4 $\pm$ 1.9 years, range 18-87; 67% female), representing robust community response. All participants self-identified as non-Hispanic White. Mean BMI was 29.2 $\pm$ 0.6 kg/m² (range 15.8-44.9) with 2.3% underweight, 21.6% normal weight, 30.7% overweight, and 45.5% obese. Alcohol consumption: 65.9% drank < 4 times weekly, 19.3% never drank, and 8% consumed alcohol ≥ 4 times weekly. Mean liver enzyme levels remained within normal ranges in 79 participants with available data: ALT 17.5 $\pm$ 0.8 U/L (range 6-48 U/L) and AST 20.8 $\pm$ 0.9 U/L (range 11-75 U/L). Using sex-specific thresholds, 29.6% of women had ALT ≥ 19 U/L and 9.3% had AST ≥ 25 U/L, while 12% of men had ALT ≥ 30 U/L and 12% had AST ≥ 30 U/L. Notably, a 53-year-old female participant living less than 0.25 miles from the derailment site (highest exposure proximity) presented with normal ALT (12 U/L) and AST (15 U/L), normal BMI (22.57 kg/m²), and no alcohol use, yet demonstrated elevated liver stiffness (10.8 kPa) on FibroScan suggesting F2-F3 fibrosis and moderate hepatic steatosis (280 dB/m). This case highlights the potential inadequacy of traditional liver enzymes in detecting early hepatotoxicity from environmental exposures. As recruitment remains active, these preliminary findings may evolve. **Conclusions:** This pioneering surveillance study reveals critical findings: despite potential vinyl chloride exposure, the majority demonstrate normal or near-normal liver enzyme levels using sex-specific thresholds, yet FibroScan analysis uncovered significant liver abnormalities in a participant with highest exposure proximity and normal enzymes, demonstrating that traditional biomarkers ALT and AST may not detect early hepatotoxicity. The case of F2-F3 fibrosis with moderate steatosis in a young, normal-weight, non-drinking individual living nearest the derailment site raises urgent concerns about subclinical liver damage that standard screening misses. Continued vigilance remains essential. Future analyses of the bio banked samples will include CK-18, which has demonstrated superior predictive value for vinyl chloride-related liver damage. These baseline data enable longitudinal tracking of hepatic health in this uniquely exposed population. This study represents a critical model for environmental health response with implications extending beyond East Palestine to communities worldwide facing similar toxicant exposure threats, emphasizing the necessity of advanced imaging techniques alongside traditional biomarkers for comprehensive liver health assessment.

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Background and Purpose: Cyanobacterial blooms are increasing in frequency and magnitude due to rising water temperatures and nutrient enrichment. These blooms produce over 300 hepatotoxic microcystin (MC) congeners. Among these, microcystin-LR (MCLR) is one of the most hepatotoxic, common and studied. MCLR has been classified by the International Agency for Research on Cancer as a group 2B carcinogen. Recent preclinical evidence indicates MCLR can exacerbate the chronic liver disease, metabolic dysfunction-associated steatotic liver disease (MASLD), which has a global prevalence of approximately 25%. This study investigated the effects of MCLR on liver cell type populations in healthy mice and a MASLD model. **Methods:** C57BL/6J mice received either a control diet or a MASLD-inducing high fat/high cholesterol (HFHC) diet for 14 weeks, followed by an oral gavage with vehicle or 0.8 mg/kg MCLR every 24 h for 14 days, resulting in 4 treatment groups: control diet + vehicle (CV), control diet + MCLR (CM), HFHC diet + vehicle (HV), and HFHC diet + MCLR (HM). Animals were euthanized after final MCLR dose and liver samples were collected for histology and single nuclei RNA-sequencing (snRNA-seq) analysis. H&E staining was scored by a veterinary pathologist. snRNA-seq identified 10 distinct cell populations: hepatocytes, cholangiocytes, hepatic stellate cells (HSCs), liver sinusoidal endothelial cells (LSECs), macrophages, neutrophils, B cells, T cells, plasmacytoid dendritic cells (pDCs), and an undefined immune cells population. Gene expression counts were pseudobulked by cell type, dose, and diet for differential expression analysis. Marker genes identified hepatocytes as centrilobular, midzonal, or periportal. Zonation inference used trajectory analysis, with center of expression (CoE) calculated as the pseudospacial center-of-mass for gene expression along the portal-to-central axis. Gene set enrichment analysis (GSEA) applied the Kyoto encyclopedia of genes and genomes, Gene ontology, and Reactome libraries. CellChat was used to assess ligand-receptor signaling between cell types. **Results:** Hepatocyte and immune cell proportions were altered by MCLR and HFHC diet. Hepatocytes decreased from 70.0% in CV to 55.2%, 49.2%, and 26.9% in CM, HV, and HM groups, respectively. In contrast, immune cell proportions increased from 11.6% in CV to 26.3%, 30.0%, and 43.2% in CM, HV, and HM groups, respectively. The number of unique differentially expressed genes in hepatocytes was higher in CM compared to HM (3,693 vs. 2,393, respectively), whereas immune cells, such as macrophages, showed the opposite trend (740 vs. 1,247, respectively). The top 50 genes with the most variable CoE revealed MCLR and HFHC diet elicited a shift in zonation from centrilobular to periportal. Specifically, MCLR in both diets caused a substantial periportal shift, and the HFHC diet moved CoE closer to an intermediate phenotype between centrilobular and periportal. Approximately 680 pathways were enriched across all cell types in the different dose and diet groups based on GSEA. In hepatocytes, 79 pathways were enriched, including some associated with MASLD progression and carcinogenesis such as the negative regulation of gluconeogenesis, anti-apoptotic response (Birc5), and cell cycle check points (Bub1). CellChat revealed increased cell-to-cell communication in the HM compared to the CV group (1,213 connections to 374 connections, respectively). **Conclusions:** The greater effect of MCLR on immune cells, liver zonation, and intracellular communication in the context of the HFHC diet indicate increased immune response and loss of hepatocyte zonal structure may be cellular mechanisms that contribute to how MCLR exposure exacerbates MASLD. Overall, these data highlight important differences in liver cell type-specific responses to MCLR in healthy versus MASLD livers and suggest increased risk of MCLR hepatotoxicity in populations with pre-existing liver disease.

PS 3001 Sex-specific roles of LKB1 in acetaminophen hepatotoxicity in mice

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Background and Purpose: Acute liver failure (ALF) is a rare but devastating condition with high mortality. Liver transplantation, the only life-saving treatment for ALF, is limited by an enduring shortage of healthy donor livers. In addition, transplant recipients face numerous life-threatening risks such as graft rejection and infection. New treatments are clearly needed to improve transplant-free survival and reduce dependence on transplantation. Interestingly, our prior work revealed that LKB1 signaling is strongly activated in non-survivors compared to survivors in patients with APAP-induced ALF. Based on that, we hypothesized that LKB1 is detrimental during APAP hepatotoxicity and inhibiting LKB1 may be a novel therapeutic strategy. To test that hypothesis, we generated liver-specific LKB1 KO mice, overdosed them with APAP, and measured liver injury. **Methods:** LKB1 KO and control animals were generated by treating LKB1 floxed mice with adeno-associated viruses carrying plasmids encoding Cre recombinase or eGFP, respectively, under the hepatocyte-specific thyroglobulin promoter. After 2 weeks, male and female mice were treated with 300 mg/kg APAP or 500 mg/kg APAP, respectively. The higher dose in females is necessary to achieve the same extent of liver injury as the males because females are less susceptible to APAP toxicity. Liver and blood were then collected at 2, 6, and 24 h post-APAP. Liver injury was assessed at all time points by measuring serum alanine transferase (ALT). JNK activation was measured by immunoblotting for phosphorylated

JNK. Cyp2e1 levels were measured by both immunoblotting and Cyp2e1 activity. NAPQI formation and protein binding in the liver were assessed by measuring total glutathione and APAP-protein adducts. Oxidative stress in the liver was assessed by measuring oxidized glutathione. **Results:** Serum ALT was reduced in LKB1 KO mice compared to WT animals at 6 and 24 h post-APAP (6 h mean±SE: 1269±365 vs. 3693±747 U/L, p<0.05; 24 h mean±SE: 1614±747 vs. 8491±946 U/L, p<0.05). JNK activation was also reduced in the KO mice. Total glutathione was increased in male KO mice compared to male WT animals (mean±SE: 0.51±0.06 vs. 0.27±0.02 μmol/g, respectively, p<0.05), indicating inhibition of NAPQI formation in the males. Consistent with that, Cyp2e1 protein and activity were lower in the male KO mice, as well as APAP-protein adducts (mean±SE: 0.2606±0.07834 vs. 1.251±0.1159 nmol/g, p<0.05). However, there was no difference in total glutathione, Cyp2e1, or APAP-protein adducts between female KO and WT mice. Instead, oxidative stress was significantly reduced in the KO females (%GSSG mean±SE: 1.8±0.2 vs. 2.9±0.3, p<0.05), indicating an antioxidant effect. **Conclusions:** Consistent with our hypothesis, LKB1 KO protected against APAP-induced liver injury. However, the mechanisms of protection are sex-specific: Male mice have reduced Cyp2e1 expression, while female mice have less oxidative stress. Additional research is needed to determine how LKB1 regulates Cyp2e1 and reactive oxygen species in males and females, respectively. (Support: NIH R01 DK135752 (PI: McGill)).

PS 3002 Hepatic stellate cell-specific HuR deficiency protects female mice from CCl4-induced liver fibrosis development

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Background and Purpose: Chronic liver injury drives hepatic stellate cell (HSC)-mediated fibrosis, ultimately progressing to liver cirrhosis, the leading cause of liver-related mortality. Thus, developing liver-targeted anti-fibrotic therapies is imperative. In cultured HSCs, the RNA-binding protein Human antigen R (HuR) exhibits pro-fibrotic activity; however, its role in liver-wide fibrogenesis remains incompletely defined. Furthermore, fibrosis mechanisms in females are underexplored. To address these gaps, we characterized CCl4-induced liver injury in female HSC-specific HuR deficient (HuRHSC-/-) mice. **Methods:** Female HuRHSC-/- mice and their wildtype (WT) counterparts were administered 0.5 μl/g CCl4 either as a single dose (acute injury) or biweekly for six-weeks (chronic injury); corn oil served as control. Livers and serum were collected 48-hours post treatment. **Results:** In oil injection controls, HuRHSC-/- livers showed a 1.5-fold decrease in HuR mRNA, while cytochrome P450 2e1 expression was similar between genotypes. Acute CCl4 exposure caused comparable serum ALT elevations and centrilobular necrosis in both groups. Contrastingly, pericentral F4/80+ macrophage accumulation and pro-inflammatory and pro-fibrotic gene induction occurred only in WT livers and were blunted in HuRHSC-/- livers. Following chronic treatment, WT livers exhibited extensive collagen deposition and macrophage infiltration, whereas these features were largely absent in HuRHSC-/- livers. Correspondingly, the induction of pro-fibrotic gene expression decreased in HuRHSC-/- livers. **Conclusions:** In summary, HSC-specific HuR deletion protects females from CCl4-induced inflammation, macrophage accumulation, and fibrosis without altering CCl4 metabolism. These findings identify HSC HuR as a key regulator linking hepatotoxic injury to macrophage recruitment and collagen deposition, highlighting HuR as a promising therapeutic target.

PS 3003 Combined Liver-Specific Deletion of RNA-Binding Proteins, ZFP36L1 and ZFP36L2, Promote Hepatocarcinogenesis

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Background and Purpose: Environmental carcinogens often promote endogenous mutagenic processes in human liver cancer. While transcriptional regulation and mutational landscape have been studied in this process, the role of post-transcriptional gene regulation mediated by RNA-binding proteins (RBP) remains poorly understood. Our previous study showed that combined liver-specific deficiency of two RBPs, Zinc Finger Protein 36 Like 1 (ZFP36L1) and Zinc Finger Protein 36 Like 2 (ZFP36L2), results in spontaneous hepatic inflammation and activation of oncogenic pathways. Since inflammation drives liver cancer, we hypothesized that ZFP36L1 and ZFP36L2 regulate hepatocarcinogenesis. We tested this hypothesis using a diethylnitrosamine (DEN)-induced mouse model of hepatocarcinogenesis. **Methods:** Liver-specific ZFP36L1 and ZFP36L2 double knockout (L1/L2^{dKO}; AlbCre⁺/Zfp36l1^{fllox/fllox}/Zfp36l2^{fllox/fllox}) and flox-only control (L1/L2^{FLX}; AlbCre⁺/Zfp36l1^{fllox/fllox}/Zfp36l2^{fllox/fllox}) mice of both sexes were administered DEN (25mg/Kg BW) intraperitoneally at postnatal day 14 and euthanized at 24 h and 6 months post-injection to assess DEN-induced DNA damage and hepatocarcinogenesis, respectively. Additionally, we assessed the expression of these RBPs in hepatocellular carcinoma cohorts from the TCGA database. **Results:** The expression of yH2AX, a DNA damage marker, was comparable between groups at 24 h post-DEN administration, suggesting no difference in the initiation of DEN-induced carcinogenesis. Interestingly, a higher percentage of DEN-treated L1/L2^{dKO} mice (~85%) developed liver tumors compared with DEN-treated L1/L2^{FLX} mice (~16.6%) at 6 months post-exposure. L1/L2^{dKO} livers

exhibited a greater tumor burden and predominantly high-grade lesions characteristic of hepatocellular carcinoma (HCC), whereas L1/L2^{FLX} livers displayed only a few low-grade preneoplastic foci. Additionally, L1/L2^{dko} livers had higher immune cell infiltration and collagen deposition - two key drivers of HCC - compared to L1/L2^{FLX} livers. Furthermore, the expression of HCC-promoting target genes, including *Bcl2*, *Cybb*, *Fgf21*, *Igf1p1*, *Mmp13*, *Plau*, and *Zeb2*, was significantly upregulated in the livers of DEN-treated L1/L2^{dko} mice compared with those of DEN-treated L1/L2^{FLX} mice. Finally, ZFP36L1 and ZFP36L2 were significantly downregulated in the human liver cancer cohort (n=374) compared to controls (n=50) retrieved from the TCGA database. Overall, the combined liver-specific deletions of ZFP36L1 and ZFP36L2 promoted hepatocarcinogenesis by augmenting inflammation, fibrosis, and stabilizing HCC-associated target genes. **Conclusions:** Our findings uncover a novel tumor-suppressive role of ZFP36L1 and ZFP36L2 in liver cancer and provide a mechanistic association between post-transcriptional gene regulation and environmental carcinogenesis.

PS 3004 Enhancement of acetaminophen-induced hepatotoxicity in Spontaneously Diabetic Torii-Lepr^{fa} (SDT fatty) rats

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Background and Purpose: Acetaminophen (APAP) is widely used as a relatively safe antipyretic and analgesic. However, when administered at high doses, it can cause liver injury due to the binding of a reactive metabolite, NAPQI, produced via cytochrome P450 metabolism, to proteins. In most cases, NAPQI is detoxified through conjugation with glutathione (GSH). However, when the amino acid supply is interrupted, even low doses of APAP can induce liver injury. In patients with type 2 diabetes, GSH levels are reported to be lower than in healthy individuals, suggesting a higher susceptibility to APAP-induced liver injury. However, the relationship between diabetes-induced enhancement of gluconeogenesis and GSH synthesis capacity remains unclear. Therefore, we investigated the impact of the diabetic condition on hepatic GSH synthesis capacity and APAP metabolism using Spontaneously Diabetic Torii-Lepr^{fa} (SDT fatty) rats, a spontaneous non-obese type 2 diabetes model, and sought to determine whether SDT fatty rats exhibit increased susceptibility to APAP-induced liver injury and to elucidate the underlying mechanisms. **Methods:** APAP was administered orally, once daily, to Sprague-Dawley (SD) and Spontaneously Diabetic Torii-Lepr^{fa} (SDT fatty) male rats (6 weeks of age, 6 or 7 animals per group) at the dose levels of 300 and 500 mg/kg for 42 days. The animals in the matched control groups (6 animals of both sexes) were administered only the vehicle (0.5% methylcellulose aqueous solution). Plasma hepatic-function parameters (aspartate aminotransferase (AST), alanine aminotransferase (ALT), glutamate dehydrogenase (GLDH)) were measured before the initiation of dosing, and on days 14, 28 and 42. At the end of the dosing period, hepatic glutathione (GSH) levels and hepatic mRNA expression of gluconeogenesis-related gene (*Pck1*, *Fbp1* and *G6pc*), drug metabolizing enzyme-related gene (*Ugt1a6*, *Cyp1a2* and *Cyp2e1*), drug metabolizing enzyme activity (CYP2E1) and GSH synthesis-related gene (*Gclc* and *Gclm*) were measured. **Results:** In SD rats, there were no changes in plasma AST, ALT, or GLDH activities at any dose level. In contrast, in SDT fatty rats, the activities increased during the latter half of the administration period at 500 mg/kg. In comparison between the matched control groups, the mRNA levels of *Cyp1a2* and *Cyp2e1* were higher in SDT fatty rats, whereas no strain differences were observed for *Ugt1a6*. Upon APAP administration, *Ugt1a6* mRNA levels significantly increased in both strains in a dose-dependent manner; however, the degree of increase was smaller in the SDT fatty rats compared to SD rats. There were no changes in hepatic CYP2E1 activity at any dose level in the SD rats, but in the SDT fatty rats, a significant decrease in activity was observed at 500 mg/kg compared to the control group. Hepatic GSH concentrations significantly increased in the SD rats at 300 and 500 mg/kg, whereas in the SDT fatty rats, hepatic GSH levels decreased at 500 mg/kg. Although mRNA levels of *Gclm* and *Gclc*, which encode subunits of γ-glutamylcysteine synthetase, the rate-limiting enzyme in GSH synthesis, did not change in response to APAP administration in either strain, comparison of control groups revealed that SDT fatty rats had lower mRNA levels of both genes compared to SD rats. **Conclusions:** SDT fatty rats were shown to have a higher susceptibility to APAP-induced liver injury compared to SD rats, through decreased glutathione conjugation capacity and reduced GSH synthesis by enhanced gluconeogenesis and lower gene expression levels of GSH-synthesizing enzymes.

PS 3005 Cholesterol mediated endotoxic liver injury is enhanced by sitagliptin in Sprague-Dawley rats.

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Background and Purpose: Animal products like dairy, poultry, and meat are rich in methionine (Met) and cholesterol (Cho) and therefore are consumed together. Homocysteine (formed from Met) and Cho are documented risk factors for liver and heart disease, to mention some. Emerging evidence suggests an association between high cholesterol and dysbiosis, leading to endotoxemia and liver damage, among others. In view of the known pleiotropic effects of sitagliptin (an anti-type

2 diabetic drug), studies were conducted to investigate its role in reducing endotoxemia. **Methods:** After taking an initial blood sample, adult male Sprague Dawley rats were divided into four groups. These were fed *ad libitum* a) control (Con) diet, b) enriched with 1.5% Methionine, c) 2.0% Cholesterol, and d) the combination (1.5% Methionine + 2.0% Cholesterol) for 35 days. From day 10, an aqueous suspension of sitagliptin (100mg/kg) was orally gavaged till the end of the experiment. On day 36, after overnight fasting, the rats were euthanized for collecting blood, liver, heart, and other tissues. Liver samples were analyzed for markers of endotoxemia by qPCR and substantiated by other methods. **Results:** Preliminary results suggest significant increases in the markers of endotoxemia (**Lipocalin-2 (LCN-2)**, **Cyclooxygenase-2 (COX-2)**, and **Hemoxygenase-1 (HMOX-1)**) in the liver samples of rats fed high-cholesterol diet. Such increases were not seen in the liver of high methionine-fed rats. In the combination diet, these parameters were significantly reduced and could be attributed to the presence of methionine. Sitagliptin, when tested, exacerbated endotoxemia markers in high cholesterol-fed rats instead of attenuating them. Interestingly, methionine was able to counter even the exacerbated responses due to sitagliptin in high cholesterol-fed rats. **Conclusions:** Hepatic endotoxemic markers were significantly increased in rats fed high-cholesterol diet, and these were exacerbated by sitagliptin. These results substantiate our previous findings of increases in hepatic inflammation, oxidative stress, and fibrotic responses (typical of Non-alcoholic steatohepatitis-NASH) in high cholesterol-fed rats which were further enhanced by sitagliptin (Pathak R et al. (2019, 2022), Kumar A et al. (2020), Palfrey et al. (2024)). Therefore, the present studies help in bridging the understanding of the association between hepatic endotoxemia, (which is increased due to high cholesterol) and sitagliptin-mediated enhancement of NASH responses. Additional studies will likely yield a better understanding about the role of sitagliptin in endotoxemia, especially during hypercholesterolemia.

PS 3006 Application of immunofluorescence for analysis of zone-selective hepatic steatosis driven by perfluorooctane sulfonate (PFOS) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

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Background and Purpose: Liver toxicity by environmental contaminants often develops in a zone-selective manner, driven by physiological and functional gradients along the periportal to centrilobular axis. Perfluorooctane sulfonate (PFOS) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), two persistent environmental pollutants, are reported to elicit zone-selective lipid accumulation by H&E. Given the importance of cellular and spatial heterogeneity of the liver in response to chemicals and drugs, there is a need for definitive methods to characterize molecular changes associated with liver zonation. Our goal was to develop a multiplexed immunofluorescence-based method that anchors changes such as hepatic lipid accumulation and inflammation, to established markers of zonation. **Methods:** We developed a workflow based on the Iterative Bleaching Extends Multiplicity (IBEX) method to evaluate the zonation of hepatic features. The method was modified to enable the staining of neutral lipids using BODIPY, paired with zonation markers ASS1 (portal hepatocytes) and GLUL (central hepatocytes), as well as cell type markers GFAP (quiescent hepatic stellate cells), ADGRE1 (F4/80; Kupffer cells), and ACTA2 (αSMA; vascular smooth muscle cells and activated hepatic stellate cells). Using these markers, hepatic lobules were computationally inferred to establish a zonation map and subsequently used to quantitatively assess zonation of immunofluorescence signal. Livers from male C57BL/6J mice treated with PFOS (0.3 or 1 mg/kg/day in 0.5% Tween-20 drinking water for 84 days) or fed a Gubra Amylin NASH (GAN) diet for 12 weeks were used as models of centrilobular steatosis, while those treated with TCDD (3 μg/kg in sesame oil via oral gavage every 4 days for 84 days) represented a periportal steatosis. **Results:** BODIPY staining revealed dose-dependent lipid accumulation in the centrilobular regions of PFOS treated mice, beginning with small droplets at 0.3 mg/kg/day and progressing to large droplet accumulation at 1 mg/kg/day. In contrast, lipid accumulation in livers of TCDD treated mice was more skewed towards the midzonal and periportal region. After 12 weeks on a GAN diet, livers exhibited a more homogenous lipid accumulation with larger droplets localized near centrilobular vessels. Each of these models showed increased lipid accumulation compared to vehicle control where minimal or no lipid accumulation was observed. While no treatment related changes were seen for GFAP or ACTA2, each showed zone-selective distribution. ADGRE1 showed enrichment in the periportal region consistent with their role in responding to cues entering the liver via the portal vein. **Conclusions:** Through development of a multiplexed immunofluorescence imaging workflow, this study was able to more definitively characterize the zone-selective changes in livers of mice treated with the environmental contaminants PFOS and TCDD, or a steatogenic diet. Notably, incorporation of BODIPY staining revealed clear differences in lipid accumulation patterns between PFOS and TCDD reflecting two different mechanisms. Ultimately, this strategy represents a versatile framework for studying spatially resolved toxicological responses in the liver.

PS 3007 Potential mechanisms of hepatic Cyp2e1 over-expression in diet-induced obesity mouse model, associated with aggravated acetaminophen-induced liver injury

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Background and Purpose: Obese individuals are known to have different drug metabolism profiles compared to lean populations due to altered hepatic CYP450s expression, which makes them prone to drug-induced liver injury (DILI). Among these enzymes, hepatic Cyp2e1 plays central role in metabolizing numerous drugs and has been reported to modify drug toxicokinetics particularly in obesity. Consequently, Cyp2e1 may contribute to higher risk of liver-related drug adverse effects in obese populations. However, the exact cause-and-effect relationship of obesity, Cyp2e1, and DILI is not fully understood. Moreover, the molecular mechanisms underlying Cyp2e1 alterations are poorly defined. Here in this study, we confirmed the elevation of Cyp2e1 in diet-induced obesity (DIO) mouse model and demonstrated its causal involvement in aggravated acetaminophen-induced liver injury (AILI) through a unique liver-biopsy-based approach, which enabled individual tracking of Cyp2e1's contribution to drug hepatotoxicity. In addition, we shed light on the potential mechanisms that may be responsible for Cyp2e1 elevation in obesity. **Methods:** Male C57BL/6J mice were fed either a high-fat diet (HFD) or a low-fat diet (Normal Diet, ND) for 24 weeks to establish DIO model and its lean control. Then, liver biopsy was performed to obtain liver samples prior to drug administration and Cyp2e1 expression level was analyzed. After a 3-week recovery period, acetaminophen (300 mg/kg, P.O.) was administered and blood, liver and fecal sample were collected. Hepatotoxicity was evaluated through serum chemistry, LC-MS/MS quantification, histopathology, RT-qPCR, and western blotting (WB). Furthermore, 16s rRNA sequencing, fecal metabolomics (LC-MS/MS), single-cell RNA sequencing (scRNA-seq), and *in vitro* fatty acid treatment using liver cells were performed to investigate the mechanisms of Cyp2e1 alteration. **Results:** After 24 weeks of HFD feeding, DIO mice showed significantly higher body weight and impaired glucose tolerance compared to their lean controls. Liver biopsy samples of DIO mice revealed marked elevation of Cyp2e1 expression. Animals selected to examine Cyp2e1 level by biopsy also exhibited marked increase in serum ALT and AST level as well. Following acetaminophen exposure, 3-cysteiny-acetaminophen (APAP-Cys), a serum biomarker reflecting N-acetyl-p-benzoquinone-imine (NAPQI) and liver protein adduct formation, also increased in DIO mice. Histopathological and molecular analysis further confirmed aggravated hepatic inflammation and oxidative stress. In addition, fecal metabolomics identified a few microbial-origin metabolites which may affect hepatic metabolizing enzyme expression level via the gut-liver axis. Cell culture and scRNA-seq results gave knowledge to possible gene networks that may regulate Cyp2e1 expression. **Conclusions:** Our findings indicate that DIO mice are more susceptible to AILI, which can be attributed to hepatic Cyp2e1 upregulation. Our study also shows the potential microbial and transcriptional mechanisms responsible for Cyp2e1 elevation in obesity. Our research emphasizes the need for further in-depth research on the mechanism behind the change of Cyp2e1 expression and expanding research interest to other drug-metabolizing enzymes as well in order to predict and prevent DILI in obese patients in the future.

PS 3008 18β-glycyrrhetic acid regulates cuproptosis by p65/STAT3-STEAP4 axis to ameliorate acetaminophen-induced liver injury

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Background and Purpose: 18β-Glycyrrhetic acid (GA) is a main metabolic product of glycyrrhizin which is a type of triterpenoid compound found in the roots and stems of the traditional Chinese medicine licorice. Modern pharmacological researches have revealed the benefit of GA in alleviating acute liver injury (ALI), but the mechanism is still obscure. We first discovered that GA downregulated STEAP4 protein level which is a metal reductase that reduces Cu²⁺ to Cu⁺ to promote copper absorption and utilization in a model of alpha-naphthylisothiocyanate (ANIT)-induced cholestasis by mass spectrometry, implicating a potential role for GA in the regulation of copper homeostasis. As reported, there is a potential association between cuproptosis and acetaminophen-induced liver injury (AILI). However, the correlation between GA alleviation of AILI and cuproptosis has not been touched. **Methods:** The effect of GA on cuproptosis induced by Es-Cu in HepG2 cells was examined by CCK8 assay for cell viability. The AILI mouse model was constructed by an intraperitoneal injection (i.p.) of APAP (350 mg/kg) in C57BL/6 mice, and the alleviating effect of GA (20 and 50 mg/kg) on AILI was evaluated by serum biochemical analysis and H&E staining. The correlation between the treatment of AILI with GA and cuproptosis was investigated by copper measurement, RT-qPCR, western blotting, and siRNA transfection. **Results:** GA could inhibit Es-Cu-induced cuproptosis in a concentration-dependent manner *in vitro* assay. *In vivo*, 50 mg/kg of GA improved AILI, manifested as the lower ALT, AST, and GLDH, the decreased area of hepatic necrosis around the central

vein. GA regulated the expression of genes critical to copper homeostasis (STEAP4, SDHB, COX17, ATOX1, CCS) and mitochondrial TCA cycle (LIAS, FDX1, DLAT, SDHB), accompanied by lower concentration of copper ion in cells and liver tissue. GA could bind to p65 and STAT3 and reduce the ratio of p-STAT3/STAT3 and p-p65/p65 obviously. Knockdown of p65 or inhibition of STAT3 activity could block the relief of GA in STEAP4 mRNA and cuproptosis. **Conclusions:** GA regulates the phosphorylation levels of p65 and STAT3 to further affect the transcription of STEAP4 to inhibit cuproptosis, thereby ameliorating AILI.

PS 3009 Delta-8-tetrahydrocannabinol mitigates ConA-induced hepatotoxicity through epigenetic regulation of immune dynamics

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Background and Purpose: Concanavalin A (ConA) induces immune-mediated hepatotoxicity characterized by an imbalance between effector and regulatory immune responses. Epigenetic regulation via microRNAs (miRNAs) has emerged as a key determinant of immune signaling. Cannabinoids are increasingly recognized for their immunosuppressive and anti-inflammatory properties; however, the impact of delta-8-tetrahydrocannabinol (Δ⁸-THC), a minor cannabinoid with reduced psychoactivity compared to Δ⁹-THC, remains entirely unexplored in the context of immune-mediated hepatotoxicity. To address this gap, we investigated whether Δ⁸-THC protects against ConA-induced hepatotoxicity and delineated the underlying cellular and epigenetic mechanisms. **Methods:** miRNA sequencing and flow cytometric analysis were performed using hepatic immune cells from female C57BL/6 mice treated with ConA (12.5 mg/kg, i.v.) and Δ⁸-THC (20 mg/kg, i.p.). Differential miRNA expressions were analyzed using the edgeR, and miRNA targets were predicted using Ingenuity Pathway Analysis. Target gene expression was validated by qRT-PCR and Western blot, with functional assays using miRNA mimics/inhibitor transfection and CD4⁺ T-cell differentiation assays. **Results:** We found that Δ⁸-THC effectively ameliorated ConA-induced hepatotoxicity by restoring intrahepatic innate and adaptive immune homeostasis through the preservation of tolerogenic Kupffer cells (KCs) and the suppression of inflammatory monocytes, neutrophils, and natural killer cells (NKs). Moreover, Δ⁸-THC reversed the inflammatory lymphoid microenvironment in the liver caused by ConA by decreasing inflammatory CD4⁺ T cells and promoting the expansion of regulatory T cells. These effects were associated with the preservation of hepatoprotective miR-199a-3p and miR-100-5p expression in hepatic immune cells, which were markedly downregulated in ConA-treated mice. Ingenuity Pathway Analysis identified mTOR, a key immune regulator of T-cell differentiation, as a target of these miRNAs. Consistently, Δ⁸-THC treatment reduced hepatic mTOR expression *in vivo*. Functional assays using miRNA mimics and inhibitors confirmed direct regulation of mTOR by these miRNAs, whereby miR-199a-3p/miR-100-5p-mediated mTOR suppression promoted regulatory T cells differentiation and inhibited inflammatory Th1 and Th17 responses. **Conclusions:** Δ⁸-THC protects against immune-mediated hepatotoxicity by restoring hepatic immune balance through epigenetic regulation of microRNA-mTOR signaling and promoting tolerance by enhancing regulatory T-cell responses. By preserving anti-inflammatory miR-199a-3p and miR-100-5p and suppressing mTOR-driven inflammatory responses, Δ⁸-THC reduces hepatocellular injury and immune dysregulation, revealing a novel epigenetic mechanism and highlighting its potential as a therapeutic candidate for immune-related liver toxicity.

PS 3010 Sex-Dependent Hepatotoxicity of Trichloroethylene in Humanized PPARα Mice Fed a Western Diet

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Background and Purpose: Consumption of a high fat western-style diet is a major risk factor for metabolic dysfunction-associated steatotic liver disease (MASLD). Environmental exposures to toxic chemicals may contribute to the progression of this disease. Trichloroethylene (TCE) is a commonly used industrial solvent and environmental contaminant that has been linked to metabolic dysfunction and hepatic steatosis. Metabolites of TCE activate the transcription factor PPARα, a major regulator of lipid metabolism in the liver. Both diet and sex have been identified as important modifying factors in MASLD development. Therefore, we sought to test the hypothesis that TCE exposure exacerbates western diet-induced metabolic dysfunction and liver injury in a sex-dependent manner using a humanized PPARα mouse model. **Methods:** Mice expressing human PPARα were used in this study because they are a better model for human responses to PPARα agonists. Male and female humanized PPARα C57BL/6J mice aged 9-12 weeks were placed on either a low-fat diet (LFD; 10% fat) or a western diet (WD; 42% fat) with concurrent exposure to 0.5 mg/mL of TCE or 1% ethoxylated castor oil (used to emulsify TCE) in drinking water for 12 weeks. Body weight and water consumption were measured each week. A glucose tolerance test was performed at week 10, and EchoMRI was performed at week 11. At week 12, blood and tissues were collected for measurement of plasma AST and ALT, tissue weights, and analysis of liver histopathology with H&E and Oil Red O. Statistical analyses were performed using two- and three-way ANOVA followed by Tukey's post hoc test, with significance set at p ≤ 0.05. **Results:** Male

mice on WD, independent of TCE exposure, exhibited increased body weight, fat mass, white adipose tissue, and liver weight compared to their female counterparts. Interestingly, the female mice on WD and TCE exposed exhibited a significant decrease in body weight, white adipose tissue and fat mass relative to WD-fed females. TCE exposure relative to body weight was greater in females than in males. Glucose tolerance was impaired in male mice on a WD diet, independent of TCE exposure, whereas their female counterparts maintained glucose tolerance. Plasma AST and ALT levels were elevated in WD-fed males, independent of TCE exposure, but remained unchanged in females. Histological analysis revealed enhanced steatosis in WD-fed males, regardless of TCE exposure, as confirmed by H&E and Oil Red O staining, compared with their female counterparts. **Conclusions:** This study revealed marked sexual dimorphism in diet- and TCE-induced hepatotoxicity in humanized PPAR α mice. Male mice were more susceptible to liver injury and toxicity, whereas female mice showed alterations in systemic fat deposition.

PS 3011 Inhibition of Apoptosis-Mediated Kidney Cell Death Improves Survival Following Acetaminophen Overdose

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Background and Purpose: Acetaminophen (APAP), commonly called Tylenol, is widely used to relieve pain and reduce fever. However, an APAP overdose can lead to acute liver failure (ALF) and acute kidney injury (AKI). The current standard treatment, N-acetyl-L-cysteine (NAC), only prevents liver injury and does not influence kidney damage. Although the liver is the primary target organ for APAP-induced injury, renal dysfunction occurs in over 50% of APAP-induced ALF patients and contributes significantly to mortality. Clinical data indicate that APAP-induced ALF is associated with more severe kidney injury than ALF of other etiologies, suggesting a direct nephrotoxic effect of APAP. Our recent observations revealed elevated plasma creatinine and renal failure in APAP overdose patients, predominantly in patients with severe liver injury. Though mechanisms of renal cell death are distinct from those in the liver (apoptosis in the kidney vs necrosis in the liver), it remains unclear whether concurrent kidney damage after an APAP overdose worsens liver injury and accelerates ALF progression. In this study, we investigated whether selectively inhibiting kidney cell death by blocking apoptosis with a caspase inhibitor could improve survival after a severe APAP overdose in the human-relevant mouse model. **Methods:** Male C57BL/6J mice were fasted overnight and given 600 mg/kg of APAP intraperitoneally (n=9), a dose sufficient to induce both liver and kidney injury. A subset (n=7) of mice received 3 mg/kg of Ac-DEVD-CHO, a caspase inhibitor, thirty minutes before APAP. Mice were euthanized 48 hours after APAP injection, and blood, liver, and kidney samples were collected for further analysis. **Results:** Mice treated with both APAP and the caspase inhibitor showed a significantly higher survival rate (71%) than those receiving APAP alone (22%). Plasma ALT levels were comparable in both groups, indicating similar degrees of liver injury. However, mice in the caspase-inhibitor group exhibited significantly lower blood urea nitrogen (BUN) and kidney injury molecule 1 (KIM-1) levels, suggesting preservation of kidney function and reduced kidney injury. Liver histology showed a significant decrease in areas of necrosis around central veins in the caspase-inhibitor group, suggesting enhanced liver regeneration at 48 hours post APAP-exposure. Consistent with this observation, liver proliferating cell nuclear antigen (PCNA) staining demonstrated significantly greater numbers of PCNA-positive hepatocytes in the perinecrotic regions of the caspase-inhibitor group, further confirming improved liver regeneration compared with APAP alone. **Conclusions:** Selective inhibition of apoptosis-mediated kidney cell death improves survival following APAP overdose by preserving renal function and promoting hepatic regeneration, even without directly reducing initial liver injury. These findings highlight the therapeutic potential of targeting kidney apoptosis to improve outcomes after APAP toxicity.

PS 3012 Rust Belt Toxicants Amplify Diet-Induced Hepatotoxicity in Mice

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Background and Purpose: Metabolic dysfunction-associated steatotic liver disease (MASLD) affects over 30% of Western populations with disproportionate impact on lower socioeconomic communities. While early-stage MASLD prevalence approaches 100% in at-risk individuals, progression to severe forms is not inevitable, suggesting additional risk factors drive disease severity. Environmental chemical mixtures may be such a risk factor and transform manageable dietary stress into severe liver injury. "Rust to Resilience" (R2R) chemical mixtures contain legacy toxicants (lead, cadmium, arsenic) and emerging PFAS relevant to industrialized countries and of high priority concern in southwestern Pennsylvania. **Methods:** This study investigated whether R2R-mixture exposure transforms Western diet-induced metabolic stress into severe hepatotoxicity. C57BL/6J mice were fed Western or control diets for 12 weeks, with subgroups receiving R2R mixtures at 1X and 100X concentrations. Analysis included plasma biochemistry, histopathological assessment, and RNA-sequencing with integrated pathway analysis to determine molecular mechanisms of toxicant-diet interactions. **Results:** The combination of R2R-mixture exposure with Western diet escalated diet-induced liver injury, causing metabolic

dysfunction, severe inflammation, and portal expansion. RNA-seq analysis revealed dysregulation of key regulators of hepatic lipid metabolism inflammation. IHC staining for activated macrophages and endothelial and epithelial markers demonstrated a unique, lipotoxicity-associated immune response as well as portal expansion and endothelial cell damage, all hallmarks of more severe hepatic pathogenesis. These results were not replicated in control diet mice with equal R2R-mixture exposure or western diet mice with vehicle exposure, indicating that the combination of both factors leads to more severe disease progression. **Conclusions:** Western diet transformed manageable environmental exposure into severe hepatotoxicity characterized by metabolic dysregulation, activation of pro-inflammatory pathways, and endothelial cell dysfunction.

PS 3013 Utilizing Clinically Approved Epidermal Growth Factor Receptor Inhibitors for the Treatment of Acetaminophen-Induced Liver Injury

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Background and Purpose: Acetaminophen (APAP) overdose is the leading cause of acute liver failure (ALF) in the United States. Despite this, APAP-induced liver injury (ALI) currently has only one widely accepted treatment, N-acetylcysteine (NAC), which only works within a limited time window. Epidermal growth factor receptor (EGFR) has been previously established to bolster regeneration of the liver following partial hepatectomy. Our recent work demonstrated that hepatocyte-specific deletion of EGFR leads to a surprising alleviation of ALI, indicating an injury promoting facet of EGFR. Here, we seek to investigate the therapeutic relevance of this paradoxical finding by employing clinically approved EGFR inhibitors (EGFRi), afatinib and osimertinib, with the aim of discovering novel treatment strategies for countering ALI. **Methods:** C57BL/6J mice were first administered either a moderately toxic (300 mg/kg) or severely toxic (500 mg/kg) dose of APAP. Following this, these mice were treated with afatinib, osimertinib or their vehicle (PBS) at 1 or 12 hours post APAP administration. These mice were then sacrificed at 6, 24 or 48 hours post APAP administration to investigate liver injury and regeneration. Osimertinib was also tested in combination with NAC, being given 4 hours after APAP administration, a timepoint at which NAC does not work alone. Additional analysis was conducted on publicly available single nucleus RNA sequencing and spatial transcriptomics databases. **Results:** Treatment with EGFRi significantly decreased ALI when administered 1 hour after both moderately and severely toxic doses of APAP. Of the two, osimertinib proved to be consistently more effective, reducing ALT and necrosis more than afatinib. EGFRi treatment did not impair the initial mechanisms driving APAP hepatotoxicity such as depletion of glutathione (GSH), APAP protein adduct formation and JNK activation at 6 hours post APAP. However, decreased activation of JNK and increased rate of GSH replenishment were observed in EGFRi treated mice 24 hours post APAP, potentially contributing to restricted progression of ALI. In addition to this, EGFRi treated groups displayed increased markers of autophagy and mitophagy, which are known to decrease ALI through degradation of dysfunctional mitochondria. Mechanistically, EGFRi treatment decreased activation of MTOR signaling resulting in increased levels of TFEB, a master transcriptional regulator of autophagy. These results are all consistent with our previous findings in hepatocyte-specific EGFR-KO mice. In addition to working if given 1 hour after APAP overdose, osimertinib also decreased ALI when administered either alone or, more effectively, in combination with NAC 4 hours after APAP when NAC by itself was ineffective. Furthermore, EGFRi treatment did not inhibit liver regeneration, whether given early or after injury was fully established (12 hours post APAP), as indicated by PCNA staining and immunoblotting. Lastly, analysis of single nucleus RNA sequencing and spatial transcriptomics data revealed that EGFR signaling and several EGFR ligands were significantly upregulated in human ALF patients, signifying the human relevance of our findings. **Conclusions:** Overall, our studies suggest that EGFR inhibition using clinically approved inhibitors is a viable treatment strategy for targeting ALI, significantly reducing hepatotoxicity via inhibiting mitochondrial damage and inducing autophagy, without impairing regeneration.

PS 3014 Pharmacological inhibition of MET impairs hepatocyte regeneration in a murine model of acetaminophen-induced liver injury

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Background and Purpose: Hepatocyte growth factor receptor (MET) is the key driver of liver regeneration following partial hepatectomy (PHx) but remains poorly understood in the context of acetaminophen-induced liver injury (ALI). ALI is the foremost cause of acute liver failure (ALF) in the US and the Western world. Our previous studies utilized a hepatocyte-specific MET knockout (KO) mouse model to explore its function in ALI. Due to severe injury and high mortality in MET KO mice, even at moderate overdose levels where spontaneous recovery typically occurs, the direct contribution of MET to liver regeneration could not be assessed. To overcome this limitation, the present study investigates the regenerative potential of MET in ALI using a pharmacological approach with Capmatinib (CAP), a selective MET inhibitor.

Methods: Male C57BL/6J mice received either a moderate (300 mg/kg) or severe (500 mg/kg) overdose of APAP, followed by CAP (15 mg/kg) or vehicle treatment 2 hours later. Serum and liver tissue were subsequently collected at 12, 24, and 48 hr post-APAP administration to assess the extent of liver injury, regeneration and recovery. The time points utilized covered both the peak injury (12 and 24 hr) and recovery phases (48 hr) of AILI. Publicly available bulk and spatial transcriptomic data from APAP-induced acute liver failure patients were also analyzed. Lastly, freshly isolated primary human hepatocytes (PHH) were treated with APAP (10 mM) alone and in combination with CAP (0.1 and 1 μ M), and cell death was assessed using propidium iodide staining. **Results:** The moderate APAP overdose led to elevated serum ALT and centrilobular necrosis, with similar peak injury levels observed in CAP-treated mice. This similar extent of peak damage allowed us to evaluate the direct role of MET specifically in compensatory regeneration and recovery independent of injury. MET inhibition markedly reduced key cell cycle markers (p-ERK1/2, Cyclin D1, and PCNA) at 24 and 48 hr, indicating impaired regeneration with CAP treatment. Further, MET inhibition resulted in no significant difference in peak ALT levels or necrosis at 24 hr after severe APAP overdose. Both these injury parameters declined at 48 hr in vehicle-treated mice but remained markedly higher in CAP-treated mice, indicating impaired regression of injury following MET inhibition. This impaired recovery was accompanied by a reduced regenerative capacity, as evidenced by decreased expression of PCNA and Cyclin D1 at 48 hr in CAP-treated mice. Furthermore, a comprehensive analysis of publicly available bulk and single-nucleus RNA sequencing datasets demonstrated that the upregulation of proliferation and cell cycle-associated genes and pathways, indicative of an active regenerative response, was accompanied by robust activation of the HGF/MET signaling axis in APAP-induced ALF patients. Interestingly, spatial transcriptomic analysis revealed a marked elevation of HGF expression in peri-necrotic regions of APAP-induced ALF patients. Notably, pharmacological inhibition of MET exacerbated APAP-induced hepatotoxicity in primary human hepatocytes in a concentration-dependent manner, highlighting the critical protective role of MET signaling in a human model of AILI. **Conclusions:** Overall, MET inhibition using Capmatinib suppressed liver regeneration and impaired recovery following AILI especially after severe APAP overdose. These findings highlight MET's direct role in coordinating timely regenerative responses during recovery from AILI.

PS 3015 Hepatic stellate cell-specific HuR deficiency protects mice from CCl₄-induced liver fibrosis development

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Background and Purpose: Liver fibrosis is a progressive condition characterized by excessive extracellular matrix (ECM) accumulation and chronic inflammation resulting from hepatocellular injury. Hepatic stellate cells (HSCs) are the principal drivers of this process, transitioning from a quiescent to an activated myofibroblast-like state that produces collagen and pro-inflammatory mediators. The RNA-binding protein HuR (ELAVL1) stabilizes and regulates mRNAs involved in inflammation, stress response, and fibrogenesis, but its cell-type-specific role in liver fibrosis remains unclear. This study investigates how HSC-specific *HuR* loss alters fibrogenic and inflammatory responses during acute and chronic liver injury. **Methods:** *HuR^{fllox/flox}* mice were bred with LRAT-Cre mice to generate hepatic stellate cell-specific *HuR* knockout (*HuR^{HSC-/-}*) mice, alongside wild-type (WT) littermate controls. Male mice at least 2-months old were administered carbon tetrachloride (CCl₄) via intraperitoneal injection at 0.5 μ g/g to induce acute (single dose) or chronic (twice weekly for six weeks) liver injury. Serum alanine aminotransferase (ALT) activity was measured to assess hepatocellular damage. Liver tissue was collected 48 hours after the final exposure for histological (H&E, Picro Sirius Red), immunohistochemical (HuR, PCNA, F4/80), and immunofluorescent (HuR, SMA) analysis. Fibrogenic and inflammatory gene expression was quantified by qPCR for *Acta2*, *Col1a1*, *Adgre1*, *Ccl2*, and *Tnfa*. Single-nucleus RNA sequencing (snRNA-seq) was additionally performed on chronic injury samples to explore cell-type specific changes associated with HSC-*HuR* loss. **Results:** *HuR^{HSC-/-}* mice exhibited similar serum ALT levels to WT controls under both acute and chronic conditions, confirming equivalent hepatocyte injury. Histological assessment revealed reduced necrotic regions and diminished collagen deposition in *HuR^{HSC-/-}* mice after chronic CCl₄ treatment. Picro Sirius Red staining demonstrated less fibrotic bridging in knockout livers, while immunofluorescence confirmed loss of HuR expression in SMA-positive HSCs, validating efficient and specific deletion. qPCR analysis showed that fibrogenic gene expression (*Acta2*, *Col1a1*) was elevated following CCl₄ exposure but was significantly reduced in *HuR^{HSC-/-}* mice compared to WT. Inflammatory markers (*Adgre1*, *Ccl2*, *Tnfa*) followed a similar pattern, indicating reduced macrophage activation and cytokine signaling. Immunohistochemistry for F4/80 supported these findings, revealing dense macrophage clustering along collagen bridges in WT livers, while *HuR^{HSC-/-}* tissue exhibited markedly fewer macrophages, particularly after chronic exposure. Preliminary snRNA-seq analysis of chronic injury samples suggested transcriptional shifts consistent with altered activation of HSC, macrophages, and endothelial cells, along with dampened inflammatory signaling in *HuR^{HSC-/-}* livers. Early findings also point toward dysregulated expression of ECM-remodeling genes and immune response pathways, warranting further investigation into how HSC-specific *HuR* regulates cell-to-cell communication in fibrosis. **Conclusions:** Loss of *HuR* in HSCs reduces fibrogenic activation and inflammatory crosstalk during both acute and chronic liver injury. Despite comparable hepatocyte damage, *HuR^{HSC-/-}* mice show attenuated collagen deposition, decreased macrophage recruitment, and lower expression of fibrogenic and inflam-

matory genes. Initial snRNA-seq analyses support a model in which HuR modulates HSC activation and paracrine signaling within the fibrotic microenvironment. These findings identify HuR as a key post-transcriptional regulator of liver fibrosis and a potential therapeutic target for chronic liver disease.

PS 3016 Plasminogen and plasminogen deficiency in acetaminophen-induced acute liver injury

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Background and Purpose: Acetaminophen/paracetamol (APAP) induced liver injury is a leading cause of acute liver injury/failure (ALI/ALF). Challenging mice with a hepatotoxic dose of APAP recapitulates fundamental mechanisms and histopathological consequences of APAP overdose in humans, including alterations in blood coagulation and fibrinolysis. Prior studies report that APAP-induced liver damage is reduced in mice lacking plasmin(ogen), the terminal effector of the fibrinolytic system, but the precise link between plasminogen and APAP-induced liver injury is unclear. **Methods:** We determined the impact of APAP overdose on plasma and hepatic plasminogen levels in experimental APAP-induced ALI (300mg/kg) and ALF (600mg/kg) and tested the hypothesis that inducible plasminogen deficiency reduces APAP hepatotoxicity in mice. We used a novel siRNA approach to deplete plasminogen, and both immunohistochemistry and western blotting to measure plasminogen levels in plasma and liver. **Results:** APAP challenge in wild-type mice significantly decreased plasma plasminogen levels after 6 and 24 hours, and increased plasma plasmin-antiplasmin complex levels after 6 hours. This was paired with deposition of plasmin(ogen) within the injured liver, indicated by western blotting and immunohistochemical staining of plasmin(ogen); plasmin(ogen) accumulation was evident within areas of hepatocellular necrosis. Administration of lipid nanoparticles containing siRNA targeting plasminogen (siPlg) dramatically reduced plasma and hepatic plasminogen levels. Surprisingly, siRNA-induced plasminogen deficiency did not affect early (6h) or peak (24h) APAP-induced liver injury in mice challenged with either 300 or 600mg/kg APAP, as indicated by plasma alanine aminotransferase activity and hepatic necrosis. **Conclusions:** The results indicate that hepatic plasminogen levels increase significantly after APAP challenge in association with a reduction in plasma plasminogen concentration, but in contrast to congenital plasminogen deficiency in mice, siRNA-induced plasminogen deficiency does not inhibit APAP-induced liver damage. The results suggest a need to revisit the link between plasminogen and APAP hepatotoxicity, potentially due to differences in germline and knockdown plasminogen deficiency, as well as a need to further investigate hemostatic mechanisms in acute liver injury.

PS 3017 FXIII-B deficiency increases APAP-induced liver injury without impacting hepatic fibrin(ogen) crosslinking

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Background and Purpose: Hepatic deposition of the coagulation protein fibrin(ogen) is a hallmark of acetaminophen (APAP)-induced hepatotoxicity in mice and humans. The transglutaminase coagulation factor XIII (FXIII) covalently crosslinks the majority of fibrin(ogen) deposits in the APAP-injured liver. FXIII circulates in plasma as a heterotetramer (FXIII-A₂B₂) composed of two catalytic (FXIII-A) subunits stabilized by two regulatory (FXIII-B) subunits, the latter synthesized by the liver. FXIII-A₂ is also stored intracellularly in certain cell types (e.g., platelets, macrophages). We tested the hypothesis that plasma FXIII-A₂B₂ drives hepatic fibrin(ogen) crosslinking in the APAP-injured liver. **Methods:** To test this hypothesis, we generated novel mice with a selective reduction in plasma FXIII-A₂B₂ by deleting the gene encoding FXIII-B (*F13b^{-/-}* mice). *F13b^{-/-}* mice were then challenged with APAP (300 mg/kg, i.p.) and analyzed after 24 hours. **Results:** FXIII-B was not detectable in plasma of *F13b^{-/-}* mice. As anticipated, plasma FXIII-A levels were reduced to less than 3%, while platelet intracellular FXIII-A levels were not impacted. Wild-type mice challenged with APAP developed acute hepatocellular injury indicated by centrilobular necrosis and elevated plasma alanine aminotransferase activity. Hepatic deposition of fibrin(ogen) with abundant Fibrin- γ crosslinking, a modification imposed by FXIII-A, was evident in the liver of APAP-challenged wild-type mice. Unexpectedly, FXIII-B deficiency dramatically increased APAP-induced hepatic necrosis and sinusoidal congestion, an observation that was not present in *F13a1^{-/-}* mice. This increase in injury was not attributable to an increase in APAP adduct formation. Additionally, extravascular fibrin(ogen) deposition and crosslinking (Fibrin- γ) was preserved in the APAP-injured liver of *F13b^{-/-}* mice. **Conclusions:** The results indicate that selective loss of plasma FXIII-A (in *F13b^{-/-}* mice) does not impact extravascular fibrin(ogen) crosslinking in experimental hepatocellular necrosis, suggesting a potential role of cellular FXIII-A in fibrin(ogen) crosslinking. Furthermore, the results suggest a hepatoprotective effect of FXIII after APAP overdose that is mediated by mechanisms independent of traditional fibrin crosslinking.

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Background and Purpose: Liver injury is associated with activation of the blood coagulation cascade. Hepatocytes express low levels of tissue factor (TF), the transmembrane receptor and co-factor for coagulation Factor VIIa (FVIIa). The TF-FVIIa complex activates the extrinsic coagulation pathway and plays a key role in hemostasis. In various settings of liver injury, such as acetaminophen overdose, hepatocyte-derived TF has been shown to drive coagulation activation. This study tested the hypothesis that hepatocyte TF drives coagulation activation following CCl₄-induced liver injury. **Methods:** In acute challenge, wild-type mice, mice with whole-body TF deficiency (LowTF), and mice lacking TF specifically in hepatocytes (HPC^{ΔTF}) were challenged once with carbon tetrachloride (CCl₄, 1 mL/kg, i.p.). Liver injury and coagulation were evaluated various times after challenge. In separate studies, to establish chronic injury, mice were challenged twice weekly with CCl₄ for 4-6 weeks, and samples were collected 24-72 h after the final challenge. **Results:** Acute CCl₄ challenge caused hepatic injury (ALT, necrosis) and robust coagulation activation, measured by plasma thrombin-antithrombin (TAT) complexes. Whole-body TF deficiency (~1% of normal) significantly reduced coagulation activation and attenuated peak hepatocellular necrosis. Interestingly, hepatocyte TF deficiency had no effect on acute liver injury. *In situ* hybridization identified cells in inflammatory foci expressing TF (F3) mRNA in the necrotic region of the CCl₄-challenged liver. Similarly in chronic CCl₄ challenge, hepatocyte TF deficiency did not impact coagulation activation or liver fibrosis, but whole-body TF deficiency significantly reduced coagulation activation and modestly decreased hepatic myofibroblast (hepatic stellate cell) activation. **Conclusions:** Together, these data suggest that TF-expressing non-parenchymal cells mediate coagulation activation during acute and chronic CCl₄-induced liver injury. Our results indicate that hepatocellular necrosis is not sufficient to trigger hepatocyte TF-dependent coagulation in all contexts; rather, coagulation activation is likely dependent on the mechanism of cell death.

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Background and Purpose: Liver disease remains a significant global health concern, and drug-induced liver injury (DILI) often progresses to acute liver failure (ALF). Acetaminophen (APAP) overdose induces hepatotoxicity primarily through the formation of the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI), leading to severe oxidative stress, glutathione depletion, and subsequent hepatocyte necrosis. *Cornus kousa* (CK) is a tree widely distributed across East Asia, has recently been reported to exhibit antioxidant and anti-lipid peroxidation effects. Thus, CK is considered a potential agent for mitigating drug-induced liver damage. **Methods:** The powder of CK was obtained through hot water extraction, concentration, and subsequent freeze-drying. For the *in vivo* study, ICR mice were orally administered CK for 3 days. Following the final CK administration on the third day, APAP (400 mg/kg) was intraperitoneally injected. The mice were sacrificed on the fourth day. Serum samples were collected to assess liver function by measuring aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. Liver tissues were analyzed for malondialdehyde (MDA) content to evaluate lipid peroxidation, and fixed tissues were subjected to hematoxylin and eosin (H&E) staining for histopathological observation of liver injury. **Results:** In the *in vivo* study, oral administration of CK resulted in decreased serum levels of ALT and AST. Furthermore, CK treatment reduced the hepatic MDA content compared to the APAP-induced injury group. Histopathological examination via H&E staining provided evidence of tissue recovery in the liver structure following CK administration. **Conclusions:** CK could be a beneficial hepatoprotective agent for the prevention and amelioration of APAP-induced liver injury *in vivo*. These findings warrant further investigation into the specific cellular and molecular mechanisms of CK within liver cells to fully understand its therapeutic potential.

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Background and Purpose: Cisplatin is a chemotherapeutic drug prescribed for the treatment of solid tumors, but its use is limited in part by nephrotoxicity. Current methods of detecting cisplatin-induced acute kidney injury (AKI) are inadequate for revealing the progression from subclinical to clinical injury. Our team recently performed an untargeted aptamer-based discovery of urinary proteins in patients who received cisplatin-containing chemotherapy within the prior 48 h and identified over 800 differentially enriched proteins. **Methods:** In this study, 21 potential protein biomarkers from our previous study were prioritized for validation at the mRNA level using: 1) the human AKI mRNA dataset in the Kidney Tissue Atlas and 2) kidneys of mice treated with cisplatin. The human AKI mRNA dataset consisted of single cell kidney RNAseq data from 14 AKI cases of variable etiology and 28 healthy references. For the latter approach, total RNA was extracted from frozen kidneys of saline-treated control mice (n=5) and cisplatin (20 mg/kg, ip)-treated mice (n=7) at 4 days, and qPCR was performed for 24 genes, including 3 known kidney injury biomarkers (Kim-1, Timp-1, and Lcn-2) as positive controls. **Results:** Cisplatin treatment increased mRNA expression of all three established kidney injury biomarkers, Kim-1 (287-fold), Timp-1 (60-fold), and Lcn-2 (893-fold), in mice at 4 days. Of the 21 genes selected for potential validation, 6 were up-regulated, 2 were down-regulated, and 13 were not significantly changed in the kidneys of cisplatin-treated mice. Increased mRNA expression was observed for the apoptosis receptor Tnfrsf10b (8-fold), RNA-binding Carhsp1 (3-fold), calcium-binding S100a11 (6.5-fold), serine protease Prss22 (44-fold), and cytokines Cxcl5 (82-fold) and Ccl5 (3-fold). Notably, Tnfrsf10b, Cxcl5, Carhsp1, S100a11, and Prss22 showed significant positive correlation (Pearson r: 0.7-0.9) with the established injury markers. Decreased mRNA expression was observed for ubiquitin complex receptor Khlh13 (47% of control) and glycosyltransferase St8sia4 (42% of control). Overall, there was low concordance between the directional change (up-regulation, down-regulation, no change) between the untargeted protein biomarker analysis, the human AKI mRNA dataset, and changes in the regulation of these aforementioned genes in the kidneys of cisplatin-treated mice. Nonetheless, the upregulation of Tnfrsf10b mRNA in the kidneys of cisplatin-treated mice corresponded with increased protein levels in the urine of patients receiving cisplatin-containing chemotherapy. **Conclusions:** The lack of similarity in regulation of biomarkers between the untargeted analysis and the mRNA profiles in human and mouse kidneys likely reflects differences in the matrix analyzed, degree of injury, and regulation of the targets at the mRNA versus protein level. Nonetheless, Tnfrsf10b, a TNF-related receptor involved in apoptosis signaling, emerged as a potential novel biomarker of cisplatin-induced nephrotoxicity. Future studies aim to perform longitudinal evaluations of this and other potential kidney injury biomarkers in the entire patient cohort. Supported by NIH R25ES020721, R01GM123330, P30CA072720, and the American Society for Pharmacology and Experimental Therapeutics.

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Background and Purpose: Exposure to particulate matter (PM) has been associated with diseases of the respiratory and cardiovascular systems. Owing to the dense vasculature, the kidney has also been identified as a target organ to fine particulate matter of <2.5 µm in diameter (PM_{2.5}). The mechanisms through which PM affects the tissues involved in xenobiotic removal, such as the liver and kidneys, are still unclear. The aim of this study was to determine the effect of PM_{2.5} on human kidney proximal tubular epithelial cell line (HK-2 cells), an *in vitro* model of kidney function. **Methods:** For this purpose we analyzed RNA-seq data (GSE208331) of HK-2 cells treated with PM_{2.5} for 24 hours. We used our proprietary database and software for data interpretation and visualization. We generated the database to include internally curated and various publicly available data on proteins, biologically-active chemicals, their interactions, pathways and pathologies. In order to support data integration and capture different levels of histopathological observations, we developed toxicology ontology with nearly 9,000 diseases and 800 pathology endpoints. Over 30 million references were catalogued, supporting each database entry. Molecule interactions were hyperlinked to appropriate PubMed articles, proteins were hyperlinked to corresponding NCBI Gene and UniProt Protein pages, and chemicals to corresponding PubChem pages. **Results:** From the whole genome analysis, using the cut off criteria of log₂ fold change ≥ ±2, and padj<0.05, 427 differentially expressed genes (DEGs) were identified. Our system recognized 315 DEGs, with 132 of them being upregulated and 183 downregulated. Mapping DEGs to Gene function (GO) and Signaling and Metabolic pathways clusters within our system revealed that in kidney, PM_{2.5} upregulated genes involved in response to xenobiotic stimulus/xenobiotic metabolic process, Phase I-functionalization of compounds and NFE2L2 regulating antioxidant/detoxification enzymes, and downregulated genes involved in renal sodium excre-

tion, RHOA GTPase cycle and DNA damage response. Mapping of DEGs to Organ and tissue pathologies and Diseases clusters in our system revealed that upregulated genes were associated to kidney proximal tubule necrosis, kidney apoptosis, renal hypoplasia and renal fibrosis, whereas the downregulated genes were associated with renal fibrosis and renal tubular transport disease. Vast amount of processed data in our system presents the potential for generation of valuable mechanistic hypothesis. To illustrate this we chose the renal fibrosis. Detailed interrogation of the renal fibrosis network from our proprietary database, combined with DEGs from the experimental dataset, revealed PM2.5 exposure led to upregulation of the genes involved in maintaining homeostasis in the presence of oxidative stress (HMOX1). It also upregulated complement C5a receptor 1 (C5AR1). It is known from the literature that the C5a/C5aR1 axis promotes progression of renal tubulointerstitial fibrosis in a mouse model of renal ischemia/reperfusion injury. PM2.5 exposure led to down-regulation of a WNT signaling pathway inhibitor DKK1, indicating potential activation of WNT signaling pathway. For WNT signaling pathway is known, particularly its beta-catenin route, to be a driver of fibrogenesis. Regarding DKK1 specifically, the literature shows that in animal models, enhanced expression of DKK1 had a clear suppressive effect on fibrosis. **Conclusions:** By applying bioinformatics tools on dysregulated gene set from *in vitro* model of kidney function we have shown that PM2.5 dysregulates genes/proteins involved in xenobiotic responses in kidney. In addition, we have identified potential renal system toxicities following PM2.5 exposure and generated the mechanistic hypothesis showing how PM2.5 exposure can dysregulate genes leading to renal fibrosis.

PS 3022 THY1/CD90 as a Novel Biomarker of Subclinical Drug-Induced Kidney Injury in Humans

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Background and Purpose: While cisplatin is a long-standing treatment for solid tumors, clinical use is limited by kidney toxicity. THY1 (thymocyte differentiation antigen-1, known as CD90) is a small cell surface protein expressed on the brush border of human proximal tubule cells, and has been targeted experimentally to induce glomerulonephritis in rodents. In our recent untargeted proteomic assessment using aptamer-based Somascan technology, THY1 was one of the top proteins increased in the urine of 20 patients who had received cisplatin-containing chemotherapy within the preceding 48 hours. In the current study, we sought to 1) profile time-dependent changes in urinary THY1 protein levels following cisplatin infusion in a larger set of patients and 2) compare urinary THY1 excretion patterns to established kidney injury biomarkers. **Methods:** Urine samples were collected at baseline, 4-24, 24-48, 48-72 h and 10 days from cancer patients receiving their first or second round of cisplatin-containing chemotherapy (≥ 25 mg/m²; 0.5-2-h infusion, n=41). THY1 levels were measured in urine supernatant using ELISA and normalized to urinary creatinine concentrations. Patterns of THY1 secretion over time were compared to established kidney injury biomarkers, including kidney injury molecule-1 (KIM-1), trefoil factor 3 (TFF3), beta-2 microglobulin (B2M), and calbindin D_{28k} (CALB1), also measured by ELISA. **Results:** Consistent with the previous Somascan analysis findings, urine THY1 concentrations (mean $\pm$ SD) increased following cisplatin treatment, showing increases at 4-24 h (4.3 $\pm$ 6.5-fold), 24-48 h (3.0 $\pm$ 4.4-fold), 48-72 h (2.8 $\pm$ 4.7-fold) and 10 days (2.6 $\pm$ 3.5-fold), relative to baseline. THY1 was increased by at least 2-fold in 30-45% of subjects across all time points. B2M and TFF3 were increased in 63-65% and 51-60% of subjects between 4 h and 72 h with lesser change at 10 days. By comparison, less than 30% of subjects exhibited a 2-fold change in CALB1 between 4h and 72 h whereas 59% displayed elevated levels at 10 days. About 43-57% of subjects showed a 2-fold increase in KIM-1 levels between 24 hours and 10 days, while 32.5% exhibited such increases between 4 and 24 hours. **Conclusions:** Urinary THY1 levels increased significantly following cisplatin treatment, peaking within the first 24 hours and remaining elevated for up to 10 days. This pattern of increase at early time points was most similar to KIM1, B2M, and TFF3 although in a smaller subset of subjects treated with cisplatin compared to the established kidney injury biomarkers. Supported by NIH R25ES020721, R01GM123330, P30CA072720, and the American Society for Pharmacology and Experimental Therapeutics.

PS 3023 *In vitro* evaluation of drug-induced kidney injury using cell painting assay in 2D and 3D kidney models

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Background and Purpose: Drug-induced nephrotoxicity remains a leading cause of clinical trial failures and post-market withdrawals, with renal proximal tubule epithelial cells (RPTECs) being particularly vulnerable due to their role in drug secretion and reabsorption. Current preclinical models for renal toxicity screening, such as 2D monolayer cultures, often lack physiological relevance, resulting in poor predictivity of *in vivo* outcomes. 3D spheroid models, which more faithfully recapitulate RPTEC polarization, tight junction assembly, and metabolic functionality, emerge as a promising alternative. However, their performance in toxicity prediction has

not been systematically benchmarked against that of 2D models. Cell painting, a multiplex fluorescence microscopy technique, captures hundreds of cellular features (including morphology, organelle distribution, and cytoskeletal architecture) to generate "phenotypic fingerprints" of drug-induced effects, enabling unbiased toxicity profiling beyond the scope of traditional viability assays. This study aims to: 1) Compare the renal toxicity predictive capacity of 2D and 3D RPTEC models using an FDA-curated compound library; 2) Integrate cell viability data (IC₅₀) with cell painting-derived phenotypic profiles to develop predictive toxicity models; 3) Assess the sensitivity and specificity of both models in distinguishing nephrotoxic from non-nephrotoxic compounds. **Methods:** Human renal proximal tubule epithelial cells (RPTECs) were cultured as a monolayer in renal epithelial growth medium (REGM) on collagen-coated plates. For 3D spheroid formation, RPTECs were seeded in ultra-low attachment plates and cultured in REGM supplemented with 2% Matrigel. A total of 24 compounds were selected from the FDA's Drug-Induced Kidney Injury (DIKI) database and nephrotoxicity-specific databases, comprising 12 known nephrotoxins and 12 non-nephrotoxins. Both 2D RPTEC monolayers and 3D spheroids were treated with each compound at 8 concentrations (ranging from 0.1 μ M to 1 mM) for two exposure durations: 24 hours and 72 hours. Vehicle controls (DMSO, $\leq 0.1\%$) and positive controls (10 mM H₂O₂) were included. Toxicity was assessed using two complementary approaches: 1) Cell Viability Assay (CellTiter-Glo, CTG Assay) were conducted and half-maximal inhibitory concentration (IC₅₀) values for each compound were calculated using non-linear regression analysis. 2) Cell Painting Assay: Cells were stained with a panel of 6 fluorescent dyes to target distinct cellular compartments: nuclei (Hoechst 33342), cytoplasm (CellTracker Green CMFDA), mitochondria (MitoTracker Red CMXRos), endoplasmic reticulum (ER-Tracker Blue-White DPX), actin cytoskeleton (Phalloidin-Atto 647N), and Golgi apparatus (Wheat Germ Agglutinin, WGA-Alexa Fluor 555). Fluorescent images were acquired using a high-content imaging system (Operetta CLS, PerkinElmer) at 20x magnification. **Results:** Cell painting revealed distinct clustering of nephrotoxins vs. non-nephrotoxins in both models. Nephrotoxins induced consistent phenotypic changes: reduced mitochondrial intensity, fragmented ER, and actin cytoskeleton disorganization—more pronounced in 3D spheroids. Good correlation showed between cell painting assay and cell viability assay. **Conclusions:** This study demonstrates that 3D RPTEC spheroids integrated with cell painting assay yield a superior predictive approach for renal toxicity relative to 2D monolayer cultures. Key findings are as follows: 1) 3D spheroid models exhibit enhanced sensitivity and specificity in differentiating nephrotoxic from non-nephrotoxic compounds. 2) The cell painting assay captures subtle phenotypic alterations that complement viability readouts, thereby augmenting the predictive accuracy of the model. The integration of 3D RPTEC spheroids and cell painting-based analysis provides a robust *in vitro* platform for the early prediction of drug-induced nephrotoxicity during preclinical development. This approach reduces reliance on animal models and facilitates more reliable clinical translation of candidate therapeutics.

PS 3024 Retrograde Ureteral Catheterization for Renal Pelvis Access. A Feasibility Study in Cynomolgus Macaques.

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Background and Purpose: Preclinical testing of novel drugs for kidney disorders is hampered due to the rapid excretion of systemically delivered drugs through the kidneys. Accurate determination of therapeutic efficacy of systemically delivered test compounds is extremely difficult. A noninvasive method to deliver drugs to the kidneys in cynomolgus macaque, using clinically available equipment was developed. **Methods:** Female cynomolgus macaques were sedated with intramuscular ketamine and subsequently maintained with inhaled isoflurane. A veterinary endoscope (Karl Storz, Germany) was introduced via the urethra into the bladder, allowing visualization of the ureteral orifices. A guidewire was then inserted into the ureteral orifice, followed by the advancement of a balloon catheter along the wire. The catheter tip was positioned near the renal pelvis, and its location was confirmed using a C-arm fluoroscope (GE, USA). Upon successful placement, the balloon at the catheter tip was inflated to prevent retrograde leakage of iohexol, a non-ionic iodinated contrast agent. About 1.5 ml of contrast agent was injected through the catheter into the renal pelvis, and fluoroscopic imaging was used to assess the distribution and retention of the agent within the kidney. **Results:** Retro-ureter catheter insertion was achieved without complications in three macaques. Fluoroscopic imaging confirmed positioning of the catheter tip near the renal pelvis. Anatomical structure of the renal pelvis varied among individuals, resulting in distinct imaging patterns and possible differences between subjects of volume of compound that can be dosed. The procedure was well tolerated in all subjects, with no significant bleeding during the procedure and no postoperative hemorrhage or health abnormalities observed. The use of balloon inflation effectively prevented leakage into the ureter, ensuring exposure of contrast to most of the kidney. **Conclusions:** Clinically utilized retro-ureteral catheterization for targeted drug delivery to the kidney was successfully scaled down for preclinical use in the cynomolgus macaque. The method enables localized drug administration while preserving renal perfusion, offering a promising strategy for preclinical testing of treatments for renal disorders.

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Background and Purpose: Cisplatin, CDDP is metal based antineoplastic still widely used for treatment of solid tumors. Pronounced side effects are numerous, among which nephrotoxicity is the most limiting. Problem of fast decreased renal function either ends in fibrosis or through recovery is additionally burdened with potential of secondary renal derived neoplasms. Aim of this work was to determine the functional changes in the expression of primary NaK-ATPase pump throughout nephron tubules and cortically expressed, tertiary active organic anion transporters Oat1&3 responsible for para-aminohippurate, PAH secretion known to be decreased with CDDP treatment. Expected changes in nephron epithelial cells cytosolic structural and functional components beta-actin and alpha-tubulin of cytoskeleton, together with inducible metallothioneins, MT1&2 that respond to metal challenge were not addressed before. Also, we follow intermediate filament member, vimentin that specifically increase in proliferation of fibroblasts and possible indicate epithelial to mesenchymal transition, EMT in damaged cells. Whereas, cyclooxygenase, COX1&2 as regulating enzymes of immune system were also followed for purpose of multilayers in here presented picture of animal CDDP nephrotoxicity model. **Methods:** After a single treatment of male Wistar rats with cisplatin (i.p. dose of 5 mg CDDP/kg) the most pronounced toxicity effect occurs on the 5th day, a point of here presented results in observed protein expression changes, while distribution of some proteins were followed each day after treatment. Kidney samples from 4 animals as homogenates and total cell membranes were used for western blot, WB for cytoskeleton and membrane proteins, respectively and cryosections were made for immunohistochemistry, IHC; while expression of mRNA for analyzed proteins was determined by the end-point RT-PCR. Specific antibodies were used for WB and IHC parallel with custom-made primers for targeted proteins mRNA expression analysis. **Results:** WB results fifth day showed decrease in NaK-ATPase and transporters expression with Oat1 affected more than Oat3, that did correspond to IHC but not to end-point RT-PCR results, whereas all applied methods showed increase in cytoskeleton proteins especially in prominently damaged outer stripe of outer medulla kidney zone. Fibroblast specific vimentin together with MT1&3 showed increased expression and even change in distribution. Fibroblasts increased in proliferation and move toward damaged epithelia of outer stripe while Mt1&2 showed prominent expression in inner stripe and papilla, absent in the control. **Conclusions:** Decrease in expression of NaK-ATPase Oat1 and Oat3 correlates with disturbed renal function. Increase of overall cytoskeleton and MT1&2 together with vimentin followed by changed distribution indicates disruption of renal structure accompanied by proliferation and invasiveness of fibroblasts from papilla to the most damaged kidney zone of outer stripe but exclude EMT in repair. Late indicate increased potential of regeneration that can lead to fibrosis but also revealing importance of inner stripe and papilla in recovery response to CDDP treatment. Further research is needed of potentially new neoplasms growth in outer stripe epithelial cells regenerative process. *Funded by the European Union - Next Generation EU (Class: 643-02/23-01/00016, Reg. no. 533-03-23-0006)-Project: Evaluation of efficacy and toxicity of biologically active substances (BioMolTox).*

PS 3026 Differentiating Hepatic and Renal Toxicity Reveals Cyp-Independent Mechanisms of Acetaminophen-Induced Acute Kidney Injury

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Background and Purpose: Acetaminophen (APAP) overdose is the leading cause of acute liver failure (ALF) in the U.S. Hepatotoxicity is initiated by cytochrome P450 2E1 (CYP2E1)-mediated conversion of APAP to the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI), which depletes glutathione (GSH), triggers mitochondrial dysfunction, and causes necrotic cell death. Although the liver is the primary target, APAP overdose also causes substantial renal dysfunction, with acute kidney injury (AKI) occurring in more than 50% of patients with ALF, where it worsens clinical outcomes and independently predicts mortality. Despite this strong clinical association, most studies of APAP-ALF have focused primarily on hepatic injury, and the mechanisms underlying APAP-induced nephrotoxicity remain poorly defined—particularly regarding whether renal injury is a direct effect or secondary to liver injury. In addition, N-acetylcysteine (NAC), the only approved antidote, replenishes hepatic GSH and prevents liver injury but fails to protect the kidney, underscoring a mechanistic difference between hepatic and renal injury. This study investigates whether APAP-induced AKI occurs independently of ALF by using liver-specific Cyp2e1 knockout (KO) mice generated via AAV8-Cre delivery to distinguish hepatic versus renal contributions to toxicity. **Methods:** Fasted male and female C57BL/6J mice received APAP (600 mg/kg, i.p.) and were euthanized at various time points up to 48 hours post-dose. For hepatic deletion, Cyp2e1 flox/flox mice were injected with AAV8-Cre intravenously two weeks before APAP treatment and sacrificed at 24-48 hours. Hepatic injury was assessed by serum alanine aminotransferase (ALT) and necrotic area quantification in histology. Renal injury was evaluated by blood urea nitro-

gen (BUN), serum creatinine, periodic acid schiff (PAS) staining, and tubular injury markers (KIM-1 and osteopontin). Apoptosis was determined by the TUNEL assay, while CYP2E1 and osteopontin protein levels were measured by western blotting and Immunohistochemistry (IHC). APAP-protein adducts were quantified by HPLC with electrochemical detection. **Results:** In male mice, APAP overdose (600 mg/kg) caused a sharp increase in serum ALT ($\approx 10,000$ U/L), BUN (≈ 60 mg/dL) and creatinine (≈ 0.4 mg/dL), with extensive centrilobular hepatic necrosis and renal cortical damage marked by proximal tubular vacuolation and strong KIM-1 and osteopontin staining. CYP2E1 IHC revealed robust expression in the renal cortex of both control and APAP-treated males. In contrast, APAP overdose resulted in a smaller elevation in ALT (≈ 6000 U/L) and BUN (≈ 40 mg/dL) in females, where liver sections showed marked centrilobular necrosis, but renal architecture remained largely preserved. IHC demonstrated minimal KIM-1 and osteopontin staining, and TUNEL assays showed few apoptotic cells in the female kidney. No positive CYP2E1 staining was observed in the kidney in both control and APAP-treated females, and protein adducts were undetectable at 3, 6, and 24 hours, suggesting limited renal CYP2E1 expression and reduced APAP bioactivation in the kidney. In liver-specific Cyp2e1 KO male mice, hepatic injury was completely prevented, with mice surviving to 48h, confirming efficient and liver-restricted deletion of CYP2E1 as verified by IHC. Despite the absence of hepatic injury, renal damage progressively worsened over time, characterized by tubular cast formation, brush border loss, and strong induction of KIM-1, osteopontin, and apoptotic cell death at 48 hours. Interestingly, in liver-specific Cyp2e1 KO female mice, renal injury still progressed despite the absence of CYP2E1 in both liver and kidney. This was evidenced by extensive tubular vacuolation on H&E staining and strong TUNEL, KIM-1, and osteopontin staining. **Conclusions:** The persistence of AKI in Cyp2e1-deficient mice, despite absent hepatic CYP2E1, demonstrates that APAP-induced nephrotoxicity can occur independently of liver metabolism. In female mice, which do not express renal CYP2E1 and show no protein adduct formation, even after hepatic CYP2E1 deletion, kidney injury still progressed, indicating that APAP-induced AKI can occur through a CYP-independent mechanism. These findings highlight the need for kidney-targeted therapies beyond NAC.

PS 3027 A proximal tubule on-chip model for nephrotoxicity and drug safety evaluation

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Background and Purpose: Renal toxicity remains a major challenge in drug development due to the kidney's role in filtering and concentrating external compounds, which can lead to adverse effects. Renal proximal tubule epithelial cells (RPTEC) are central to drug absorption and metabolism, making them particularly vulnerable to drug-induced kidney injury (DIKI). To better mimic the kidney's complex architecture and dynamic environment, proximal tubule-on-chip systems have emerged as advanced platforms for drug screening and toxicity testing. **Methods:** This study introduces and characterizes the ^{AX}PToC model, a Proximal Tubule-on-Chip system built using AlveoliX's ^{AX}Barrier-on-Chip technology. RPTEC were cultured on a soft, porous membrane within the chip, and their differentiation was monitored over time via gene expression analysis (RT-qPCR). Barrier integrity was evaluated using Transbarrier Electrical Resistance (TER) and permeability assays. Cell morphology was examined through immunofluorescence staining, and albumin uptake via Megalin/Cubilin was assessed using fluorescently-labelled BSA. **Results:** RPTEC cultured on-chip formed a functional epithelial barrier, demonstrated by permeability to both small (10 kDa) and large (70 kDa) molecules, while maintaining low TER values, closely reflecting *in vivo* kidney physiology. Over time, RPTEC differentiated and expressed key markers such as CUBN, LRP2 (Megalin), CD13, ATP1A1 (Na/K ATPase), and transporters involved in drug metabolism and resistance (ABCC2, OAT1). Albumin uptake was confirmed through FITC-albumin assays and shown to be specific, as it decreased significantly in the presence of unlabelled albumin. Immunofluorescence revealed characteristic dome-like structures and P-Glycoprotein (MDR1) expression in RPTEC on-chip. When challenged with cancer therapeutics such as Cisplatin, the ^{AX}PToC model showed hallmarks of DIKI such as cytotoxicity and cell layer impairment. **Conclusions:** The ^{AX}PToC model successfully replicates essential features and functions of the human proximal tubule, including marker expression and transport activity. This robust and physiologically relevant system offers a promising platform for evaluating nephrotoxicity and drug safety, particularly in the context of DIKI.

PS 3028 Exosomes Secreted from Human Proximal Tubular Cells are Sources of Biomarkers for Exposure to Nephrotoxic Environmental Chemical and Drugs

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Background and Purpose: In previous work, we showed that exposure of human PT (hPT) cells to subtoxic or mildly toxic concentrations of nephrotoxicants resulted in release of proteins and metabolites associated with mitochondrial and other pathways and that these could be used as biomarkers. We further hypothesized that

hPT cells would also release exosomes and that these could serve as an enriched source of biomarkers for the same exposures. **Methods:** hPT cells were grown to approx. 80% confluence and incubated for 0.5, 1, or 4 h with either media (= Control), the trichloroethylene metabolite S-(1,2-dichlorovinyl)-L-cysteine (DCVC) [10, 50 µM], HgCl₂ [0.2, 0.5 µM], cisplatin (CDDP) [10, 50 µM], polymyxin B (PmxB) [10, 50 µM], or tenofovir disoproxil fumarate (TDF) [5, 20 µM]. Exosomes were isolated from extracellular media samples by differential centrifugation and ultracentrifugation steps and were analyzed by both metabolomics and proteomics. Metabolomics analysis was conducted by high resolution mass spectrometry coupled with ultrahigh performance liquid chromatography to determine pathways altered by the diverse exposures. Abundance of proteins released into exosomes by cells was analyzed by labeling with isobaric TMT mass tag reagents. Peptides were identified by MS2 spectra using an Eclipse Orbitrap mass spectrometer and abundances were quantified from the TMT tags in MS3 spectra using Real Time Search. Protein MS/MS data were evaluated using Proteome Discoverer 3.1 (PD) and then proteins and peptides were quantified based on the TMT reporter ion signal. Quantitative values were evaluated in a PCA plot to visualize segregation of the sample groups. Data were then submitted to a moderated t-test to detect differences between groups and proteins that were different between groups were identified. Proteome Discoverer was used to perform protein enrichment analysis and to identify biological processes and pathways altered by treatment. Reactome and GO Metabolic Pathway analyses were conducted to identify pathways altered by the exposures. **Results:** Metabolomics analyses of exosomes released from hPT cells exposed to low concentrations of the nephrotoxic environmental chemicals or clinically used drugs whose efficacy is dose-limited by nephrotoxicity demonstrated significant alterations in several pathways, many of which are associated with mitochondria. Further, many of these pathways were the same ones identified by study of the total secretome from the hPT cells. These include prostaglandin metabolism from arachidonate, fatty acid beta-oxidation, several amino acid metabolism pathways, pentose phosphate and related vitamin metabolism pathways, and glutathione metabolism. Proteomics analysis of the whole secretome confirmed the presence of exosomes by detection of 9 marker proteins and found 8 of 9 to be increased 1.3- to 3.2-fold in association with exposures. Reactome pathway analysis showed many of the same pathways altered by exposures in the whole secretome as well as additional pathways. Common pathways that were shared amongst exosomes from hPT cells incubated with the two toxicants and the three drugs included pathways derived from mitochondria, cytoskeleton, and cytoplasm for metabolism of several amino acids, pathways indicative of immune function modulation, including cytokine signaling and neutrophil degranulation, purine and pyrimidine metabolism, glutathione conjugation, signaling and metabolism of retinoic acid (vitamin A), vitamin D metabolism, biotin transport and metabolism, malate-aspartate shuttle, citric acid cycle, and keratin metabolism and differentiation of keratinocytes. **Conclusions:** These results support the overall hypothesis that mitochondria are a critical source of potential biomarkers of toxicant exposure and identify putative proteins and metabolic pathways that may serve as these biomarkers for both nephrotoxicant exposure and metabolic reprogramming for repair and regeneration. Isolation of exosomes from the secretome of hPT cells supports the hypothesis that secreted vesicles may serve as an additional and specific source of biomarkers of effect. This research was funded by NIH Grant R01-ES031584 and the CURES Grant Number P30-ES036084.

PS 3029 1,4-Dichlorobenzene Nephrotoxicity *In Vitro*: Effect of Electron-withdrawing Groups

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Background and Purpose: 1,4-Dichlorobenzene (1,4-DCB) is a common chemical used in mothballs and as a building block for pesticides and other commercial products. 1,4-DCB is also found in the environment as a pollutant. Exposure to 1,4-DCB in the air can cause respiratory issues, while prolonged exposure can lead to liver and kidney injury. In *in vitro* studies, 1,4-DCB was the most cytotoxic of the three dichlorobenzene isomers to isolated kidney cells (IKC) from Fischer 344 rats. The purpose of this study was to examine the effects of various electron-withdrawing groups added to 1,4-DCB on 1,4-DCB nephrotoxicity as part of a structure activity study on the *in vitro* nephrotoxicity of halogenated benzenes. **Methods:** IKCs (~4 million cells/ml; 3 ml) from male Fischer 344 rats were incubated with 1,4-DCB or a 1,4-DCB derivative (1,4-dichloro-2-nitrobenzene, 2,5-dichlorobenzoic acid, and 2,5-dichloroacetophenone; 0.25-1.0 mM) or dimethyl sulfoxide (DMSO, vehicle) for 30 or 60 minutes at 37 °C in a 95% oxygen/ 5% carbon dioxide atmosphere. The release of lactate dehydrogenase (LDH) was utilized to determine cytotoxicity. **Results:** All the examined electron-withdrawing groups produced compounds with a reduced level of cytotoxicity compared to 1,4-DCB. 1,4-Dichloro-2-nitrobenzene caused the least cytotoxicity in IKC, while 2,5-dichloroacetophenone induced the highest cytotoxicity of the tested 1,4-DCB derivatives. 2,5-Dichlorobenzoic acid produced intermediate cytotoxicity among the 1,4-DCB derivatives. **Conclusions:** The results of this study indicate that the stronger the electron-withdrawing group, the greater the reduction in 1,4-DCB derivative nephrotoxicity. Previous studies have indicated that 1,4-DCB is oxidized to form a toxic metabolite(s). Since oxidation rates would be reduced by the addition of an electron-withdrawing group, the order of decreased cytotoxicity relative to 1,4-DCB supports those findings.

PS 3030 Menthol Induced Decline in Mitochondrial Function and Autophagy in Renal Epithelial Cells

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Background and Purpose: Vaping device use is increasing in teenagers and young adults. The health effects of the numerous agents released into the atmosphere during vaping is relatively unknown. Menthol is a flavoring agent contained in vaping products and detectable plasma levels of menthol have been measured following vaping. The health effects beyond the lung are not widely explored. The purpose of this project was to evaluate the effect of menthol on renal proximal tubular epithelial cells. The overall hypothesis was that menthol is cytotoxic to HK-2 cells by impairing mitochondrial function and autophagy. **Methods:** Human HK-2 cells were plated and equilibrated for 48h. Cells were exposed to a final concentration of 0-1500 uM menthol for 24h. The vehicle for menthol was water. All experiments were conducted as a minimum of 4 independent experiments with different cell passages. Cytotoxicity was assessed using the MTT assay after the 24h exposure. MTT in viable cells was converted to formazan. Upon completion of the exposure period, cells were collected, lysed and samples prepared for Western blot analysis. Differences between groups were evaluated using ANOVA followed by a post hoc test at a 95% confidence interval. **Results:** Viability was not impacted in HK-2 cells incubated with 0-200 uM menthol. Further studies examined 0-1500 uM menthol. Menthol was cytotoxic between 200-1500uM compared to vehicle control (p<0.05). Studies examined autophagy markers LC3BII and II and SIRT1 by western blot which showed no difference between groups to 0-200 uM menthol. LC3BII levels were increased by 1500 uM menthol when compared to control. Additional studies examined mitochondrial function following a 24 h exposure to menthol. **Conclusions:** In summary, menthol was cytotoxic to HK-2 cells within 24h when compared to vehicle controls. Mitochondrial impairment of basal and maximal respiration was detected at lower concentrations than impacted by MTT and cell membrane integrity. Menthol also increased autophagy. (Supported by NIH Grant P20GM103434).

PS 3031 Blocking complement as a preventative measure for kidney ischemia reperfusion injury: Insights from a human kidney-on-a-chip study

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Background and Purpose: Delayed graft function (DGF), a prevalent complication following kidney transplantation, is often driven by ischemia reperfusion injury (IRI), with complement activation playing a key role. Despite this recognition and its significant impact on transplant outcomes, preventative treatments are currently not available. In this study, we investigated the therapeutic potential of ARGX-117, a novel humanized anti-C2 antibody, in diminishing complement-induced IRI using a human kidney on-a-chip model. **Methods:** We established a 3D kidney-on-a-chip co-culture model in the OrganoPlate platform that incorporates human renal proximal tubule epithelial cells (RPTECs) and human umbilical vein endothelial cells (HUVECs) cultured under flow and in direct contact with a collagen-I ECM gel layer. Ischemia-reperfusion (IR) conditions were established by culturing the cells under low oxygen and without perfusion flow for 48 hours, followed by restoring normoxic conditions for 24 hours representing reperfusion. **Results:** Under IR conditions, the model exhibited hallmark features of IRI, including altered RPTEC morphology, decreased barrier function, and IgM-mediated complement activation, as evidenced by C3 deposition. Furthermore, complement-driven upregulation of ICAM-1 expression on RPTECs was observed, indicative of an inflammatory response. Therapeutic intervention with ARGX-117 effectively prevented IR-induced complement activation in a concentration-dependent manner, as demonstrated by reduced C3 deposition, while preserving IgM binding. Finally, ARGX-117 attenuated the IR-induced upregulation of ICAM-1 on RPTECs, suggesting reduced inflammation and subsequent tissue damage. **Conclusions:** These findings highlight the crucial role of complement activation in IRI pathogenesis and demonstrate the efficacy of ARGX-117 using a preclinical human kidney IRI model. By preventing complement-driven inflammation and kidney damage, ARGX-117 offers a promising strategy to prevent DGF and to improve kidney transplantation outcomes. This study emphasizes the importance of advanced human *in vitro* models for preclinical evaluation of targeted therapies, paving the way for better management of transplantation-associated IRI.

PS 3032 Evaluating the NRF2-KEAP1 Responses of Melatonin in Human Kidney Proximal Tubule Cells

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Background and Purpose: Melatonin is an endogenous neurohormone and dietary supplement used to treat sleep disturbances. Melatonin acts as an antioxidant and reactive oxygen species scavenger, with documented protective effects against diseases in the immune, metabolic, neurological, and renal systems. We have previ-

ously reported the anti-apoptotic effect of melatonin against vancomycin-induced cytotoxicity in human kidney proximal tubule HK-2 cells. The mechanism of this protection remains unclear although preliminary studies have suggested that melatonin can promote antioxidant signaling by favoring the release of NRF2, the master regulator of oxidative stress response, from the cytoplasmic regulatory protein KEAP1. This allows NRF2 to translocate to the nucleus and activate a coordinated network of antioxidant genes. Therefore, the purpose of this study was to compare the mRNA levels of NRF2-target genes in HK-2 cells genetically manipulated to have low, normal, or high NRF2 signaling following treatment with melatonin or the prototypical NRF2 activator sulforaphane. **Methods:** Three HK-2 cell lines, empty vector (EV), KEAP1-knockdown (KD), and NRF2-KD, were generated using stable transfection with short hairpin RNAs and treated with melatonin (32 μ M) or a known NRF2 activator, sulforaphane (SFN; 10 μ M), either alone or in co-treatment with a non-cytotoxic concentration of vancomycin (VAN; 1 mM) for 24 hours. qPCR was performed to assess the expression of kidney injury biomarker KIM-1, the pro-inflammatory cytokine TNF- α , and NRF2-ARE pathway genes. **Results:** As expected NRF2 mRNA was lowest in the NRF2 knockdown cells with little difference in mRNA levels between EV and KEAP1-KD cells. Likewise, SFN induced the expression of NRF2 and its target genes NQO1 and HO-1 in EV and KEAP1-KD cells, but to a lesser extent or not at all in NRF2-KD cells. In general, melatonin and vancomycin had little to no impact on the mRNA levels of NRF2, NQO1, or HO-1 in any of the three cell lines. Notably, VAN treatment did not alter KIM-1 mRNA levels, but it did increase TNF- α mRNA by 145%. Co-treatment of cells with vancomycin and SFN, but not melatonin, mitigated the induction of TNF- α mRNA regardless of NRF2 expression. **Conclusions:** The results indicated the attenuation of kidney injury and inflammation biomarkers by sulforaphane may involve both NRF2-dependent and independent mechanisms. By comparison, melatonin had minimal impact on NRF2-KEAP1 signaling in HK-2 cells. Ongoing studies are investigating additional pathways and transporters regulated by melatonin to further elucidate its mechanisms of renoprotection. Supported by R01DK131214, T32ES007148, and F31DK141202.

PS 3033 Silica deposits in drinking water and renal biopsies from a Mexico hotspot region of Chronic Kidney Disease of Unknown etiology (CKDu)

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Background and Purpose: Chronic kidney disease of unknown etiology (CKDu) is a worldwide epidemic primarily affecting healthy male young adults. These individuals lack traditional co-morbidities such as hypertension or diabetes. The cause of the disease remains unknown, however, current understanding suggests an environmental etiology including heat stress, particulate matter, and pesticide exposure from agricultural practices. In Aguascalientes, Mexico, a hotspot for CKDu, the disease disproportionately affects adolescents, presenting primarily with focal segmental glomerulosclerosis (FSGS). Our previous experimental and clinical studies have implicated silica particle exposure from particulate matter as a potential contributor to CKDu leading to oxidative and mitochondrial injury in the kidney. The goal of this study was to assess the presence of silica particles in renal tissue from adolescents with adaptive podocytopathy and adults with FSGS and explore possible environmental sources of silica exposure in Aguascalientes, Mexico. In addition, we examined the effect of silica particles collected from water samples in Aguascalientes on mitochondrial respiration via human renal cell models and functional assays. **Methods:** This case-control study included kidney biopsies from four groups (median age 30 years, IQR 18-42; 50.9% female): [1] adolescents with adaptive podocytopathy (n = 15), [2] adults with adaptive FSGS (n = 19), [3] adults with other glomerulopathies (n = 10), and [4] renal tissue from trauma nephrectomy controls (n = 10). We used single-particle inductively coupled plasma mass spectrometry (SP-ICP-MS) to measure silica particles in all samples. **Results:** Analysis of kidney biopsies (n=54) detected silica particles in 66% of adolescents and 65% of adults with FSGS, compared with 0% and 10% in the control groups (p < 0.01). Mean silica particle density was highest in adolescents (1.7 $\pm$ 0.4 particles/mm³ tissue) and FSGS adults (1.26 $\pm$ 0.1), versus 0.23 $\pm$ 0.04 and 0.29 $\pm$ 0.12 in disease and controls, respectively. Silica was also detected in water from non-commercial purification plants but was absent in the public water system (72,272.8 vs. 0 particles/ml, p < 0.001). Lastly, we found that silica particles disrupted mitochondrial respiration and caused oxidative stress in human renal cells. **Conclusions:** Elevated silica levels in both renal samples from affected individuals and local water sources supports silica exposure as a potential contributor to CKDu pathogenesis in Aguascalientes, Mexico.

PS 3034 Modified Albumin Elicits AKI-Like Transcriptomic Programs in Human Proximal Tubule Microphysiological Systems

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Background and Purpose: Proteinuria is a key predictor of chronic kidney disease (CKD) progression, yet whether direct tubular exposure to serum proteins exacerbates injury remains debated. We have previously shown that normal human serum, rather than purified human serum albumin (HSA), activates pro-inflammatory and remodeling pathways in human proximal tubule microphysiological systems (MPS). In CKD and diabetes, albumin is subject to post-translational modifications such as carbamylation and glycation, which alter structure and bioactivity. Carbamylated albumin (CA) accumulates with uremia in advanced disease states, whereas glycated albumin (GA) arises in hyperglycemia and may engage inflammatory receptors such as RAGE. The present study aimed to determine whether these modified albumin species independently drive tubular injury and to define associated molecular pathways. **Methods:** Primary human proximal tubule epithelial cells from three donors were cultured in Nortis ParVivo microphysiological devices under continuous perfusion. Tubules were exposed for 48 h to control media, 2% pooled human serum, or 720 μ g/mL of HSA, bovine serum albumin (BSA), CA-BSA, or GA-HSA. Effluent media were analyzed for cytokines, and tubule lysates underwent bulk RNA-seq with differential expression and pathway analyses performed using iPathwayGuide. Transcriptomic profiles were then compared with Kidney Precision Medicine Project (KPMP) single-cell datasets from healthy, AKI, and CKD proximal tubule samples. **Results:** Carbamylated albumin and human serum induced transcriptional changes (516 and 157 DEGs, respectively, FDR < 0.05), whereas native or glycated albumin elicited no significant transcriptional change. Gene Ontology enrichment identified perturbation of plasma-membrane, transporter, and oxidative-stress pathways. Consistent downregulation of Metallothionein-1 family genes (MT1E/G/H/X/M/F) by CA-BSA was also observed in AKI transcriptomes. CA-BSA exposure upregulated SMURF2, FSTL1, and TNFRSF11B (osteoprotegerin) while suppressing CISH, GPC3, and FKBP5, a pattern consistent with enhanced TGF- β and cytokine signaling which was observed in AKI and CKD transcriptomes as well. While not significant, GA-HSA demonstrated a trend of MAPK-cascade activation with donor-to-donor variability. **Conclusions:** Carbamylated, but not native, albumin invokes an AKI-like transcriptional response in human proximal tubule MPS, characterized by activation of TGF- β remodeling, cytokine signaling, and metallothionein depletion. These findings suggest that post-translational albumin modifications associated with advanced kidney disease can directly perturb tubular processes, linking proteinuria to epithelial stress and fibrotic remodeling. The data provides a mechanistic bridge between modified albumin exposure and pathways driving CKD progression along the AKI-to-CKD continuum.

PS 3035 Spatially Resolved MultiOmics Reveals Localized Renal Responses to Cisplatin Nephrotoxicity

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Background and Purpose: Cisplatin, a cornerstone in bladder cancer chemotherapy, faces a significant clinical hurdle due to cisplatin nephrotoxicity (CN). This adverse renal effect necessitates innovative therapeutic approaches to safeguard kidney function without diminishing cisplatin's anticancer potency. Our research employs a relevant bladder cancer model to elucidate the spatial metabolic alterations induced by cisplatin within the renal microenvironment, thereby paving the way for protective strategies against CN that maintain cisplatin's therapeutic efficacy. **Methods:** Male C57Bl/6J mice were induced with bladder cancer via 16 weeks of N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN) in drinking water, followed by a 3-week treatment of 9 mg/kg cisplatin weekly. Outcomes assessed included general health, survival, renal injury (as determined by plasma and kidney samples), and cancer progression (as assessed by bladder tissue). Renal injury and cancer progression were further analyzed using Desorption Electrospray Ionization-Mass Spectrometry Imaging (DESI-MSI) on 5 μ M frozen kidney sections, followed by spatially resolved cell population transcriptomics with the GeoMx digital spatial profiler. **Results:** Mice treated with BBN developed various stages of bladder cancer, including muscle-invasive bladder cancer, leading to a significant decline in survival rates due to rapid tumor growth and weight loss. Cisplatin treatment, while improving survival by reducing tumor weight and progression, also induced considerable nephrotoxicity, as indicated by elevated serum creatinine and BUN levels. Histological analysis revealed that cisplatin primarily induced tubular damage within the renal cortex. DESI-MSI further demonstrates that cisplatin disturbs the localization of specific lipids, as indicated by the regional variations of lipids like SM (d34:1) and phosphatidylcholine PC (38:4). **Conclusions:** The direct correlation between metabolite abundance and cisplatin-induced renal gene expression changes offers crucial insights into potential molecular mediators that could be therapeutically targeted to prevent CN.

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Background and Purpose: Drug-induced kidney injury affects millions of individuals annually across the globe. One particular culprit is vancomycin, a glycopeptide antibiotic, that is essential for treating serious *Staphylococcus aureus* infections. The molecular mechanisms contributing to the toxicity of vancomycin are not fully understood, but preliminary studies suggest that oxidative stress and mitochondrial dysfunction damage proximal tubule cells. As an endogenous antioxidant, melatonin offers cellular protection from oxidative damage by scavenging reactive oxygen species and enhancing cellular defense pathways. In the current study, we sought to 1) evaluate the ability of vancomycin to alter mitochondrial function in cultured human proximal tubule cells and 2) determine whether melatonin can mitigate the vancomycin-induced changes. **Methods:** Human proximal tubule epithelial cells (HK-2, N=8/treatment group) were exposed to vancomycin at pharmacologically-relevant concentrations of 0, 1, 2, 4, or 8 mM with or without concomitant treatment with melatonin (16 μ M) for 24 hours. Mitochondrial health was assessed with the Mito Stress assay performed on the Agilent Seahorse XF Analyzer. This assay measures bioenergetic parameters, including basal respiration and maximal respiratory capacity. The Bioenergetic Health Index (BHI), a quantitative measure of energy production capacity and mitochondrial stress, was calculated using the bioenergetic parameters acquired from the Mito Stress assay. A lower BHI is reflective of increased mitochondrial damage and decreased metabolic function. **Results:** Vancomycin treatment caused a concentration-dependent decrease in mitochondrial metabolic function. Compared to control HK-2 cells, the basal and maximal respiration of cells exposed to 4 mM vancomycin was reduced by 88% and 83%, respectively. Likewise, the BHI in HK-2 cells treated with 4 mM vancomycin was 44% lower than that of the control, indicating decreased mitochondrial health. The BHI of HK-2 cells treated with 8mM vancomycin was 167% lower than that of the control. Similar to vancomycin, treatment of HK-2 cells with melatonin alone reduced the basal and maximal respiration of cells between 48-61%. Together, cotreatment with melatonin further decreased both the basal and maximal respiration of cells by 29% relative to vancomycin treatment alone. By comparison, melatonin did improve the decline in BHI associated with vancomycin. **Conclusions:** Exposure of HK-2 cells to vancomycin significantly reduced mitochondrial respiration and metabolic function indicated by the decrease in basal and maximal respiration, suggesting compromised mitochondrial integrity. Despite our team previously demonstrating the ability of melatonin to protect against the cytotoxicity of vancomycin in HK-2 cells, we observed mixed mitochondrial responses to the co-treatment. Further studies using real-time monitoring of mitochondrial respiration and changes in morphology and fusion/fission are needed. Supported by R01DK131214; F31DK141202

PS 3037 AI-Driven Histology and Urinary Biomarkers to Assess Adverse Effect of Heat Stress on the Kidney in a Rat Model

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Background and Purpose: Chronic kidney disease of unknown etiology (CKDu) is an urgent health issue with increased mortality among farmworkers across the globe. Geographic data suggested a cluster of reported CKDu in agricultural communities from regions with hot and humid climates, like Central America and South Asia. Multiple factors, including heat exposure, have been reported to contribute to the development of CKDu, but the underlying cause(s) of the disease remain unclear. Previously, we reported that young adult male Sprague-Dawley (SD) rats that were repeatedly exposed (4 hours a day, 5 days a week) to heat stress at 35 °C with 50±10% humidity which increased core body temperature (CBT) by ~ 1 °C, comparable to the hyperthermia observed in farm workers, showed impaired growth and decreased body weight gain over a 4 week period. During each 4-hour heat exposure with free access to water, the animals lost ~3% of their body weight, despite significantly higher water intake as compared to the control. **Methods:** To further assess the effect of repeated chronic heat exposure on the kidney, we gathered urine samples in metabolic cages and measured a panel of urinary protein biomarkers for kidney toxicity (Thermo Fisher Scientific) by Luminex multiplex assay. We assessed kidney histopathology changes at 2-week intervals using customized AI and deep learning algorithms developed in the HALO AI platform (Indica Labs). We carried out a holistic assessment of rat kidney histopathologic changes, which we term Whole Slide Imaging (WSI) analysis, by combining quantitative structural analysis with functional staining-based objective quantification. **Results:** Our quantification of urinary biomarkers indicated that KIM-1, Calbindin, and NGAL were increased in the heat-exposed animals. We validated increased expression of *Calb1* mRNA in the kidney, and further demonstrated increased levels of the acute-phase protein, haptoglobin (Hp), which has been previously associated with heat stress in the liver. Furthermore, the whole slide histology analysis indicated glomerular and tubular injuries, as well as evidence of renal interstitial fibrosis in the heat-exposed animals. Specifically, we noted glomerular shrinkage and increased intratubular proteinuria, accompanied by significant intraglomerular accumulation of IBA-1 indicative macrophages and PAS+/Trichrome+ deposits in the kidney. **Conclusions:** Our present study reveals that heat stress increased urine biomarker levels and was associated with histopathological

changes in the kidney, highlighting the importance of applying different approaches in assessing renal damage, which may provide insights into understanding the cause and development of the disease. These findings may have relevance to the development of chronic kidney disease of unknown etiology in heat-stressed individuals.

PS 3038 Novel Role of Lipins in Aminoglycoside-Induced Renal Cell Death

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Background and Purpose: Aminoglycoside antibiotics (AGs) remain an essential class of drugs for treating life-threatening gram-negative infections, particularly in hospital and emergency settings; however, their clinical utility is limited by dose-dependent nephrotoxicity, which can lead to acute kidney injury (AKI). Lipins are phosphatidic acid phosphatases, enzymes that convert phosphatidic acid to diacylglycerol. Recent studies have suggested novel roles for lipins in renal cell death, but their ability to mediate AG-induced nephrotoxicity has not been studied. As such, this study investigated the role of lipins in AG-induced renal cell death. **Methods:** The toxicity of AGs was studied in cultures of human renal proximal epithelial tubule (RPTEC) and human kidney-2 (HK-2) cells using MTT staining, cell morphology, and annexinV/PI flow cytometry. Autophagy was also assessed using flow cytometry and western blot analysis. The expression of lipin isoforms was determined using immunoblot analyses and lipin activity was inhibited using the non-selective inhibitor propranolol. **Results:** Exposure of RPTECs and HK-2 cell to AGs for 48 hr resulted in concentration-dependent cytotoxicity. High concentrations (8 mM) of multiple AGs produced marked morphological changes indicative of cell death, while gentamicin exhibited the greatest toxicity even at 2 mM. Quantification of MTT staining demonstrated dose-dependent toxicity across the AG panel. Annexin V and propidium iodide staining revealed increased early and late apoptosis in AG-treated HK-2 cells relative to control. Autophagy red staining demonstrated modest increases in autophagic flux following AG exposure, as compared to rapamycin, which was used as a positive control. Immunoblot analysis of LC3-II expression supported these findings, with rapamycin and tobramycin producing the most pronounced increases in LC3-II expression. Treatment of cells with propranolol increased RPTEC cell death as determined by cell morphology and MTT staining. **Conclusions:** These data show that AGs induced concentration-dependent cytotoxicity in both RPTEC and HK-2 cells via mechanisms that involved apoptosis. Select AGs induced low levels of autophagy. Propranolol increased the toxicity of select AGs in RPTEC suggesting the novel finding that lipin mediates the mechanism of toxicity of AG's.

PS 3039 Organophosphate Flame Retardants Induce Renal Cell Proliferation In Vitro

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Background and Purpose: Organophosphate flame retardants (OPFRs) are widely used in consumer products as alternatives to brominated flame retardants (BFRs). Despite their increasing prevalence, the renal toxicity of OPFRs remains poorly understood. This study investigated the effects of select OPFRs—triphenyl phosphate (TPP), tris(2-ethylhexyl) phosphate (TEHP), tris(1,3-dichloro-2-propyl) phosphate (TDCPP), and tris(2-butoxyethyl) phosphate (TBEP)—on renal proximal tubule epithelial cells (RPTECs) by assessing viability, proliferation, wound repair, autophagy, and transcriptomics. **Methods:** Cell viability was assessed using MTT staining after 24 and 48 hours of OPFR exposure (5-100 μ M). Proliferation was measured by fluorescent DNA-binding dye across serial seeding densities following TPP and TEHP exposure (5 and 25 μ M). Wound healing capacity was evaluated using a scratch assay at 24 and 48 hours. Ki67 immunocytochemistry was used to assess cellular proliferation. Autophagy was examined via autophagy red staining, flow cytometry, and LC3-II immunoblotting. For transcriptomic analysis, migrating cells (those repopulating the denuded region) and non-migrating cells (those remaining after wound initiation) were harvested after DMSO or TEHP (5 μ M) treatment and processed for RNA-sequencing. **Results:** None of the OPFRs significantly decreased MTT staining at 24 or 48 hours; however, morphological alterations were observed at concentrations above 50 μ M after 48 hours. Both TPP and TEHP increased MTT staining and RPTEC number at concentrations below 25 μ M, consistent with enhanced proliferation at low seeding densities. TPP at 25 μ M inhibited wound closure in RPTEC cells, whereas TEHP enhanced wound closure after 48 hours. Ki67 staining was increased in cells located at the wound periphery following TEHP exposure, aligning with its pro-migratory phenotype. Additionally, TPP—but not TEHP—induced autophagy, as indicated by autophagy staining, flow cytometry, and elevated LC3-II levels. RNA-sequencing of migrating versus non-migrating RPTECs exposed to TEHP identified 206 upregulated and 107 downregulated genes, with enriched KEGG pathways that included cell cycle, lysosomal processes, DNA replication, and metabolic and DNA repair pathways, indicating a TEHP-driven transcriptional program supporting migration and proliferation. **Conclusions:** These findings show that OPFRs caused distinct, compound-specific effects on RPTEC growth and repair. TEHP promoted proliferation and migration, whereas TPP hindered wound repair through autophagy induction.

PS 3040 Multiple doses of elesclomol-copper is beneficial and does not trigger cuproptosis in a mouse model of Menkes disease

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Background and Purpose: Menkes disease is an X-linked disorder of copper (Cu) deficiency, caused by loss-of-function mutations in the Cu transporter ATP7A. The disease is characterized by progressive neurodegeneration and connective tissue defects, with death occurring at three years of age. Currently, no FDA-approved drug is available for the treatment of this fatal pediatric disease. We recently discovered that an investigational chemotherapy drug, elesclomol (ES), when complexed with Cu (ES-Cu), rescues Cu deficiency, activates cuproenzymes, and prevents perinatal lethality in a *mottled-brindled* (*mo-br*) mouse model of Menkes disease. We showed that a mere two doses of ES-Cu can rescue *mo-br* mice from early mortality; however, within a few months of life, the Cu deficiency remains visible, as reflected by the decrease in Cu-dependent proteins, such as COX1, in the mitochondria. **Methods:** The current study was conducted to evaluate the feasibility of administering extra doses of ES-Cu at later stages of life to correct Cu deficiency without causing unwanted toxic effects. To evaluate this we have used mottled-brindled (*mo-br*) mouse model of Menkes disease. **Results:** Here, we report that multiple ES-Cu doses in *mo-br* mice, at various stages of growth, are beneficial in preserving the mitochondrial COX1 without triggering cuproptosis. **Conclusions:** Our results support the concept of repurposing ES for the treatment of Cu deficiency disorders.

PS 3041 Assay-Dependent Artifactual Prolongation of Activated Partial Thromboplastin Time (APTT) Observed in Cynomolgus Monkeys

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Background and Purpose: This study characterizes an assay-dependent, artifactual prolongation of activated partial thromboplastin time (APTT) observed in nonclinical safety studies of a polymer-conjugated enzyme biologic in cynomolgus monkeys. **Methods:** Two 8- and 13-week repeat-dose toxicology studies were conducted in cynomolgus monkeys. The animals received subcutaneous injections twice weekly. Study endpoints included pharmacokinetic/toxicokinetic (PK/TK) and pharmacodynamic (PD) assessments, clinical observations, body weight, clinical pathology, gross and histopathology. The 8-week study utilized a single silica-based APTT assay, whereas the 13-week study employed both silica- and kaolin-based assays. Additional *ex vivo* APTT assays were performed using plasma from naïve cynomolgus monkeys spiked with either the full biologic or the conjugated polymer. **Results:** In the 8-week study, a time- and dose-dependent prolongation of APTT was observed using the silica-based assay at Weeks 4 and 8. PK/PD effects were prominent during the first two weeks but diminished thereafter, likely due to immunogenicity, and did not correlate with APTT prolongation. No abnormalities were observed in other coagulation parameters (e.g., prothrombin time, fibrinogen), nor were there histopathological findings indicative of bleeding or coagulopathy. *Ex vivo* spiking of plasma with the biologic or polymer did not result in APTT prolongation. In the 13-week study, APTT prolongation was again observed only with the silica-based assay, while the kaolin-based assay showed no such effect. **Conclusions:** The APTT prolongation observed was specific to the silica-based assay and was not associated with any clinical or pathological evidence of hemorrhage or coagulopathy. These findings support the conclusion that the APTT changes were assay-dependent artifacts related to the silica activator, rather than true pharmacologic or toxicologic effects.

PS 3042 From Monospecific to Bispecific: Comparative Safety of T-Cell Engagers and Dual-Receptor BsAbs Versus Monospecific Agents Using Clinical Outcomes and FAERS Data

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Background and Purpose: **Background:** The shift from monospecific monoclonal antibodies (mAbs) to bispecific monoclonal antibodies (BsAbs) enables simultaneous engagement of two targets, allowing immune cell redirection or dual-pathway blockade. This mechanistic versatility can enhance therapeutic efficacy and overcome resistance, but it introduces unique safety challenges distinct from those of mAbs. The ability to engage two antigens drives both enhanced potency and complex adverse event (AE) profiles. As of September 2025, PharmaPendium lists 18 BsAbs approved in Europe and the United States, spanning two mechanistic classes: T-cell engagers (TCEs) and dual-receptor modulators. BsAbs range from Bi-specific T-cell engager (BiTE)-like fragments to full IgG-like constructs. This study compares the safety of TCEs and dual-receptor BsAbs against monospecific antibodies targeting similar antigens, integrating clinical regulatory and postmarketing safety data. **Methods:** Clinical data from PharmaPendium were analyzed using the *Tox Navigator* (developed in collaboration with Pfizer) to extract structured toxicity information for both monospecific and bispecific monoclonal antibodies. Postmarketing data from

the FDA Adverse Event Reporting System (FAERS) were evaluated for real-world safety signals and disproportionality. Clinical outcomes were aligned with FAERS data to triangulate safety trends. BsAbs were classified as (1) T-cell-engaging BsAbs (TCEs) or (2) dual-receptor BsAbs that modulate two signaling pathways without direct T-cell engagement. Monospecific comparators targeting the same antigens were included to enable mechanism-based safety contrasts. Adverse drug reactions (ADRs) were mapped to MedDRA terminology and aggregated by System Organ Class (SOC) and preferred terms. Endpoints included cytokine release syndrome (CRS), neurotoxicity/ICANS, cytopenias, infections, hepatic and renal dysfunction, cardiovascular events, and ocular or dermatologic toxicities. Descriptive statistics by mechanistic class, molecular format (BiTE-like vs IgG-like), target, indication, and line of therapy were applied to identify and interpret safety signals. **Results:** Integrated analyses using *Tox Navigator* revealed mechanism-consistent and class-specific safety profiles. For TCEs, safety signals aligned with T-cell-mediated toxicities, including higher frequencies of CRS and immune effector cell-associated neurotoxicity syndrome (ICANS) compared with monospecific antibodies targeting the same antigens. Cytopenia and infections were frequent, varying by timing and treatment line. Dual-receptor BsAbs exhibited safety profiles consistent with dual-pathway modulation, including overlapping toxicities such as hepatic enzyme elevations, edema, and hypertension, along with class-related cytopenias and infections. Integration of FAERS and clinical data strengthened the detection of mechanism-driven AE patterns, distinguishing inflammatory versus dual-pathway safety liabilities. **Conclusions:** This integrated framework demonstrates that TCEs exhibit inflammatory toxicity patterns (CRS, ICANS), while dual-receptor BsAbs present broader but overlapping adverse events linked to dual-target blockade. Triangulation of clinical and postmarketing data provides translational insight into mechanistic toxicities and informs risk mitigation strategies such as antibody design optimization, dose modulation, premedication, and patient monitoring. Leveraging structured datasets via the *Tox Navigator* supports evidence-based risk assessment to guide safer development and clinical use of next-generation bispecific antibodies.

PS 3043 Corrosive Exposures in Clinical Toxicology: Poison Prevention Strategies for Hydrofluoric Acid

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Background and Purpose: Both acids and bases can exert corrosive tissue effects through routes of ingestion, inhalation, dermal, and optic exposures. As with other toxic exposures, symptoms may be related to dose, route, and individual characteristics. Hydrofluoric (HF) acid is an inorganic acid and dehydrating agent that is used for industrial and automotive purposes. HF is used in metal processing and glass etching and can be found in rust removers and cleaners. The purpose of this study is to examine HF exposure and describe poison prevention strategies. **Methods:** Using the National Poison Data System (NPDS) annual reports from poison control centers in the United States (years 2018 to 2023), HF poisoning exposures and fatalities were reviewed and described. In addition, accident reports from the Occupational Safety and Health Administration (OSHA) for HF were reviewed (years 2018 to 2021). Poison prevention information was accessed from Health Resources & Services Administration (HRSA) Poison Help and the National Capital Poison Center. **Results:** During the 6 years of NPDS reports, HF exposures were reported either as a chemical acid ($N = 3,085$), or a household cleaning substance in wheel cleaners ($N = 227$) or rust removers ($N = 1,184$). Most of the HF exposures were for populations 20 years and older, 83.92%, 73.13%, and 60.6%, for the three sources, respectively. Overall, 96.92% of exposures to HF as a chemical acid and 95.15% of exposures to HF through wheel cleaners were unintentional. In contrast, only 76.44% of HF exposures through rust removers were reported as unintentional. NPDS reported HF-related fatalities steadily decreased from 2018 ($N = 5$), 2019 ($N = 2$), 2020 ($N = 2$), 2021 ($N = 1$), 2022 ($N = 1$), to 2023 ($N = 1$). Based on the OSHA Accident Reports for industrial exposures for HF (2018-2021), 11 cases were reported, including one fatality. Two cases were reported in 2018, six cases in 2019, one case in 2020, and two cases in 2021. Descriptions of these exposure cases included chemical burns, illness from inhalation, splash injuries to skin and face, injuries from an arc flash in an electrical panel, and death from falling into industrial paint remover. **Conclusions:** HF exposure was documented in chemical acids, wheel cleaners, and rust removers from annual and accident reports of the NPDS and OSHA, respectively. Unintentional exposures dominated the intent of exposures, with variations in site and routes of exposure. Due to the significant adverse health outcomes from exposure, including fatalities, preventive measures should be emphasized, reviewed, and implemented routinely with product use. In order to limit toxic exposures and promote proper handling for safe usage, consistent practices for poison prevention should include the following: keeping products in original containers, with proper labelling; placing products in locked cabinets, and away from children and cognitively disabled persons; providing appropriate protective equipment and environmental conditions in occupational settings; and accessing available resources from the Poison helpline, America's Poison Centers, and HRSA Poison Help Prevention Tips. With continuing preventative and educational efforts in both community and workplace settings, residential and occupational risks of HF exposure can be mitigated.

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Background and Purpose: A significant portion of disease-relevant proteins remain “undruggable” by traditional therapeutic approaches, representing a major hurdle in drug discovery. Targeted protein degradation (TPD) is rapidly advancing as a strategy to overcome this challenge, enabling the selective elimination of previously inaccessible proteins. Protein degraders have potential safety benefits due to their targeted nature but also pose risks like long-lasting toxicity from prolonged degradation and off-target effects. Pre-clinical safety data for TPD is limited due to a combination of their novel mechanism, structural complexity and evolving evaluation framework. Loss-of-Function (LoF) variants could be used as analogues to the effects of TPDs to reveal long-term consequences of reduced or absent protein function. Similarly, Knockout (KO) models especially humanized versions are playing a crucial role in anticipating and evaluating potential toxicities associated with targeted protein degraders (TPDs) such as PROTACs and molecular glues. This systematic review of literature, conferences, clinical trials, pathway maps compare the safety and selectivity of clinical TPDs against pharmacological inhibitor, as well as reported LoF and KO models. **Methods:** Meta-analysis of TPDs in development from clinical to pre-registration using Cortellis Drug Discovery Intelligence (CDDI) and OFF-X Safety Intelligence. CDDI incorporates information from journals, conferences, patents, press releases, regulatory agencies and company websites on the scientific development of drugs and targets from biological testing to launch. OFF-X monitors all adverse events (AEs) from pre-clinical, clinical and post-marketing studies from regulatory agencies, biomedical literature, congresses and scientific conferences, major clinical trial registries, company communications and pharmacovigilance databases (i.e. FAERS, JADER). To assess the potential of degraders as an alternative technology for target modulation, we utilized these databases to compare the safety profile of degraders that are in late clinical phases with traditional inhibitors and reported LoF and KO models. **Results:** There are currently 12 TPDs that were included in this analysis, four of them are in Phase 2, seven in Phase 3 and one Pre-registered. Common targets for these degraders are androgen receptor (AR), IKAROS family and Bruton tyrosine Kinase (BTK). Commonly observed safety issues across all the compared degraders included hematologic, gastrointestinal and skin disorders. Both BTK degraders and inhibitors show infections and vascular and cardiac disorders as common issues. AR pharmacologic inhibition and degradation, commonly manifests systemic gastrointestinal, metabolic and cardiovascular risk. However, no signs of hepatotoxicity have been identified with any of these degraders yet, in line with the preclinical data that has been reported for BTK and AR degraders. **Conclusions:** Observed safety issues are not unique to TPDs but are particularly relevant due to their potent and irreversible mechanism of degrading target proteins. When BTK is degraded or inhibited, it disrupts several critical immune pathways-this leads to an increased risk of infections, such as upper respiratory tract infections (URTIs). Loss of BTK function, such as in X-linked agammaglobulinemia, was also linked to increasing susceptibility to infections. AR is a systemic regulator of metabolism, cardiovascular health, and tissue homeostasis. Disrupting these pathways, potentially is leading to a cascade of metabolic, cardiovascular, and GI symptoms. TPDs that degrade transcription factors like Ikaros, which are key regulators of lymphocyte development and immune homeostasis can lead to hematologic toxicity like neutropenia and thrombocytopenia. Preclinical toxicology studies with degraders need to be designed with the aim to anticipate potential safety liabilities, including those identified with pharmacology inhibitors, to de-risk development programs. To do so, individual target evaluation, considering context-specific biology, genetics and pharmacology need to be considered to help identify mechanistic toxicity drivers.

PS 3045 Cigarette Smoking, Cytokines, and Immune Evasion in Lung Cancer

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Background and Purpose: Cigarette smoking introduces a complex mixture of toxicants into the airways and remains the leading cause of lung cancer, while also contributing to the development of several other malignancies. A recent study indicates that roughly 25% of cancer patients are active smokers when diagnosed, and up to 58% maintain smoking behavior during therapy. Malignant pleural effusion (MPE) is a common complication of advanced cancers, especially lung cancer. Research indicates that crosstalk between tumor cells and the immune system contributes to the development of MPE, establishing a unique tumor microenvironment (TME) enriched in immune cells. In our preliminary studies using a personalized immunotherapy approach on an NSCLC patient from the UNC-Chapel Hill’s Carolina Center for Pleural Diseases (CCPD) biobank, we detected antigen-specific CD8⁺ T cell populations in the patient’s MPE. However, despite their presence, effective intrapleural anti-tumor immunity was not achieved, suggesting that factors within the MPE microenvironment may actively suppress CD8⁺ T cell function or impair their cytotoxic potential. A few studies describe overall elevation of immunosuppressive cytokines in pleural fluid of lung cancer patients but do not differentiate between smokers and non-smokers. Evidence from recent research suggests that, smokers exhibit significantly higher levels of pro-inflammatory cytokines in blood serum compared to non-smokers. Therefore, we sought to determine whether cigarette smoking similarly influences the cytokine profile and the immunosuppressive mechanisms within the MPE microenvironment. **Methods:** This study utilized

matched MPE fluid and plasma samples from lung cancer patients categorized as cigarette smokers or non-smokers. To examine whether smoking similarly influences the cytokine landscape in other cancers, we also obtained matched MPE fluid and plasma samples from breast cancer patients, who were likewise categorized as smokers or non-smokers. Using a Human Immune Response Panel, we quantified the levels of different cytokines, chemokines, and growth factors in matched MPE fluid and plasma samples to comprehensively profile the immune landscape and identify potential smoking-related alterations in immune signaling. **Results:** Across all MPE fluid samples analyzed, several cytokines—including IL-6, IL-10, and MCP-1 (MCAF), were consistently elevated, reflecting the inflammatory and immunosuppressive nature of the pleural tumor microenvironment. These cytokines are linked to immune regulation and T-cell suppression, such as IL-6 and IL-10 that showed the most pronounced variability between groups. These early findings suggest that MPE fluid has immunosuppressive cytokine milieu within the pleural space, potentially impairing effective anti-tumor immune responses. Ongoing analyses will expand this dataset to more comprehensively compare lung and breast cancer MPE samples between smokers and non-smokers and to better understand how smoking status influences the pleural immune landscape. **Conclusions:** Our preliminary findings reveal a distinctly immunosuppressive cytokine milieu within the MPE microenvironment, which is highly intriguing and may help explain the limited effectiveness of local anti-tumor immunity. These observations warrant deeper investigation across smoking histories and cancer types to define key immune-regulatory pathways. Understanding how cigarette smoke alters cytokine signaling within the pleural space in cancer patients, provides critical insight into the immunotoxic mechanisms driving cancer progression and impaired immune surveillance.

PS 3046 A second generation PFAS, GenX, Induces Astrocytic Activation and Metabolic Defects

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Background and Purpose: Per/polyfluoroalkyl substances (PFAS) are a group of anthropogenic chemicals, characterized by a strong carbon-fluorine bond. Owing to extensive usage in industrial and consumer products such as firefighting foams, water or stain repellents, etc., and their resistance to breakdown, PFAS are persistent in the environment, and have been detected in over 98% of the USA. Use of some PFAS, such as perfluorooctanesulfonate (PFOS) and perfluorooctanoic acid (PFOA), was discontinued in 2000, and as such, these are referred to as “legacy PFAS.” Over time, legacy PFAS have been replaced by newer (so-called ‘second generation’ or emergent) PFAS, which often have shorter chain length; however, their safety to human health and the environment is still questionable. Emergent PFAS such as GenX (Ammonium perfluoro(2-methyl-3-oxahexanoate)), also known as (HFPO-DA), was synthesized in 2009 to replace the legacy PFAS, PFOA. **Methods:** Using an iPSC-derived blood-brain barrier (BBB) model, we demonstrated that GenX readily crossed the barrier. Further, rodents exposed to GenX showed that GenX was present in mouse cortex, where it induced pronounced astrogliosis (GFAP⁺), suggesting that GenX can induce astrocytic activation. To understand the effect of GenX on glial cells, we treated astrocytic and microglial cell lines with GenX. Interestingly, GenX reduced metabolic activity and ATP production in human astrocytes, but not in microglia, indicating cell-type-specific toxicity. Mitochondrial impairment manifested as decreased basal respiration and ATP production, accompanied by A1-like astrocytic activation (C3⁺, IL-6⁺) and senescence markers (γH2AX⁺, p53⁺), phenotypes associated with neurodegeneration. **Results:** Mechanistically, PFAS have been linked to lipid dysregulation in humans. Apolipoprotein E4 (APOE4) is the primary genetic risk for sporadic AD and is expressed in ~40-65% of AD patients. Apolipoprotein E is involved in neuronal proliferation, synapse formation, and myelination, affecting cellular functions such as inflammation. Astrocytes are the main source of APOE in the brain and are also involved in lipid metabolism. GenX exposure caused marked mitochondrial dysfunction and IL-6 up-regulation in primary astrocytes from humanized APOE4, but not APOE3 mice, implicating a strong gene-environment interaction between GenX and ApoE. **Conclusions:** In summary, GenX acts as an astrocyte-specific neurotoxicant that perturbs mitochondrial metabolism, driving inflammatory and senescent phenotypes. This toxicity is strongly amplified by the APOE4 genotype, providing an interaction between environmental PFAS exposure and genetic risk factor. These findings establish a framework for evaluating emerging PFAS in the context of gene-environment interactions underlying brain aging.

PS 3047 First clinical case of N-pyrrolidino protonitazene intoxication in Brazil: detection and quantification in serum by micro-QuEChERS and LC-MS/MS

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Background and Purpose: Nitazenes are a class of novel synthetic opioids derived from 2-benzylbenzimidazole, several of which display potencies greater than fentanyl, raising significant concerns for public health. Their rapid emergence in

global drug markets poses a major challenge to forensic and clinical toxicology laboratories. *N*-pyrrolidino protonitazene is among the most recent nitazene analogues associated with severe opioid toxicity. In 2025, the first clinical case of *N*-pyrrolidino protonitazene intoxication was confirmed in Brazil, prompting a national alert. This study describes the analytical detection and quantification of *N*-pyrrolidino protonitazene in human serum using a micro-QuEChERS extraction procedure coupled to a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system and reports the associated clinical manifestations. **Methods:** Serum aliquot (100 μ L) was extracted using a micro-QuEChERS approach. The sample was mixed with 400 μ L of acetonitrile and 100 mg of partitioning salts (NaAc:MgSO₄, 1:4 w/w), vortexed for 5 minutes (2500 rpm), and centrifuged for 5 minutes (14000 rpm). The supernatant was collected and evaporated under a nitrogen stream for 10 minutes at 40 °C. Finally, the content was reconstituted using 100 μ L of methanol. A 5 μ L aliquot was injected into an LC-MS/MS system operating in positive electrospray ionization (ESI) mode. Separation was performed with a C18 column (50 mm $\times$ 3 mm, 2.6 μ m) using gradient elution with 0.1% formic acid and 2 mM ammonium formate both in water (mobile phase A) and methanol (mobile phase B). Detection was performed in multiple reaction monitoring (MRM) mode using a certified *N*-pyrrolidino protonitazene reference standard. The estimated limit of detection was 0.025 ng/mL and the limit of quantification 0.05 ng/mL. **Results:** *N*-pyrrolidino protonitazene was detected and quantified in the patient's serum at 1.37 ng/mL. The patient had reportedly the consumption of an alcoholic beverage and one tablet before developing symptoms of severe opioid intoxication, including marked somnolence, miosis, apnea, oxygen saturation of 30%, and a Glasgow Coma Scale score of 3, indicating profound central nervous system depression. Administration of three ampoules of naloxone was required to reverse respiratory failure. Additional compounds identified in the toxicological screening included MDA, dipentylone, pentylone, ethanol, and nicotine, suggesting polydrug exposure. **Conclusions:** This case represents the first confirmed clinical detection of *N*-pyrrolidino protonitazene in Brazil. The combination of micro-QuEChERS extraction and LC-MS/MS provided an efficient and sensitive approach for detecting highly potent synthetic opioids at trace levels. The clinical course, requiring multiple naloxone administrations, highlights the pharmacological potency and toxicological risk of nitazenes. These findings were promptly communicated to the Brazilian Rapid Alert System on Drugs, contributing to the inclusion of *N*-pyrrolidino protonitazene in the national list of controlled substances. This case underscores the need for analytical vigilance and continuous monitoring of emerging synthetic opioids in forensic and clinical settings.

PS 3048 *In silico*-driven mechanistic insights into the increased sensitivity of canines to drug-induced seizures in nonclinical safety studies

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Background and Purpose: Drug-induced seizures are serious adverse events for which adequate nonclinical testing is crucial. The translatability of drug-induced seizures in canine toxicology studies is being questioned due to neurological findings at low exposures in beagle dogs which are not observed in other standard toxicology species. The lack of mechanistic and physiologic understanding behind this outcome compromises the safety window for predicted human exposure and can lead to clinical hold or attrition. Our objective was to investigate whether there are differences in ion channel function and/or expression that explain the increased sensitivity to seizure observed in dog. **Methods:** We have previously identified and characterized a panel of 15 human ion channels involved in seizure liability using *in vitro* electrophysiology (Na_v1.1, Na_v1.2, Na_v1.6, K_v1.1, K_v2.1, K_v3.1, K_v4.2, K_{Ca}1.1, K_{Ca}4.1, K_v7.2/7.3, K_v7.3/7.5, GABA α ₁ β ₂ γ ₂, NMDA 1/2A, nicotinic α ₄ β ₂, and Ca_v2.1). In the current study, we have used a suite of computational approaches to generate alignments of human versus dog protein sequences and analyze the differences in amino acids. We identified sites of drug- or ligand- binding, sites of known epilepsy mutations, residues with post-translational modifications, variants located in or near functional domains, and nonconservative amino acid changes. Based on these parameters, the canine ion channels were prioritized from highest to lowest interest for further investigation. Preliminary transcriptomic analysis of seizure-associated ion channels and their auxiliary subunits revealed their absolute gene expression levels across multiple CNS regions in humans versus dogs. **Results:** Our data indicated that the nicotinic α ₄ β ₂, K_v7.2/7.3, Ca_v2.1, K_v7.3/7.5, GABA α ₁ β ₂ γ ₂, and NMDA 1/2A channels were of highest interest after the sequence alignment analysis. Examples of the findings include the following: the nicotinic α ₄ β ₂ channel had the lowest sequence homology (89.75%) between humans and dogs and the lowest number of conserved residues at phosphorylation sites, ligand-binding and functional domains. K_v7.2/7.3 had the highest number of altered residues at pathogenic/likely pathogenic sites identified in UniProt. GABA α ₁ β ₂ γ ₂ had the lowest Blosum score (-1.0) of all the channels, meaning that its amino acid changes were on average the most unlikely to occur by chance. Expression level differences were examined in seizure- and ataxia-relevant tissues - cerebellum and spinal cord. Initial investigations of transcriptomic data showed no notable differences in spinal cord. Cerebellar expression was more varied, with human cerebellum showing the greatest increases in KCNQ2 and CACNA1A expression compared to dogs. **Conclusions:** Our initial findings provide insights into the complex question of increased seizure liability in canine toxicology by highlighting inherent differences in ion channel sequence

and expression level between dogs and humans. Next steps will be to generate cell lines expressing the relevant canine ion channels to assess relative sensitivity via automated patch clamp electrophysiology.

PS 3049 Screening Novel Radioprotective Compounds for Salivary Glands Using Microbubble Chip Array

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Background and Purpose: Head and neck cancers (HNCs) are a group of malignancies accounting for approximately 900,000 new cases and over 400,000 deaths annually. According to the World Health Organization (WHO), HNCs are the 7th most common cancer globally. Currently, the most effective cytotoxic therapy for localized solid tumors is ionizing radiation (IR). However, this approach comes at a cost with 80% of patients experiencing some degree of salivary gland damage or permanent loss of secretory function as an off-target effect. This dysfunction decreases the production of saliva and increases the risk for infection, difficulty speaking, eating, or swallowing, which ultimately lowers quality of life. Amifostine is an antioxidant drug and the only FDA approved radioprotective compound used for clinical indications against IR therapy. This compound however, has a myriad of limitations including high doses, short half-life (~15min), high toxicity, severe side effects and low efficacy. For these reasons, its use is often not recommended. Thus, there is a critical unmet need to develop a new radioprotective compounds which will decrease the severity of off-target radiation injury. Discovery of new drugs has however, been hindered by the lack of functional salivary gland tissue models. Recently, the DeLouise lab pioneered the development of the first ever functional 3D salivary gland tissue chip array platform which was used successfully to conduct high-content screening of a Selleck Chemicals library. This screen identified 7 non-antioxidant top hits, three of which were validated in a murine model. This project seeks to investigate analogs of these hits to investigate structure function relationships, to develop more potent drugs and provide insight into their mechanism of action. **Methods:** Female SKH1 hairless mice backcrossed 6 generations with C57BL/6J mice, aged 6-12 weeks were used in this study. Only female mice were used due to known sex differences in rodent salivary glands, with female glands more accurately emulating human salivary gland structure and function. Primary murine submandibular gland cells were isolated and introduced into the salivary gland tissue chip and irradiated at 15Gy. Injury response to IR was quantified using the reduced glutathione and cell senescence assays that were developed to conduct the initial radioprotective drug screen. The established protocol was followed by conducting the glutathione assay first. Hits that showed indistinguishable radioprotection from 0 Gy controls were then tested with the senescence assay. Drug analogs were screened at 10 μ M. **Results:** In this study, drug analogs of enoxacin (fluoroquinolone antibiotic) and doripenem (carbapenem antibiotic) were tested. Structural analogs were selected primarily from the fluoroquinolone class, with the C=N 8-azaquinoline core that seems to be essential for enoxacin's radioprotective activity. In total 24 analogs were tested and 13 showed higher levels of reduced glutathione levels indicating some radioprotection. **Conclusions:** These preliminary results indicate that fluoroquinolone and carbapenem antibiotics exhibit radioprotective properties in the tissue chip and in preclinical models. On-going studies will seek to test the senescence (β -galactosidase) levels upon IR exposure to confirm radioprotection of the 13 additional hits. Moreover, understanding radioprotective mechanisms of antibiotics compounds is critical for clinical translation of these novel compounds that can have a significantly benefit to HNC patients

PS 3050 Dual roles of the Ah receptor (AHR) ligand 6-Formylindolo[3,2-b]carbazole (FICZ) in the induction of pulmonary cytochrome P450 (CYP)1A1 by hyperoxia, and the attenuation of hyperoxic lung injury in mice *In Vivo*

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Background and Purpose: Supplemental oxygen administration is frequently encountered in infants and adults with pulmonary insufficiency. Hyperoxia causes lung injury in experimental animals, and contributes to acute respiratory distress syndrome (ARDS) in adults and to bronchopulmonary dysplasia (BPD) in premature infants. In this investigation, we tested the hypotheses that (i) hyperoxia induces CYP1A1 via the formation of 6-Formylindolo[3,2-b]carbazole (FICZ), an endogenous ligand for the Ah receptor (AHR; and (ii) FICZ would attenuate hyperoxic lung injury *in vivo*. **Methods:** Adult (10 week old) wild type (WT) mice were maintained in room air or exposed to hyperoxia (> 95% O₂) for 6-48 h, and lungs were analyzed for the formation of FICZ. In a second set of experiments, WT mice were treated with FICZ (2 mg/kg), i.p., once daily for 3 days, and the animals were maintained in room air or exposed to hyperoxia (> 95% O₂) for 72 h, and lung injury was determined by histology. In a third set of experiments, WT, *Cyp1a1*-null, *Cyp1a2*-null, *Cyp1b1*-null, *Cyp1a1/1a2*-double null, *Cyp1a1/1a2/1b1* triple null, and AHR-null mice were treated with FICZ (2 mg/kg), i.p., and FICZ levels were measured in lung and liver. **Results:** FICZ was detected in mouse lungs exposed to hyperoxia at 6 h, but not at later

time points, as revealed by LC-MS/MS experiments. Further mechanistic studies showed formation of FICZ from tryptophan in the presence of Fe²⁺ and H₂O₂ *in vitro*, and also in H358 cells exposed to hyperoxia (80% O₂; 8 h). FICZ was detected *in vivo* in mouse lungs exposed to hyperoxia at 6 h, but not at later time points, as revealed by LC-MS/MS experiments. FICZ significantly upregulated CYP1A1 and CYP1B1 in lung and liver and CYP1A2 in liver at the mRNA, protein, and enzyme levels. Hyperoxia caused significant lung injury and inflammation as determined by increased lung weight/body weight (LW/BW) ratios and histology and neutrophil recruitment. FICZ treatment significantly suppressed lung injury mediated by hyperoxia. FICZ was detected in all types of mice, but the levels were significantly higher in *Cyp1a1*-null, *Cyp1a1/1a2*-double null, *Cyp1a1/1a2/1b1*-triple null, and AHR-null, with FICZ being the highest in AHR-null mice. These results suggest that CYP1A1, 1A2, and 1B1 play an important, differential role in the metabolic degradation of FICZ *in vivo*. **Conclusions:** Our results demonstrate (i) novel role of FICZ in the induction of CYP1A1 by hyperoxia via the AHR; (ii) protection against hyperoxic lung injury by FICZ; and (iii) role of CYP1A1, 1A2, 1B1, and AHR-mediated mechanisms in the metabolism of FICZ *in vivo*. Further studies could lead to the development of novel AHR agonists in the prevention/treatment of ARDS and BPD in humans.

PS 3051 Advanced 3D iPSC-derived cardiac model for improved drug-induced cardiotoxicity assessment

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Background and Purpose: Cardiotoxicity is a leading cause of drug attrition during development, largely due to the poor predictive capacity of traditional 2D *in vitro* models. To address this unmet need, we employed the SmartHeart® technology platform to generate a 3D co-culture system combining iPSC-derived cardiomyocytes (YBLiCardio) and human cardiac fibroblasts, which more closely mimics the *in vivo* cardiac microenvironment and enhances prediction of drug-induced toxicity. **Methods:** Cardiac fibroblasts and iPSC-derived cardiomyocytes cells were seeded on 4D plates at a 75:25 cardiomyocyte:fibroblast ratio, yielding 85,000 cells/well. Cultures were maintained for seven days before performing the experiments. Downstream analyses included microscopy, live cell imaging, flow cytometry, qPCR, calcium flux, immunocytochemistry, and western blot for cardiac markers. Cardiotoxicity drugs with known like Disopyramide, Quinidine, Nifedipine, Ibutilide, Vandetanib, Azimilide, and Bepridil—were tested and compared for toxicity in the 2D and 3D model. **Results:** Preliminary results indicate robust formation of 3D cardiac rings with viable contractile activity. Imaging and flow cytometry confirmed successful co-culture and differentiation, with cardiac markers expressed as per expected profiles. Calcium flux assays demonstrated functional responsiveness, and molecular profiles (qPCR, ICC) supported enhanced maturation and cell-cell interaction in 3D compared to 2D. Drug exposure studies revealed differential sensitivity and toxicity profiles in the 3D model. Preliminary data suggests improved predictive sensitivity of the 3D model over traditional 2D assays. **Conclusions:** The SmartHeart® technology enables the generation of complex 3D cardiac co-cultures using iPSC-derived cardiomyocytes (YBLiCardio) and human ventricular fibroblasts, substantially improving the accuracy and sensitivity of *in vitro* cardiotoxicity prediction. This approach offers a translationally relevant alternative to conventional 2D screening methods and paves the way for improved risk stratification of drugs early in the development process.

PS 3052 Investigation of the application of Hemoglobind™ to improve detection of nitrate in hemolyzed ocular fluid samples

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Background and Purpose: Nitrate toxicosis, characterized by an excess of nitrate intake, can cause significant health risks in both animals and humans. In animals, especially ruminants such as cattle, high nitrate levels can cause methemoglobinemia. This disease is characterized by its reduction in hemoglobin's oxygen-carrying abilities. This leads to respiratory distress and in severe cases death. Although ruminant animals are more susceptible to nitrate-induced methemoglobinemia, these conditions can still have dire effects on humans. In humans, specifically infants under six months, this condition is commonly known as "blue baby syndrome." The lack of oxygen in blood results in a bluish tint to the skin, especially around the eyes and mouth. If left untreated, this condition can be life-threatening. Currently testing for nitrate toxicosis in ruminants involves the use of post-mortem ocular fluid and a color-based semi-quantitative test in the form of strips. Due to the nature of this testing method the presence of high levels of hemoglobin in the samples renders inconclusive results. The post-mortem ocular fluid samples extracted can be hemolyzed, impeding the detection of nitrate. **Methods:** This study focuses on a new method to reduce the degree of hemolysis in post-mortem ocular fluid samples therefore improving the ability to test for nitrate toxicosis. A series of tests were conducted to determine if the addition of Hemoglobind™ interferes with the detect of nitrate. The study was conducted using EM Quant® Nitrate Ion Specific Test Strips, quality control samples of known concentrations of nitrate up to 100 ppm, and samples with Hemoglobind™. These were then compared using the nitrate testing strips. The procedure briefly involves one drop of ocular fluid and one drop of quality control

sample to individual strips and then incubated for approximately one minute. After incubation the strips are compared to the ppm values on the container to determine the concentration of nitrate present. After concluding that Hemoglobind™ does not interfere with the detection of nitrate, the experiment was conducted again to show repeatability. To determine the optimal ratio of ocular fluid to Hemoglobind™, a sample of ocular fluid was artificially hemolyzed and then ratios of 1 to 1, 1 to 2, and 1 to 3 were compared using the nitrate testing strips. An additional experiment was conducted using a series of 6 samples of ocular fluid in varying degrees of hemolysis to determine an optimized ratio of 1 to 2 (ocular fluid to Hemoglobind™). The last experiment conducted involved determining how much hemoglobin was removed from the ocular fluid samples using Hemoglobind™. **Results:** By utilizing Hemoglobind™ to reduce the degree of hemolysis in the samples, the detection of nitrate improved while not interfering with the detection of nitrate. Determining the percent deduction of hemoglobin was accomplished utilizing an indirect ELISA for bovine hemoglobin. A ratio of Hemoglobind™ to post-mortem ocular fluid of 2 to 1 decreases the hemoglobin levels in the ocular fluid by 45.45% to 69.07% depending on the degree of hemolysis. The higher concentration of Hemoglobind™ was shown to have no binding specificity for nitrate. **Conclusions:** This study provides a new method for improving detection of nitrate in hemolyzed ocular fluid samples in suspect cases of nitrate toxicosis.

PS 3053 Evaluation of Mutational Signatures in Rat Tumors Resulting from Chronic Inhalation Exposure to Vinyl Chloride

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Background and Purpose: Vinyl chloride (VC), used to manufacture polyvinyl chloride, is a mutagen and an IARC Group 1 carcinogen linked to hepatic angiosarcoma and hepatocellular carcinoma in humans as well as in rodent models. Mutational signatures, composite patterns of somatic substitutions, can provide mechanistic insight and enable comparisons across tissues and species. The Catalogue of Somatic Mutations in Cancers (COSMIC) maintains an extensive repository of mutational signatures with established or hypothesized mechanistic bases across various cancers. Due to the inherent multifactorial nature of the human exposome, many signatures hold unresolved etiologies. Identification of mutational signatures attributable to vinyl chloride exposure is imperative for understanding carcinogenesis and advancing environmental health risk assessments related to this exposure. We hypothesized that VC-induced rat tumors harbor distinct mutational signatures relative to spontaneous tumors. **Methods:** We performed whole-exome sequencing (WES) of tumor-normal pairs (n=88) from archival (~50-year) ethanol-fixed paraffin-embedded Sprague Dawley rat tumors (hepatocellular carcinoma, hemangiosarcoma, mammary adenocarcinoma, neuroblastoma) resulting from chronic (2 years) exposure to vinyl chloride or from unexposed chamber controls, i.e. tumors arising spontaneously. Exome enriched libraries were generated using the Agilent SureSelect System and sequenced on Illumina NovaSeq. Data analysis followed a modified GATK pipeline (FastQC, BWA-MEM) with variant calling by Mutect2. Samples with <70% target coverage at 30X were excluded, leaving 32 tumors for analysis. SigProfilerExtractor v1.2.0 (rn6) was used to extract *de novo* SBS signatures. Spontaneous (n=8) and VC-exposed (n=24) cohorts were analyzed separately to assess group-specific patterns. **Results:** Signature extraction in the VC-exposed cohort of tumors identified SBS40a and SBS22a. SBS40a occurred in 11 out of 24 VC-exposed tumors, driven primarily by hemangiosarcomas (8 out of 14) with per-sample contributions >35% of total mutations in all positive cases. SBS22a occurred in 7 out of 24 VC tumors, and were distributed between tumor types (3 hemangiosarcomas, 2 mammary adenocarcinomas, 1 hepatocellular carcinoma, and 1 neuroblastoma) with per-sample contributions 10-16% of total mutations. In the spontaneous only extraction, neither signature was detected. When both cohorts were combined in one signature extraction, SBS40a was also assigned to 5 out of 8 spontaneous tumors and SBS22a to 1 spontaneous tumor. **Conclusions:** These findings suggest SBS40a as a possible signature correlated with VC exposure based on its frequency and magnitude in the VC-exposed tumor cohort, particularly in hemangiosarcomas, and its absence in the spontaneous only cohort. Because *de novo* signature extraction can be sensitive to group effects in small WES datasets, the SBS40a assignment to spontaneous tumors in the combined run is regarded as tentative pending confirmation. Ongoing work includes cross tool extraction with an orthogonal method to confirm results, per-sample refitting, and subgroup analyses by exposure status and tumor type to assess robustness. If validated, an SBS40a association could provide a genomic indicator of VC exposure with implications for toxicological mechanism and use in environmental risk assessment.

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Background and Purpose: Topoisomerase II (TOP2) targeting drugs are widely used in chemotherapy. Most TOP2 targeting drugs are TOP2 poisons that stabilize TOP2 on DNA, leading to a toxic intermediate known as TOP2-DNA cleavage complex (TOP2cc). Bisdioxopiperazine derivatives, such as ICRF193, act as catalytic inhibitors of TOP2 without inducing TOP2cc. Although ICRF193 has been extensively used as a research tool, it has not been approved for clinical use due to the lack of antineoplastic effects itself. Its unique catalytic inhibition profile suggests that it may serve as a potentiator or modulator when combined with other drugs. The goal of this study was to discover novel drug combinations that exploit catalytic inhibition of TOP2 for improved anticancer efficacy. **Methods:** A quantitative high-throughput screening assay was optimized for the combination of ICRF193 and a total of 2,678 compounds from the NCATS Pharmaceutical Collection (the NPC library). The NPC library was screened for cytotoxicity in the presence or absence of ICRF193 in human colon cancer cell lines (HCT116). Compounds showing a left-shifted concentration curve in the presence of ICRF193, indicative of potentiation, were selected for follow-up studies. Follow-up experiments included: (1) Cell viability assays to validate synergistic cytotoxicity, (2) DNA synthesis assays using 5-ethynyl-2'-deoxyuridine (EdU) incorporation to measure DNA replication inhibition, (3) γH2AX immunofluorescence to quantify DNA double-strand breaks, and (4) Flow cytometry for G₂/M cell-cycle arrest analysis. To assess if the compounds with similar structure have similar effect, the same experiments were performed with the clinically approved bisdioxopiperazine derivative, ICRF183, and outcomes were compared to those of ICRF193. **Results:** The primary screening identified four compounds, including etoposide, trametinib, mebendazole, and oxfendazole, that demonstrated increased cytotoxicity in the presence of ICRF193. Of these, only etoposide consistently showed strong synergistic effects with ICRF193 across all tested cell lines. In HCT116 cells, the IC₅₀ for etoposide was potentiated from 1.05 μM to 278 nM with 200 nM ICRF193. Similar shifts were observed in breast cancer cell lines, MCF7 and T47D. Although ICRF193 was expected to reduce the toxicity of TOP2 poisons, our results indicated the opposite effect at low concentrations. Further tests with higher concentrations of ICRF193 demonstrated suppression of etoposide toxicity, indicating a dual effect: enhancement of etoposide-induced toxicity at low concentrations (200 nM) and suppression at high concentrations (10 μM). Etoposide co-treatment with ICRF193 significantly enhanced γH2AX foci formation and G₂-phase accumulation, indicating increased DNA damage and checkpoint activation. Consistent with these results, EdU incorporation assay confirmed the potentiation for DNA synthesis inhibition by 200 nM ICRF193. Importantly, the similar potentiation was not observed with other TOP2 poisons, including teniposide, daunorubicin, idarubicin, or aclarubicin, suggesting a unique functional interaction between etoposide and ICRF193. We also found that ICRF183 enhanced etoposide-induced cytotoxicity. **Conclusions:** Through quantitative high-throughput combination screening, we identified a specific and reproducible synergistic interaction between the catalytic TOP2 inhibitors (ICRF193 and ICRF183) and the TOP2 poison (etoposide). At a few hundred nanomolar, these catalytic inhibitors significantly potentiate etoposide-induced cytotoxicity and genotoxicity across multiple cancer cell lines, leading to enhanced DNA damage signaling and G₂-phase arrest.

PS 3055 Sinonasal Cytokine Patterns as Biomarkers of Gulf War Exposures: A VAGLAHS veteran population study

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Background and Purpose: Gulf War Illness (GWI) is a chronic multisystem disease affecting one third of Veterans from the Persian Gulf War (GWV) (1990-1991). Symptoms of chronic rhinosinusitis (CRS), including nasal congestion and fatigue, are the 1st (47%) and 3rd (41%) most common complaints associated with GWI. This study examines cytokine patterns in sinus secretions of Veterans with and without Gulf War exposures and GWI. **Methods:** We analyzed sinonasal secretions from 48 GWVs (25 with GWI, 23 without GWI) compared to those of 60 non-GWVs (23 with CRS, 37 without CRS) from the Greater Los Angeles VA. A Luminex 38-plex quantified cytokine and chemokine. Kruskal Wallis testing was used and were verified with multivariable logistic regression. **Results:** Sinus secretions of GWV with GWI had elevated levels of EGF, G-CSF, IL-8, IL-10, and TNF-beta (p<0.05) compared to GWV without GWI. A subgroup of GWI with nasal polyps (n = 7) demonstrated significantly elevated levels of EGF, IL-4, IL-5, MIP-1a, and MIP-1b compared to those with GWI without nasal polyps. Among veterans with CRS, GWI patients had elevations in G-CSF, IL-8, IL-10, IL-22 and IP-10 compared to non-GWVs with CRS (p<0.05). Among patients without CRS, GWV had elevated EGF, IL-1RA, IL-6, IL-8, IP-10, MCP1, and M-CSF (p<0.05) compared to non-GWV, suggesting that Gulf War-related exposures

influence sinonasal immune activity independent of CRS diagnosis. **Conclusions:** This study demonstrates that GWV with and without GWI have distinct cytokine profiles in their sinonasal secretions that may not only assist with detection of GWI but also differentiate between exposure-related sinonasal inflammation from conventional CRS.

PS 3056 Pathology of PD-L1 blocking therapeutics in three immune-humanized mouse models

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Background and Purpose: PD-L1 is a receptor commonly found on myeloid antigen presenting cells. PD-L1 binds PD-1, found on T cells. Such therapeutics are checkpoint inhibitors (CI) drugs and are used to treat cancer. To determine if immune-humanized mice could show immune related adverse effects (irAEs), we tested two different anti-PD-L1 drugs, atezolizumab and avelumab, in three different humanized mouse models. Avelumab is an IgG1 monoclonal antibody (mAb) with a functional Fc receptor, while atezolizumab has a non-functional IgG1 Fc receptor. This study evaluates a range of doses for anti-PD-L1 drugs to assess irAEs in immune-humanized mice. **Methods:** Immune-humanized mice were made using either NOG-hIL6 or NBSGW mouse strains. Using previously described techniques, both mouse strains received one of the following tissue implants or injections: 1) thymic fetal tissue and CD34+ hematopoietic stem cells (HSC), 2) neonatal thymus tissue and CD34+ HSC (Neo-Thy), or 3) CD34+ HSC only. Following humanization, mice were randomized to receive either atezolizumab, avelumab or saline control. Mice were treated once weekly IP with either saline or low, mid or high dose of the therapeutic. Treatment for avelumab and atezolizumab was 3, 15 or 30mg/kg, and 4, 20 or 40mg/kg, respectively. Flow cytometry was performed on days 0, 14 and 21 to assess humanization progression. Necropsies were performed on day 28, with PBMC, 2/3 spleen and bone marrow collected for flow cytometry processing. Tissues collected for pathology included heart, lungs, kidneys, adrenal gland, liver, gastrointestinal tract, mesenteric lymph nodes, reproductive tract, 1/3 of spleen, brain, femur, muscle and skin. Femur and head were decalcified for 24 hours, and all were fixed with 10% formalin and processed into slides. **Results:** Both atezolizumab and avelumab treated mice showed differences in the breadth and location of inflammatory lesions based on the method of humanization. For CD34 mice, there were no findings for atezolizumab in any tissues, whereas avelumab treated mice had mild to severe granulomatous (Gran) inflammation in the spleen, with 50% having either lymphohistiocytic (L/H) or Gran. inflammation in mesenteric lymph node or bone marrow. Mice produced with fetal thymic tissue had L/H and/or Gran. inflammation in small and large intestine, spleen and lung, with some pancreatic atrophy in atezolizumab treated; and in adrenal glands, lung, spleen, and bone marrow for avelumab treated. Neo-Thy mice also had L/H and/or Gran. inflammation, including in lungs, spleen, liver, mesenteric lymph node, pancreas, and bone marrow; which was similar in distribution for both therapeutics. **Conclusions:** Immune-humanized mice, particularly those produced with human thymic tissue, could demonstrate irAEs when treated with either anti-PD-L1 therapeutic. CD34 immune humanized mice showed no irAEs when treated with atezolizumab, and those treated with avelumab only had lesions in immune organs, suggesting they are not an appropriate model for this therapeutic. While Neo-Thy mice had a wider range of organs affected as compared to fetal-tissue produced mice, this may represent a broader immune humanization of all organs than appeared to occur with fetal-tissue produced mice. Neo-Thy mice also showed inflammation in liver, pancreas, and lung; which is consistent with some severe adverse events experienced by patients. This study suggests that Neo-Thy immune humanized mice may be an appropriate model for testing a broad range of checkpoint inhibitor therapeutics. Disclaimer: This abstract reflects the views of the author and should not be construed to represent FDA's views or policies.

PS How to use Retention Index information (NIST) to support Mass Spectral based identifications in GC/MS.

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Background and Purpose: Organic leachables surfaced by GC/MS are often identified by mass spectral matching (MSM) in which the experimental mass spectrum is matched to reference spectra contained in a spectral library. The compound is "identified" as being the compound whose reference mass spectrum best matches the experimental spectrum, which often leads to flawed identifications. A compound's Retention Index (RI) is an independent means of corroborating proposed MSM identities. A compound's analytical RI can be matched to reference RIs contained in a RI database (NIST23). The identity secured by MSM is corroborated when the experimental RI acceptably matches the reference RI of the compound proposed by MSM. Practical use of RI matching requires that acceptance criteria be established to define when an acceptable RI match has been achieved. The use of the absolute value of the difference between RI experimental and RI reference (termed $|\Delta RI|_{obs}$) is proposed as the proper characteristic for defining the goodness of an RI match. Depending on its value, $|\Delta RI|_{obs}$ can be used to corroborate (support), reject or accept the proposed identity. Acceptance criteria for supporting, rejecting, or accepting proposed identities were established using experimental values for RI secured

by Nelson Labs and reference RI values found in the commercially available NIST23 library. **Methods:** A Confusion Matrix was constructed using the available data, intending to minimize both false positive and false negative outcomes, while taking into account that false positives should have a higher weight because they have a greater impact on the outcome of a toxicological risk assessment. A full description can be found in the scientific article that was recently accepted by the PDA Journal of Science and Technology **"Use of the Retention Index to Secure Correct Identities in GC/MS"**. **Authors :** Piet Christiaens, Dennis Jenke, Jan Baeten, Philippe Verlinde, and Jean-Marie Beusen (2025) **Results:** Use of the Confusion Matrix resulted in the following outcomes: • The supporting limit is the value for $|\Delta RI|_{obs}$ below which there is a very high confidence in the proposed identity obtained via MSM. A $|\Delta RI|_{obs}$ value below the supporting limit corroborates (supports) the identity proposed by MSM. The value for the supporting limit is set at $|\Delta RI|_{obs} \leq 20$. • The rejection limit is the value for $|\Delta RI|_{obs}$ above which there is a very low confidence in the proposed identity obtained via MSM. A $|\Delta RI|_{obs}$ above the rejection limit refutes (rejects) the identity proposed by MSM. The value for the rejection limit is set at $|\Delta RI|_{obs} > 50$. $|\Delta RI|_{obs}$ values between the supporting and rejection limits neither corroborates nor refutes the proposed identity and the use of $|\Delta RI|_{obs}$ is inconclusive. This presentation reflects the content of a scientific article, written by Nelson Labs, which was submitted to the PDA Journal **Conclusions:** Chromatographic information, such as retention index, can be a very important and useful tool in the identification process and will enhance the quality of a subsequent toxicological assessment

PS 3058 Exhaustive Extractions are Exhausting: Justification of Changes in Medical Device Manufacturing through Materials Science and Toxicology

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Background and Purpose: Any manufacturing site, material, supplier, sterilization or processing change in medical device manufacturing necessitates a re-evaluation of biological safety and demonstration of biological equivalence. Biological equivalence, in turn, requires demonstration of endpoint and material equivalence. Given the challenges of generating reproducible extractables profiles, combined with the burdensome test set-up, the use of exhaustive chemical characterization to provide evidence of material equivalence can be challenging. **Methods:** Biological risk assessments for various devices with different natures and durations of contact were reviewed with a focus on those that used analytical approaches outside of exhaustive chemical characterization to demonstrate material equivalence. **Results:** Manufacturing site, supplier, and sterilization changes were justified through non-traditional methods to demonstrate material equivalence and, when combined with endpoint testing results, biological equivalence. **Conclusions:** Materials science-driven approaches utilizing non-exhaustive extraction methods were used to effectively justify changes to medical devices. A key factor in the success of these justifications was early and strategic involvement of biocompatibility experts, materials science subject matter experts, and toxicologists to assess whether the non-traditional extraction methods could still adequately support the biological and/or toxicological risk assessment.

PS 3059 Can Material Evaluations Accurately Predict Irritation Outcomes for Medical Devices?

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Background and Purpose: When preparing biological evaluation plans and evaluating the biocompatibility of medical devices, the ISO standards emphasize identifying biological hazards and harms to estimate patient risk. Irritation is a biological effect to be evaluated for all medical devices that come in direct or indirect contact to patients. We wanted to understand if only evaluating device materials of construction is sufficient to predict irritation responses. Given the diversity of materials and processes used to make medical devices, we hypothesized that a material evaluation alone would not be able to predict *in vivo* irritation failures. **Methods:** Thousands of biocompatibility tests have been conducted at NAMS for medical device manufacturers resulting in an extensive database of reports. The *in vivo* intracutaneous irritation test is the most common assay used to test for irritation potential. A Python script that targeted intracutaneous irritation reports was developed to mine the NAMS database. The script was used to automate an unbiased process of extracting the results from studies completed from 2019-2024. Reports with failing intracutaneous irritation results (score of >1.0) were manually reviewed and the device's materials of construction were tabulated (typically, only high-level material of construction information is reported). Each disclosed material was reviewed to determine if the materials are known to cause irritation, or tend to fail this study, were present (e.g., adhesives). **Results:** A total of 3,801 intracutaneous irritation reports were identified. Of those, there were a combined 55 failures from the polar ($n=23$) and non-polar ($n=32$) extracts (1.47% failure rate). Based on our analysis of the materials of construction: 29 (53%) failures could have been predicted. 6 of these devices had contact with intact skin only. 14 devices were used for wound care and typically included adhesive materials which have intact skin contact only. 10 (18%) failures may have been predicted. No obvious trends were identified amongst the devices. However, some aspect of each device was identified that could explain the

result (e.g., presence of an adhesive material, device was absorbable, physical form of the device). It is unclear if the result would have been predicted before performing the study. Noteworthy, most irritation scores were <1.9 , indicating a slight irritation response. 16 (29%) failures would not have been predicted. Based on the compositional information reported, no materials had an obvious irritant hazard. **Conclusions:** We performed a retrospective analysis to determine if material evaluations alone can predict failing results for the intracutaneous irritation study. Based on the evaluated dataset, irritation failures are not common; without performing any formal material assessment, the failure rate was 1.47%. Over half (53%) with failing results could have been predicted based on a materials assessment; in many instances, the intracutaneous irritation method was deemed inappropriate based on the type of patient contact (intact skin) with the suspect material. Additionally, 18% might have been predicted based on some aspect of the device, as described above. Lastly, 29% of the devices with failing results would not have been predicted. Considering the results of our study, we rejected our hypothesis. Although not perfect, material evaluations alone can predict failing results for the intracutaneous irritation study. It is anticipated that when material evaluations are combined with evaluations of the manufacturing processes (e.g., chemicals), predictions will improve. When planning medical device testing, consideration to all device components and its intended patient contact is important to ensure appropriate testing methods are selected.

PS 3060 A comparative regulatory analysis of substance-based medical device risk classification in ASEAN and EU

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Background and Purpose: The ASEAN Medical Device Directive (AMDD), largely modeled after the EU's former Medical Device Directive (MDD) to harmonize regional requirements, has diverged from the EU framework following the introduction of the more stringent EU Medical Device Regulation (MDR). The precise impact of this regulatory divergence on the risk classification of substance-based medical devices (SBMDs) has not yet been systematically analyzed. **Methods:** We developed decision trees as analytical tools to visually map and compare the risk classification pathways for SBMDs under both the ASEAN and EU regulatory frameworks. Case studies on eye drops, vaginal lubricants, and alginate-based reflux products were conducted to identify points of divergence in the risk classification of representative SBMDs. **Results:** The risk classification outcomes of the case studies showed that the EU MDR assigned a higher risk class (Ib, moderate-high risk) to all representative SBMDs compared with their corresponding classifications under the AMDD (Class B, low-moderate risk). **Conclusions:** Our analysis showed that Rule 21 of the EU MDR, which governs SBMDs, results in higher risk classifications for the representative products examined. In contrast, the AMDD currently lacks a rule specific to SBMDs. This divergence may affect clinical evidence and technical requirements and delay global market access due to differing risk classifications of SBMDs. Continued collaboration between ASEAN and the EU on classification criteria is essential to reduce regulatory burdens arising from these differences.

PS 3061 Scientific Confidence in the Monocyte Activation Test: A Human-Relevant Pyrogenicity Test for Medical Devices

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Background and Purpose: Pyrogenicity is an important safety consideration in the evaluation of medical devices. This response is mediated by immune recognition of foreign substances, leading to cytokine release and hypothalamic modulation of body temperature. Pyrogens are broadly classified as endotoxins, which are primarily lipopolysaccharides (LPS) from gram-negative bacteria, and non-endotoxin pyrogens (NEPs), which include a diverse array of biological and chemical agents. Historically, the Rabbit Pyrogen Test (RPT) has been the standard assay for detecting pyrogens; however, regulatory agencies are encouraging transition to the use of more human-relevant and quantitative methods. The Monocyte Activation Test (MAT) is a quantitative *in vitro* assay using human peripheral blood mononuclear cells (PBMCs) that offers a reliable and mechanistically relevant approach. As such, there is interest in conducting case studies to further demonstrate that the MAT is capable of detecting both endotoxin and NEPs in medical devices. Additionally, there is a growing scientific consensus that so-called material mediated pyrogens (MMPs; listed in Annex G of ISO 10993-11:2017) are not relevant to the pyrogenicity testing of medical devices, which our data supports. **Methods:** Following discussion at a 2018 workshop, we evaluated six sets of dental bone screws ("spider screws") to assess the MAT's ability to detect both endotoxins and NEPs on device surfaces within this narrow context of use. These samples were spiked with known pyrogens (LPS, HKSA, R848) or MMPs. Devices were tested with and without dry-heat depyrogenation, multiple spike controls, and dilution series (LPS/HKSA/R848 dose-responses) to assess spike recovery and to distinguish microbiological contamination from material-mediated effects. Additionally, seven MMPs listed in ISO 10993-11:2017 Annex G were tested using the MAT to determine their potential to elicit a pyrogenic response. Testing was conducted by MAT BioTech using pooled PBMCs from four

donors. Cytokine release was quantified via enzyme-linked immunosorbent assay (ELISA), using an IL-6 standard to generate a four-parameter logistic curve. After data analysis, the scientific confidence framework outlined in the 2024 Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) report, *Validation, Qualification, and Regulatory Acceptance of New Approach Methodologies*, was used to evaluate the MAT for detecting surface pyrogens on medical devices. **Results:** The results demonstrated that of the six spider screw batches tested, five were found to be free of contamination, while batch 2 showed pyrogenic activity due to a microbiological source, which was successfully eliminated through dry heat treatment. Most batches showed acceptable spike recovery ranges after increasing PBMC incubation volume, though batch 1 and batch 3 exhibited outlier results that require further investigation. Overall, the testing confirmed that the MAT is effective for detecting surface pyrogens on dental bone screws. Additionally, none of the seven MMPs listed in ISO 10993-11:2017 Annex G elicited a pyrogenic response in the MAT. These findings, along with using the scientific confidence framework outlined in the 2024 ICCVAM report, support the MAT's suitability within this context of use. The test exhibits biological relevance to humans and strong technical characterization. Moreover, it has undergone extensive independent review, establishing the MAT as a fit-for-purpose new approach methodology (NAM) for surface pyrogenicity testing of medical devices. **Conclusions:** Based on the study findings, the MAT demonstrates strong suitability for detecting both endotoxins and non-endotoxin pyrogens on medical device surfaces, and these results should be considered for regulatory acceptance in place of the RPT. The absence of pyrogenic response from the seven tested MMPs further supports the growing consensus that these substances are not relevant to pyrogenicity testing. Evaluated through the 2024 ICCVAM scientific confidence framework, the MAT proves to be a biologically relevant, reproducible, and valid approach. These results reinforce the MAT's role as a fit-for-purpose NAM for ensuring the safety of medical devices and support a shift toward more ethical, accurate, and mechanistically sound approaches in medical device pyrogenicity testing.

PS 3062 Case Based Insights When Assessing Carcinogenicity Risk in Biological Safety Evaluation

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Background and Purpose: ISO 10993-17 provides a framework for evaluating the risk of chemicals released from medical devices, including specific guidance for assessing carcinogens, both known and suspected. The US NTP, IARC, and ECHA published lists of carcinogens. However, many of listed chemicals are based on exposure scenarios irrelevant to devices. This creates challenges in characterizing cancer risk under clinical use condition. To illustrate the complexities of TRA for carcinogens in devices, here are case studies evaluating some device related chemicals identified as carcinogenic by those organizations to demonstrate a fit for purpose approach to refine TI with the context of clinical relevance. **Methods:** Published data on chemicals associated with materials and manufacturing were reviewed against 3 major carcinogens databases, US NTP Report on Carcinogens, IARC Monographs, and EU CMR substances. Five common chemicals in devices were selected: Co in stainless steel alloys; Ni in Nitinol; DEHP, a plasticizer in PVC; tetrahydrofuran (THF), a solvent & polymer precursor; benzophenone, a photosensitizer & plastic stabilizer. A comprehensive literature review was conducted to assess the carcinogenicity risk of these chemicals, focusing on the applicability, reliability, and quality of publicly available data and using key criteria: exposure route, cancer site/type, mode of action and genotoxicity evidence to establish TI values for the carcinogenicity endpoint. **Results:** These chemicals were classified carcinogenic potential by US NTP, IARC, and EU CMR: Ni as a known human carcinogen by NTP, Group 1 by IARC, & Category 1A under CMR; Co as reasonably anticipated to be a human carcinogen (RAHC) by NTP, Group 2A by IARC, & Category 1B under CMR; DEHP as RAHC by NTP, Group 2B by IARC, but not classified under CMR; THF & benzophenone as Group 2B by IARC, not listed by NTP; benzophenone as Category 1B, while THF not under CMR. Both Ni and Co are endogenous elements and trace nutrients consumed daily through diet. Ni intake from food is estimated at 100-300 µg/day for adults. It functions as a cofactor in metalloenzymes. Co also plays a vital biological role with median blood conc. around 0.39 µg/L and recommended daily intake up to 0.03 mg/kg bw. Carcinogenicity associated with Ni and Co is primarily linked to inhalation of their insoluble salts, not oral exposure to soluble ones. Lifetime oral intake of soluble Ni salts in rodents showed no increased cancer incidence at doses up to 0.75 mg/kg bw/d. Similarly, Co carcinogenicity was observed only in inhalation studies, with increased lung tumors in rodents exposed to Co powder or sulfate salts. Given the route specific nature of these findings and the lack of evidence for carcinogenicity via systemic exposure, Ni and Co are unlikely to be carcinogenic in cardiovascular devices. DEHP, THF, and benzophenone are not classified as known human carcinogens. Available evidence suggests that their carcinogenic effects in animals occur through non-genotoxic mechanism. For all three chemicals, cancer TI values could not be established using the slope factor or TD₅₀ approaches outlined in ISO 10993-17 due to limited data. Alternative scientifically justified approaches were applied to reflect their non-genotoxic carcinogen mechanism with dose- and duration-dependent effects. For DEHP, a TI of 2.02 µg/kg bw/d was derived using the benchmark dose lower confidence limit for a 10% response (BMDL₁₀) based on hepatocarcinogenesis data in rats. A TI of 71.65 µg/kg bw/d for THF was established from the POD using the lowest NOAEL identified in 2-year inhalation carcinogenicity

studies from rodents and a TI of 5 µg/kg bw/d for benzophenone was derived from the chronic tolerable daily intake established by EFSA. **Conclusions:** These case studies highlight the importance of TRA using a scientifically robust, context specific framework. Variability in carcinogenic classifications across regulatory databases along with differences in exposure routes and mechanisms of action emphasize the application of specific risk assessment. Extractables that are either endogenous or act via non-genotoxic mechanisms challenge the applicability of traditional cancer risk methods (slope factor or TD₅₀). Justifiable approaches offer a more appropriate basis for evaluating cancer risk. It is critical to conduct TRA integrating key factors, including exposure route, bioavailability, mechanistic relevance, and clinical context.

PS 3063 The Influence of Extractable and Leachable (E&L) Study Design on Biocompatibility Outcomes of Skin Patch Devices used in Ambulatory ECG Monitoring

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Background and Purpose: This presentation examines how the design of extractable and leachable (E&L) studies impacts chemical characterization and biocompatibility of a wearable skin patch ECG device (SPD). The biocompatibility evaluation plan (BEP) ensures SPD safety prior to clinical studies and market release. As a single-use, skin-contacting device with an ECG sensor, SPD enables noninvasive heart monitoring under clinical supervision in home and healthcare settings. Our risk assessment shows SPD's skin-contacting components present low biocompatibility risk for use up to 30 days. **Methods:** Following ISO 10993-1, we performed physical and chemical characterization of SPD materials, leveraging supplier data and tests on the finished product. SPD is designed for prolonged (24 hours to under 30 days) intact skin contact, not for mucosal or compromised surfaces. Key biological endpoints included cytotoxicity, skin irritation, and skin sensitization per ISO 10993-1:2025 were conducted alongside a material assessment of patient-contacting components. The E&L study design is in accordance with the ISO 10993-18 to simulate skin contact through saline solution extraction of patient contacting components. Two temperatures were chosen: 37±1°C to match typical skin conditions, and 50 ±1 °C to represent an exaggerated scenario. The extraction periods included 3 days (72 hours), 14 days (336 hours), and 30 days. The 72-hour interval complies with ISO 10993-12 standards, while the 14-day period mirrors actual patient wear time. Extended time points were selected to better understand the chemical profiles of extractables during prolonged use. The ratio of surface area to saline solution varied according to the material's structure and the thickness of its layers. Compound identification was done via Gas Chromatography - Mass Spectroscopy (GC-MS). Extraction took place at 37°C for 24 hours, with one SPD used for every 10 mL of 0.9% saline solution. **Results: Chemical Characterization** No compounds above detection limits were found in saline extracts, except in one SPD's 336-hour extract at 37°C, containing cosmetic-related compounds from manufacturing. ICP-MS showed approximately 5 µg silver per SPD migrated every 24 hours from conductive tracks. **Biological Testing** Initial cytotoxicity and irritation responses were observed under ISO 10993, with no skin sensitization detected. Additional cytotoxicity tests on the patient contacting parts, Transfer Adhesive, Hydrogel, and Non-woven components, clarified causes of test failures. The Hydrogel's properties likely caused pH and osmolality shifts, and the potential silver migration from non-patient contacting part, contributing to the cytotoxicity results. Additional primary skin irritation patch test, more clinically relevant for SPD, showed passing results. Hydrogel's established safety, relevant testing outcomes, and SPD's intended intact skin usage together support SPD's low biological risk and biocompatibility. **Conclusions:** Understanding the composition of medical device materials and their potential leachable compounds, alongside biological safety testing results, offers predictive insight into possible toxicological risks to patients. Our material characterization and safety assessments demonstrate that SPD's skin-contacting components pose minimal risk over a 30-day period, supporting their safe application in ambulatory ECG monitoring.

PS 3064 A Proposed Framework for the Useful Life Evaluation of Breathing Gas Pathway Medical Devices: An Integral Component of Whole Lifecycle Assessments for Biocompatibility

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Background and Purpose: This poster presents a comprehensive yet streamlined study design framework for the useful life evaluation of breathing gas pathway devices intended for healthcare applications. The proposed framework provides for a consistent, risk-based approach to aging evaluations across diverse medical device types and use environments. These life-cycle evaluations span the entire service life of the device, from initial design through clinical therapy delivery, to ensure continued safety, performance, and regulatory compliance. Breathing gas pathway devices are typically complex and integrated with medical electrical equipment. Examples of these therapeutic systems include positive airway pressure (PAP) devices, ventilators, respiratory drug delivery devices, oxygen concentrators, and associated accessories (e.g., tubing, filters, and patient interfaces like masks). **Methods:** In accordance with ISO 10993 and ISO 18562 guidance, these medical devices can be evaluated for biocompatibility and safety throughout their entire life cycles. This may include

simulated real-time and/or accelerated aging studies designed to replicate real-world use conditions in both home and clinical environments (per ASTM F1980-21, IEC 60601-1). This proposed framework incorporates environmental exposure conditions, transportation and storage variables, and reprocessing cleaning and disinfection methods employed throughout the device's operational life. **Results:** A proposed structured framework for evaluating the whole life cycle of breathing gas pathway devices is presented, incorporating considerations for patient interface, electro-mechanical components, and device functionality. This framework considers the functional lifespan of critical to safety (CtS) components and materials, particularly those with potential toxicological risks, potential for material degradation, or patient exposure pathways, per ISO 10993 and ISO 18562 standards. **Conclusions:** Before market release, breathing gas pathway medical devices should undergo biological risk assessment, which can include aging studies. These aging studies should demonstrate acceptable results for biocompatibility, therapeutic performance, and material stability, all of which should pose negligible patient risk. These studies and datasets help meet global regulatory expectations and demonstrate conformance with international standards governing breathing gas pathway medical devices.

PS 3065 Chemical Class Emission Trends of Volatile Organic Substances Emitted from Breathing Gas Pathway Devices under ISO 18562-3:2024

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Background and Purpose: Volatile Organic Substances (VOS) released from breathing gas pathway devices constitute potential inhalation risk depending on the chemical hazard properties of the gas, emission magnitude and persistence over time. VOS emission can be evaluated under the ISO 18562-1:2024 and ISO 18562-3:2024 framework to assess potential patient exposure and establish tolerable exposure limits. The VOS emission decay curve proposed under ISO 18562-3 guidance demonstrates a typical decay curve graph, showing in theory that VOS emission from breathing gas pathway devices decreases over time. While this is widely supported by research on indoor and outdoor air pollution monitoring, no specific data on emission trends have been published on breathing gas pathway devices. An in-depth understanding of gas pathway medical device emission profiles and chemical class emission trends can assist in material selection, thereby ensuring confidence in patient safety throughout the device's lifespan. The purpose of this study is to characterize emission profiles of VOS across different chemical classes from multiple gas pathway components and determine if their emission trends are validated against the expectation of typical decay curve per the standard. **Methods:** ISO 18562-3 VOS emission tests were conducted by a third-party lab on multiple breathing gas pathway device components, constructed primarily of polycarbonate and silicones. Air was monitored continuously and sampled from the heated breathing gas stream across four discrete timepoints (0 hour, 24 hours, 72 hours, and 168 hours) under controlled conditions within the test chamber. Four representative chemical classes were selected for VOS emission trends across the multiple components tested. These classes were siloxanes (cyclic and linear), saturated hydrocarbons (aliphatic alkanes C9-C18), aliphatic aldehydes, and phenolic antioxidants. Class-summed concentrations across the four timepoints were modeled for each device and chemical class using nonlinear least-squares-fit to the first order exponential decay equation, given by: $C(t) = C_0 + A * e^{-kt}$. **Results:** The VOS emission concentrations across the four timepoints for each chemical class demonstrated a clear decay in emission, albeit with marked differences in decay profile. Modeling results indicate that Cyclic Siloxanes declined in a first-order (exponential) pattern over 168 hours. Linear siloxanes declined more rapidly than cyclic siloxanes over the same time period, yet exhibited an exponential profile during the early emission phase, consistent with first-order decay kinetics. Emissions of saturated hydrocarbons (aliphatic alkanes, C9-C18) and aliphatic alkanes declined more sharply than those of siloxanes, with a pronounced drop observed by the 24-hour timepoint. As a result, the emission pattern did not conform to first-order decay kinetics over the 168-hour period. Phenolic Antioxidants showed delayed release and gradual loss, indicating partial first-order decay after emission onset. **Conclusions:** This study demonstrates that decay profiles of volatile organic substances (VOS) emission can vary by chemical class. Furthermore, not all chemical classes exhibited first-order decay patterns over the 168-hour period, which may be partly attributed to the selection of sampling timepoints. Incorporating chemical class-specific decay patterns into toxicological risk assessments may enhance exposure estimations, inform material selection to minimize patient risk, and improve alignment with ISO 18562 framework. Future work could expand decay-based modeling across a broader range of compounds to strengthen medical device safety evaluations.

PS 3066 Determination of a medical device parenteral non-cancer TTC based on *in silico* ADME

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Background and Purpose: Threshold of toxicological concern (TTC) values are commonly used in toxicological risk assessment of medical device (MD) constituents when there is inadequate toxicity information. Builee et al. (2025) publicly made available oral non-cancer TTC values based on a curated database (Db) of MD constituents in an open-use format. The utility of the oral non-cancer TTC values can be further advanced by considering the absorption, distribution, metabolism, and elimination (ADME) properties of each constituent. Given that computer models are a valid alternative to experimental procedures, the ADMET Predictor® (AP) (from Simulations Plus, Inc) was leveraged to develop parenteral TTC values. AP is an artificial intelligence/machine learning (AI/ML) platform used in pharmaceutical development for the prediction of toxicokinetics and other endpoints. The purpose of this poster is to present a parenteral non-cancer MD TTC value for the >30 days exposure duration based on bioavailability adjustments. **Methods:** The MD Db of Builee et al. (2025) provided the source of chemical descriptors (chemical name, CAS No., Simplified Molecular-Input Line-Entry System [SMILES]) and duration-specific oral NOAELs. The chronic duration NOAELs representing 180-day to 2-year *in vivo* studies were used in this analysis. The HTPK module, a part of ADMET Predictor® 13 suite (Simulations Plus, Inc), calculated the intestinal absorption factor percentage based on SMILES entry covering the period of 24 hours after exposure. The species used in the AP model was matched, when possible, to the species in which the NOAEL was obtained. The computationally predicted intestinal absorption results were grouped into four categories: <1%, ≥1%-50%, 50%-90%, and ≥90%, with corresponding bioavailability factors (BAFs) of 100, 10, 2, and 1 (Masuda-Herrera et al., 2022). These BAFs were further used to adjust oral NOAELs into parenteral point of departures (pPOD). The lowest 5th percentile pPOD was evaluated using a log-normally fitted cumulative frequency distribution. A modifying factor of 100 (10 each for intra- and interspecies adjustment) was applied to the lowest 5th percentile pPOD to obtain the parenteral non-cancer TTC derivation. Statistical analysis and visualization were performed using standard Python scientific libraries, including NumPy (v1.26.4), SciPy (v1.14.1), and Matplotlib (v3.10.6). **Results:** The parenteral TTC value was determined from a set of 130 constituents of the MD Db. The correlation with AP by species match was approximately 95%, indicating high similarity between the toxicity study and ADME prediction species model. The distribution of predicted BAF scores was highest in low (≥1%-50%) to mid (≥50-90%) bioavailability groups. The cumulative distribution lowest 5th percentile pPOD was 0.64 mg/kg/day for the >30-day exposure duration. After applying uncertainty factors for interspecies extrapolation (10) and intraspecies variability (10), the resulting pTTC was 6.4 µg/kg/day. **Conclusions:** A non-cancer MD TTC value for the parenteral route of exposure was derived from *in silico* predictions for intestinal absorption and assigned bioavailability adjustment factors. The parenteral TTC value of 6.4 µg/kg/day obtained for the exposure duration >30 days is 6.4 times lower than the oral non-cancer TTC value derived by Builee et al. (2025) for the same exposure duration (i.e., 41 µg/kg/day). The derived pTTC replaces the default 10-fold route-to-route uncertainty factor recommended in ISO 10993-17:2023 with a more precise, data-driven adjusted value. This work constitutes an advancement in the use of non-cancer MD TTC values for the toxicological risk assessment of MD constituents for exposure scenarios other than the oral route.

PS 3067 Identification of Chemicals of Endocrine and Reproductive Concern in Medical Device Extractables and Leachables Datasets

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Background and Purpose: Medical devices are composed of a wide range of materials that have the potential to release chemical constituents during patient use. Although the biocompatibility evaluation framework outlined in ISO 10993 provides guidance for assessing various toxicological risks, there is currently no requirement by the U.S. Food and Drug Administration (US FDA) or ISO 10993-1 to specifically evaluate medical device extractables and leachables for potential endocrine-disrupting compounds (EDCs). While reproductive toxicants are evaluated in accordance with 10993-17, not every device manufacturer is required to conduct ISO 10993-17 assessments. Evaluation of reproductive risk in sensitive populations like individuals who are pregnant or may become pregnant are typically limited to reproductive devices. In contrast, the European Union's Medical Device Regulation (MDR) mandates consideration of substances with endocrine or reproductive toxicity potential when present above defined thresholds. Additionally, for pharmaceuticals, ICH S5 (R3) requires the assessment of developmental and reproductive toxicity regardless of the duration of exposure for small molecule pharmaceuticals, highlighting the recognized importance of evaluating these endpoints even for short-term patient use. As awareness grows regarding the potential for low-level chemical exposures to contribute to adverse endocrine or reproductive outcomes, there is an increasing need to characterize the presence and prevalence of such compounds in medical device extracts. By cross-referencing chemical constituents with endocrine disrupting properties or reproductive toxicity potential identified from regulatory lists

with robust, published lists of medical device extractables, we aimed to identify the frequency and distribution of these compounds in medical device materials in a 'real-world' setting. **Methods:** Compounds with endocrine-disrupting potential were identified using the European Chemicals Agency (ECHA) Substances of Very High Concern (SVHC) list, while reproductive toxicants were identified from the ECHA Annex VI Classification, Labelling, and Packaging (CLP) list. These substances were then screened against two published medical device extractables datasets: (1) extractables database reported by Builee *et al.* (2025), and (2) a compiled dataset of extractable chemicals submitted by 13 medical device manufacturers, consulting firms, and analytical laboratories at contract research organizations by Borton *et al.* (2025). **Results:** Screening of medical device extractables identified multiple compounds with known or suspected endocrine-disrupting and reproductive toxicity potential. Specifically, 16 unique EDCs and 102 unique reproductive toxicants were present across both datasets. A majority of the identified compounds were classified as reproductive toxicants 1B, and four compounds, 1,2-Dibromo-3-chloropropane, Carbon Monoxide, Ethylene glycol diethyl ether, and Warfarin, were identified as a potent Class 1A reproductive toxicants. Additional identified compounds included bisphenol A, B, and S and various per- and polyfluoroalkyl substance (PFAS) such as Perfluoroheptanoic acid (PFHpA) and Perfluorodecanoic acid (PFDA). **Conclusions:** Our results indicated that screening of chemical constituents from two representative medical device databases identified multiple compounds with potential endocrine-disrupting or reproductive toxicity concerns. While the clinical relevance of these findings is dependent on actual exposure levels, the presence of such compounds underscores the need for systematic consideration of reproductive and developmental toxicity endpoints during medical device biological and toxicological risk assessment. Current ISO 10993-1 and ISO 10993-17 risk assessment frameworks can address reproductive and developmental toxicity if these hazards are recognized; however, the absence of a requirement to proactively screen for these compounds creates a systematic gap when the device can be used in individuals that are pregnant or may become pregnant. These findings highlight the importance of incorporating reproductive and developmental toxicity considerations into medical device toxicological risk assessments as a point of departure (POD) especially for devices with up to 30 days of use.

PS 3068 A Systematic Approach to Evaluating the Biological Safety of Reusable Medical Devices: A Case Study on Vascular Surgical Tool

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Background and Purpose: Reusable medical devices pose biological safety risks that must be evaluated over their lifecycle, particularly considering repeated reprocessing cycles involving soiling, cleaning, and sterilization. ISO 10993-1:2018 Clause 4.8 states that: "For re-usable medical devices, biological safety shall be evaluated for the maximum number of validated processing cycles by the manufacturer." The Getinge Vascular Graft Tunneler Instrumentation (VGTI), constructed from surgical-grade stainless steel, is designed for repeated use when properly maintained shows no significant signs of wear. This study provides an insight into of applicable approaches for assessing biocompatibility of reusable surgical tools from the perspectives of material characteristics, biological testing, and chemical characterization. **Methods:** VGTI devices underwent 1X, 7X, and 100X validated reprocessing cycles to replicate simulated-use conditions prior to testing. Topography, morphology, and physicochemical Characterization were conducted in accordance with ISO/TS 10993-19 to identify surface features, defects, or contaminants that could influence the chemical composition in support of understanding the biocompatibility of the material. Chemical characterization of the 1x product was also performed under exaggerated extraction conditions, followed by a toxicological risk assessment to evaluate potential patient exposure to extractables during intended use. Biological tests were conducted in accordance with ISO 10993-1 testing standards. The biological safety risk of reusable VGTI was evaluated considering all available data and overall interpretation. **Results:** The applied analytical methods employed in this study demonstrated a systematic model for evaluating biological safety of reusable medical devices. The rationale for selecting reprocessing cycles and identifying the end-of-life point was provided. All sample preparations and testing processes met international standards or validated procedures in order for a satisfactory biological risk assessment. SEM and EDS analyses showed no impurities, particulates, or contaminants on the device surfaces. Toxicological risk assessment indicated negligible risks associated with the potential leachable levels expected from the intended use of the VGTI products. Total organic carbon levels showed no statistically significant difference between 7X and 100X reprocessed devices. All biological testing met the passing acceptance criteria and comparison across different reprocessing cycles was provided. Overall, this study establishes a structured framework for evaluating biological safety risks associated with reusable devices. **Conclusions:** This study demonstrates that comprehensive material, chemical, and toxicological evaluations can effectively assess the biological safety of reusable medical devices throughout their validated reprocessing lifecycle. Although this study is limited to one device type and certain reprocessing conditions, this framework can be extended to other reusable instruments leveraging a similar applicable approach. This integrated

analytical approach—combining surface characterization, biological testing, and extractables and leachables analysis—offers a robust strategy for demonstrating the biological safety of reusable surgical tools.

PS 3069 Importance of extraction vessel shape in medical device testing: a GeneTox case report

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Background and Purpose: The *In-vitro* Chromosomal Aberration Test (CAT) identifies substances called clastogens. Clastogens directly cause DNA breakage. The CAT assay plays an instrumental role in the assessment of genotoxic potential of a new medical device. Since medical devices are constructed using varying materials, their fluid extracts must first be prepared to dose, making extraction a critical factor for biological testing and risk assessment of a new device. Each device is extracted at specific temperatures / conditions as per International Organization for Standardization (ISO) guidelines. Per ISO, extraction vessels are specified to be clean, inert, closed with least dead space, but their shape is not considered. Here, we present a case report where the *shape* of the extraction vessel made a significant difference in the results (positive vs. negative) for Chromosomal Aberration Test (CAT) of a new medical device. **Methods:** Two Chromosomal Aberration Test - Confirmation Assays were conducted using Chinese Hamster Ovary (CHO) cells. The device was an implantable absorbable powder, with prolonged human contact. Extractions were performed in Ham's-F12 media at 37±1°C/72±2 hours, using 0.2g/mL ratio and extracted beyond its absorptive capacity. The vessel used for the first assay was a clean glass flat-bottomed jar and a sterile plastic conical centrifuge-tubes were used for the second assay. Post-extraction, the extracts were kept at ambient-room temperature for at least 8 hours and used within 24 hours. Cells were exposed (>20 hours) without metabolic activation and dosed at 100%, 50%, 25%, 12.5%, and 6.25% concentrations. A negative control (Hams-F12 media) and positive control (Mitomycin C/MMC, 0.5 µg/mL) were prepared parallelly. Two-hundred metaphases per condition were analyzed and Chi-square (χ^2) was performed to evaluate statistical significance ($p \leq 0.05$). **Results:** The device partially dissolved forming a biphasic extract. The biphasic extract had a diffused border with the flat-bottomed jar, whereas the conical flask extract had a well-defined border for dosing. Assay validity was confirmed with significant increase in aberrations noted in positive control compared to the negative control ($p=0.000$). The device extracted in the flat-bottomed jar induced a significant increase in aberrations versus the negative control ($p=0.000$, clastogenic), but the test article extracted in the conical centrifuge-tube did not ($p>0.411$, non-clastogenic). Notably, the clastogenicity observed with the flat-bottomed jar extract exceeded even the positive control (37% vs. 23% respectively). **Conclusions:** Under similar extraction conditions and solvent, our results show that the *shape* of the extraction vessel can produce a clastogenic (positive) versus a non-clastogenic (negative) response. This case report gives insights into whether the *shape* of the extraction vessel should be another factor to consider when performing extractions of absorbable powder-based medical devices which result in a biphasic extract. Further investigations of devices with similar characteristics should be carried out to evaluate the scope of our results.

PS 3070 Inorganic Gas and Particulate emissions from Medical devices evaluated under ISO 18562

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Background and Purpose: The ISO 18562 series of standards references inorganic gases, stating that some regulators may require evaluation of them. However, there is limited guidance on how and when to test, or otherwise assess these inorganic gasses. This lack of clarity can lead to inconsistencies in evaluation approaches and regulatory expectations. This work collates the most common sources of inorganic gases in the gas pathway of medical devices - electric motors and electronic sensors - and for each presents a high-level analysis of their risks and considerations from a toxicological perspective. The integration of electric motors and sensors into medical gas pathways introduces potential sources of inorganic emissions, including carbon monoxide (CO), carbon dioxide (CO₂), ozone (O₃), nitrogen oxides (NO_x), and also particulates. Understanding the mechanisms and triggers for these emissions is critical for toxicological risk assessment and ensuring device biocompatibility. This work is intended to provide regulators and manufacturers alike with an awareness of the engineering and science outside of, but intersecting with the field of toxicology, and how they affect the need and validity of such testing. This is to avoid both needless tests, as well as false test results. **Methods:** Inorganic gas and particulate emissions were systematically categorised using theoretical analysis and literature review. This categorisation was based on their generation mechanisms and operational triggers in common electric motor and sensor designs. Risk of inorganic gas and particulate emissions was evaluated based on component construction, operational dynamics, and material properties. For components with the potential to produce emissions, first-principles analysis was applied to derive considerations for medical device testing configurations. **Results:** Emission mechanisms were primarily associated with unintentional spark generation within electrical circuits, which can produce inorganic gases and particulates. Additional non-spark sources were

identified as high-temperature sensors and components that emit ultraviolet light, both of which can facilitate the formation of inorganic species. Importantly, brushed DC and universal motors as well as sensors that produce particulate emissions, may not have an inherent worst-case emission point, requiring characterisation of the entire life and all use cases, an assessment that extends beyond the start-of-life testing typically performed to comply with ISO 18562-2:2024. Brushed DC and universal motors, which use either a split-ring commutator or slip-rings, were found to present the highest emission risk due to spark formation and mechanical abrasion, both of which generate inorganic gases and particulates. The point of maximum emission varies between each implementation, and no single worst-case configuration can be universally defined. In contrast, brushless DC, induction, synchronous, stepper, and linear motors—all of which have passive rotors—by their innate design do not generate inorganic gases or particulates. Bearing selection also influences particulate generation. While all bearings facilitate relative motion, not all have the mechanical abrasion required to produce particulates. Bushings and seals both have directly contacting surfaces that rub, creating the mechanical abrasion that can lead to particulates. However, shielded and open ball bearings do not produce particulates. Gas sensors must interact with the gas to perform their function, but this rarely results in a toxicologically significant alteration of the gas stream. Pressure sensors, acoustic, and mass flow sensors, by their inherent design, do not produce inorganic gases or particulates. All other forms of gas sensors that were considered were found to theoretically produce trace quantities of inorganic gases. Although such emissions are expected to be below detection limits in most configurations, they still warrant empirical verification. **Conclusions:** Through appropriate selection of sensors and motor designs, the need to conduct testing for inorganic gases can often be entirely avoided. However, some sensor and motor designs can exhibit highly variable emission profiles, and testing without careful selection of parameters and device conditioning may underestimate patient risk.

PS 3071 Relevance on the Dose-Based Threshold of 120 µg/d for non-targeted analyses of non-absorbable implantable medical devices based on ISO 10993-17:2023 framework.

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Background and Purpose: ISO/TS 21726:2019 provides guidance for the application of the Threshold of Toxicological Concern (TTC) to assess biocompatibility of medical device constituents. The TTC is used to determine the Dose-Based Threshold (DBT) for the Analytical Evaluation Threshold (AET) prior the testing and to assess constituents lacking toxicological data during the Toxicological Risk Assessment (TRA). Selecting an appropriate DBT is critical to generate a conservative extractables profile for the subsequent TRA. Nevertheless, it is equally important to not select a DBT too stringent, since the non-targeted analysis may: 1) be associated with technical issues, such as obtaining an extract both compliant with the AET and compatible with the analytical instruments; 2) significantly increase the number of peaks to interpret and toxicologically evaluate, which leads to additional time and costs for the stakeholders, and 3) lead to an overly conservative assessment not relevant to assess the device's safety. In this poster, we demonstrate that the use of a DBT of 120 µg/d for non-absorbable implantable medical devices is relevant and sufficiently conservative, using the currently applicable regulatory framework.

Methods: A hypothetical non-absorbable implantable medical device (e.g. orthopedic implant or cardiovascular stent) was considered, with the assumption that a single device is used. According to the ISO 10993-18:2020, an exhaustive extraction is required. According to ISO 10993-17:2023, this exhaustive extraction allows to determine the Total Quantity (TQ) of constituents extracted from the device. Assuming release kinetics data are not available, the TQ can be used to calculate worst-case estimated exposure doses (EED_{max}) during acute (≤ 1 d), subacute (2 - 30 d), subchronic (31 - 365 d) and chronic (≥ 366 d) exposure periods, based on the fewest number of exposure days for each time period (respectively 1, 2, 31 and 366 d). Assuming a DBT of 120 µg/d was applied for the non-targeted analysis and an unrealistic Uncertainty Factor (UF) of 1 was used for the AET calculation (UF is typically higher for non-targeted analyses, which leads to detecting lower concentrations), the lowest concentration that can be detected would be 120 µg/device, which corresponds to a TQ of 120 µg in this example based on a single device. Two sets of values were used to justify that this TQ is of negligible toxicological risk: 1) The Toxicological Screening Limit (TSL), applicable to identified compound. The TQ was compared to TSL_{30d} (120 µg) and TSL_{366d} (600 µg). 2) The TTC, applicable when toxicological information is assessed to be inadequate or absent for an identified constituent. The EED_{max} expressed in µg/d, for each exposure period was compared to: TTC = 120 µg/d, for the acute (≤ 1 d) and subacute periods (2 - 30 d); TTC = 20 µg/d, for the subchronic period (31 - 365 d); TTC = 10 µg/d, for the chronic period (1 - 10 y); TTC = 1.5 µg/d, for the chronic period (> 10 y). **Results:** For all exposure periods, the TQ or EED_{max} were found to be lower or equal to the TSL or TTC, which means that the constituent can be judged to be of negligible toxicological risk and no further risk evaluation is recommended to address genotoxicity, cancer, systemic toxicity (acute, subacute, subchronic, chronic), or reproductive or developmental toxicological risks. Since the TQ is the lowest value that can be detected using a DBT of 120 µg/d, using a lower DBT is not necessary. **Conclusions:** A DBT of 120 µg/d is appropriate for non-absorbable implantable medical devices that are exhaustively extracted, considering the ISO 10993-17:2023 framework. The other TTC values from the ISO/TS 21726:2019 can be used either during the TRA or to select an appropriate

DBT prior non-targeted analysis of absorbable devices, repeatedly/single-use non-absorbable devices or devices that were extracted using exaggerated or simulated-use conditions. Before using this DBT, it is crucial to: 1) gather information on the device's composition, to ensure that compounds excluded from the TTC approach (e.g. Cohort of Concern) are not present and 2) ensure that an appropriate UF is applied for the AET calculation, since it adds a safety margin to detect potential substances of concern (such as Carcinogenic, Mutagenic and Reprotoxic 1A/1B substances or Endocrine Disruptors).

PS 3072 *In vitro* sensitization assessment of medical device extracts: Prevalidation results of GARDskin Medical Device for potential inclusion in ISO 10993-10

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Background and Purpose: Biocompatibility testing is essential to ensure the safety of medical devices (MDs), confirming their compatibility with biological systems and minimizing adverse effects. Along with cytotoxicity and irritation, skin sensitization is a key toxicological endpoint that must be assessed for all devices. While the former endpoints can be partly assessed *in vitro*, skin sensitization testing still largely relies on animal-based methods such as the Guinea Pig Maximization Test (GPMT) to meet regulatory requirements. For neat chemicals, significant progress has been made with the development, validation and regulatory integration of New Approach Methods (NAMs) into globally harmonized OECD Test Guidelines (TGs) 442C-E. These assays are increasingly replacing animal testing in several industry sectors. However, MD testing presents unique challenges due to the complex nature of polymeric materials and extractable profiles, and the regulatory adoption of NAMs in this field has been limited. To address this gap, the International Organization for Standardization (ISO) released Technical Specification (TS) 11796, providing guidance for evaluating the applicability of candidate NAMs for MD testing. The present study describes the adaptation and prevalidation of the OECD-validated GARD[®]skin assay for assessing skin sensitization potential of MD extracts in line with ISO 10993-12 and ISO TS 11796. **Methods:** To enable compatibility with MD testing, the GARDskin protocol was modified to include a pre-sample extraction step using polar (saline) and non-polar (olive or sesame oil) vehicles according to ISO 10993-12. A feasibility study followed by a prevalidation study was conducted under ISO TS 11796 to evaluate the method's reliability and applicability. In the feasibility study, silicone (MED-2000) and thermoplastic polyurethane (TPU) materials were spiked with skin sensitizers (n=4) and extracted in both polar and non-polar vehicles to serve as medical device surrogates (n=6). The prevalidation study consisted of two parts. Part 1 (GS MD method, n=16, 3 replicates): The original GARDskin Medical Device protocol was applied. Negative control material (MED-2000) was extracted in both vehicles and spiked with a panel of sensitizers and non-sensitizers. Part 2 (GS MD 0.5% method, n=29, 1 replicate): The same protocol was applied with one modification — extracts were tested at a final in-well concentration of 0.5% (reduced from 10%) to calibrate the classification threshold and compensate for the much higher sensitivity of *in vitro* assays compared to *in vivo* methods. **Results:** The feasibility study confirmed the ability of the GARDskin Medical Device protocols to correctly identify sensitizers from solid material extracts. All spiked materials (n=6) were correctly classified as sensitizers, with no false positives for un-spiked medical grade materials or extraction vehicles. Results from Part 1 of the prevalidation study demonstrated 100% reproducibility (16/16), with 94% concordance (15/16) with animal data and 88% concordance (14/16) with GARDskin data on respective neat chemical testing. With a further expanded test chemical range (n=29), Part 2 of the prevalidation study demonstrated 97% concordance (28/29) with animal data and 86% concordance (25/29) with GARDskin classifications. **Conclusions:** The GARDskin Medical Device prevalidation results demonstrate that the method is functional, reproducible, and highly accurate for assessing the skin sensitization potential of medical device extracts. The method fulfils the performance criteria to advance to an inter-laboratory ring trial, in accordance with ISO TS 11796, to further evaluate transferability and robustness prior to potential inclusion in ISO 10993-10 for *in vitro* sensitization testing.

PS 3074 Applicability and Performance of Open-Source and Paid-Access Computational Genotoxicity QSAR Models for Medical Device-Derived Extractables

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Background and Purpose: Under ISO 10993-17, toxicological risk assessments are performed on medical device extractables generated in accordance with ISO 10993-18. Such datasets are often complex and used to address systemic toxicity endpoints in lieu of animal studies. Limited availability of robust toxicological data for compounds requires use of alternative approaches, such as quantitative structure-activity relationships (QSAR), to predict toxicological endpoints. Historically, risk assessments conducted with open-source models may have resulted in conservative evaluations as out-of-domain constituents can prompt the selection of worst-case Threshold of Toxicological Concern (TTC) values. To better support the toxicological evaluation of medical device constituents, seven genotoxicity QSAR models

were evaluated for their applicability and prediction accuracy to device extractables. Genotoxicity was selected since one of the most pressing risk assessment needs is reliable data to select TTC. A comprehensive list of medical device-related constituents, both rare and common, was generated and a comparative analysis of the performance of these open-sourced and paid-access QSARs tools is presented here. **Methods:** A representative list of 120 medical device-related organic constituents was created from Boston Scientific's internal database of ~1200 medical device extractables, derived from >150 unique devices. The full list was ranked by frequency of occurrence in device risk assessments with the top 50 compounds (from 30-75 devices) and bottom 50 compounds (from <5 devices) chosen for further analysis. Additionally, 25 compounds identified in the internal database with literature-reported genotoxic, carcinogenic, or mutagenic hazards were included, resulting in a list of 120 compounds after duplicates were removed. Seven QSAR models for bacterial mutagenicity were assessed using this list to determine inclusion within each model's applicability domain. Out-of-domain compounds were further evaluated to identify any gaps in training sets specific to medical device extractables. Lastly, the accuracy of each prediction within the domain was compared with experimental data for genotoxicity from the European Chemicals Agency database. **Results:** For common, rare, and genotoxicity-related medical device extractables, open-source QSAR tools produced 48-63% in-domain calls, whereas paid-access QSAR models yielded about 91-93% in-domain calls. Siloxanes and antioxidants were among the most common extractables in this dataset but were often out of domain of open-source tools. Rare extractables were generally in domain of paid models but were in domain of only 1 of 5 open-source models. All models demonstrated high genotoxicity prediction accuracy with open-source models achieving 88-97% and paid models 95-98% accuracy compared to experimental data. Additionally, there were instances of unexpected predictions (e.g., phenol [CAS 108-95-2]), which appeared inconsistent with experimental data. Expert review identified phenol as a probable non-mutagenic genotoxicant in the literature, based on negative bacterial mutation and positive chromosomal aberration experimental results. It was predicted to be negative by bacterial mutation models, which aligns with these observations. Following the analysis of this dataset, a tiered process flow was created for genotoxicity prediction of medical device extractables to assess future data-poor compounds. **Conclusions:** Although both open-source and paid QSAR genotoxicity models cover many rare and common medical device-related compounds, larger gaps were observed in open-source training sets. When predictions could be made, accuracy was high for all models. A tiered approach is recommended, starting with open-source models and moving to paid models, as needed. Some common device extractables (e.g., siloxanes) remain outside many model domains, highlighting an area for QSAR improvement. As demonstrated by the phenol case study, constituents with non-mutagenic mechanisms of genotoxicity can result in false negatives using this tiered process flow. Moving forward, increased inclusion of medical device-related compounds in training sets would broaden usability of these programs and help bridge gaps in evaluation of medical device safety while using highly preferred non-animal approaches.

PS 3075 Solvent selection for leachables test in the condensate for gas pathway masks

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Background and Purpose: Tests for leachable in condensate are a critical part of biocompatibility assessment for gas pathway devices where patient exposure to leachable is credible. According to ISO 18562-4:2024, aqueous extraction is generally considered adequate for patient interface masks. Further, recent feedback from regulatory bodies highlights that for devices positioned close to the patient, such as patient interface masks, it is foreseeable that biological secretions from patients such as saliva, sweat, and mucus, may interact with device materials. This interaction could result in leachable compounds, which may then be reintroduced to the patient. As a result, it is recommended using water plus semi-polar and/or non-polar solvents to account for potential exposure of the inner mask surfaces with biological secretions of the oral and nasal cavities. This study was therefore designed to identify physiologically relevant solvent systems that closely replicate these biological fluids, thereby improving the accuracy and clinical relevance of Extractable and Leachable (E&L) extraction studies for face masks. **Methods:** The solvent selection was guided by Hansen Solubility Parameters (HSP) method, which describe dispersive (δ_D), polar (δ_P), and hydrogen bonding (δ_H) interactions. The chemical composition of mucus, saliva, sweat, and water was analyzed to determine their solvation characteristics. Candidate solvents were evaluated based on their HSP proximity to biological fluids. **Results:** Mucus and saliva, characterized by moderate polarity and strong hydrogen bonding capacity, showed the closest match with 20% ethanol in water (Semipolar solvent) (δ_D : ~16.0, δ_P : ~13.0, δ_H : ~26.0 MPa^{1/2}). This solvent improved extraction of semi-polar leachable while maintaining physiological relevance. PBS and saline effectively simulated the ionic strength of sweat but exhibited limited solvation power for hydrophobic compounds. Higher ethanol concentrations (>30%) led to over-extraction beyond realistic exposure conditions. Also, solvent composition of ethanol/water mixtures remained stable during extraction, with no evidence of ethanol loss or selective polymer absorption, supporting their consistency as simulating solvents. **Conclusions:** Based on HSP correlation and compositional similarity, 20% ethanol in water was identified as an optimal semi-polar solvent

for E&L extraction from face masks. It provides a balanced representation of mucus, saliva, sweat, and water environments, enabling more realistic and comprehensive detection of leachable from condensate.

PS 3076 Temporal Profiling of VOS Emissions: Accelerated Aging and Reprocessing of Breathing Gas Path Medical Devices for Biological Safety Assessments

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Background and Purpose: Volatile organic substance (VOS) emissions are a key determinant of the biological risk profile for breathing gas path medical devices. Recent updates to ISO 10993-1:2025 and ISO 18562-1:2024, have amplified focus on lifetime evaluation of breathing gas path devices as a part of the biological safety assessment. Here, we present VOS emission profile from a breathing gas path device over its useful life and additional considerations on the impact of exaggerated and clinically relevant reprocessing methods on the VOS output. **Methods:** Breathing gas path medical devices composed of polymer and metallic material classes were accelerated aged to their intended useful life, following methods outlined by ASTM F1980-21. A subset of aged devices underwent cleaning and disinfection (C&D) reprocessing following either clinically relevant or exaggerated cycle counts based on device IFUs. All aged breathing gas devices' VOS emissions were quantified using methods described in ISO 18562-3 at defined time points across the device's useful life. Emitted compounds were categorized into chemical classes and toxicologically evaluated using methods described in ISO 10993-17:2023. **Results:** Across all devices, both the number and concentration of emitted compounds decreased significantly over the simulated lifetime. Quantitative analysis demonstrated that dominant chemical classes, i.e., alkanes, organosilicon compounds, carbonyl compounds, and benzoyl derivatives decreased by 97.97%, 76.64%, 77.37%, and 89.14%, respectively, from initial (t = 0 year) to final (t = 5 year) time points. No new chemical classes were generated at the end of the device's useful life compared to initial testing. The dominant classes of chemicals detected were traceable to the materials of construction of the device, and all new compounds remained well within health-based thresholds for the intended population. For devices that received exaggerated C&D reprocessing methods (i.e., cumulative exposure to C&D agents after aging), VOS emissions were largely unchanged in chemical class composition, except for one hospital disinfecting method, where a respiratory sensitizer was identified in the VOS emission testing following an exaggerated cleaning approach. Confirmatory testing verified that the sensitizer was reduced to below quantifiable levels when clinically relevant reprocessing cycles were applied. **Conclusions:** VOS emissions significantly declined throughout the device's useful life, and exaggerated reprocessing had limited impact on chemical class composition. However, for C&D agents with the potential to cause respiratory sensitization, verification studies should be designed to ensure clinically relevant use-case scenarios are considered. These findings suggest that, with comprehensive material characterization and stability assessment, initial post-manufacture VOS emission testing can reliably inform biocompatibility risks across the full useful life of breathing gas path medical devices.

PS 3077 PFAS-Free Coverlay Material Change Assessment in SpO₂ Sensors: Biological Equivalence via E&L Study

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Background and Purpose: This presentation describes how an extractable and leachable (E&L) study, combined with cytotoxicity screening, enabled expedited biocompatibility assessment following a sudden material change in an SpO₂ sensor's (SS) intact skin contacting coverlay. In addition to adults, the intended population includes sensitive groups such as pregnant women and pediatric patients, with prolonged exposure (i.e., a typical use scenario of 7 days, up to 29 days). The coverlay was switched to a proprietary PFAS-free alternative, making immediate determination of biological equivalence challenging. To address this urgent supply chain disruption and ensure continued critical patient care, a fast-tracked biological and toxicological risk assessment was conducted. **Methods:** The overall approach was to establish biological equivalence in accordance with ISO 10993-18. The evaluated endpoints included physical and chemical characterization, cytotoxicity, irritation, and sensitization, as specified in ISO 10993-1. The risk-based assessment began with hazard identification, utilizing supplier-provided technical and regulatory compliance documentation, as well as public databases such as FDA, ECHA, and PubMed. To further establish chemical equivalence and to identify potential chemical hazards, the E&L study design was designed per ISO 10993-18, extracting the final finished SS coverlay with neat 2-propanol (100% IPA) at 50±1°C (an exaggerated condition), due to its affinity for both polar and non-polar compounds. SS extracts were collected at 0, 24, 48 and 72 hours to assess kinetic profiles (ISO 10993-12). Compounds identified Gas Chromatography-Mass Spectroscopy (GC-MS) were derived from triplicate extracts. The subsequent toxicological risk assessment was conducted in accordance with ISO 10993-17. **Results:** The new PFAS-free coverlay base resin, polyester (PET), is considered safe for intact skin use. There are no other differences in colorants, curing, or dispensing agents, all of which have previously been deemed biocompatible. To evaluate the new component, i.e. an undisclosed flame retardant (FR), the

SS exaggerated E&L profile was assessed as the worst-case exposure at 50±1°C, compared to the maximum operating temperature of 41°C. The study showed that no compound was released above the limit of quantitation (LOQ). The worst-case FR exposure estimation, based on the LOQ and assuming 100% dermal absorption, resulted in 7-day and 29-day exposures of 24.1 µg/day and 99.9 µg/day, respectively. A point of departure of 1,000 mg/kg/day (NOAEL or No-observed adverse effect level, for developmental and reproductive toxicity) was selected for the FR. The calculated margins of safety (MoS) were well above 1 (>600,000 for 7 days, and >150,000 for 29 days) based on the respective estimated exposure duration. The final finished SS extract met ISO 10993-5 requirements, with a grade 2 mild reactivity. The lack of quantifiable E&L's further supports the low toxicity risk as indicated by the mild cell reactivity. This screening also addresses any potential new manufacturing hazards. Biological equivalence with the predecessor was established given the chemical, material, physical and endpoint equivalence assessed above. Passing *in vivo* skin sensitization and irritation test results further supports the risk-based approach per the ISO 10993 series and the FDA's "3Rs" principle to replace, reduce, and/or refine animal use in testing when feasible. **Conclusions:** The SpO₂ sensor with the PFAS-free overlay is biologically equivalent to its predecessor. Therefore, the sensor is biocompatible if used as intended. The E&L and risk assessments demonstrated that the PFAS-free alternative does not introduce new or increased biological risks in the final device, supporting its safe and timely adoption.

PS 3078 Alternative Positive Control Material for the XTT and MTT Cytotoxicity Test ISO 10993-5 for Medical Devices

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Background and Purpose: Cytotoxicity testing is a key endpoint to be addressed during the biological risk assessment of medical devices. The XTT and MTT Cytotoxicity Tests are widely accepted quantitative methods recognized by regulatory agencies when performed in accordance with International Organization for Standardization (ISO 10993-5: Biological Evaluation of Medical Devices Part 5: Tests for *In Vitro* Cytotoxicity). In these biocompatibility tests, a positive control material that produces a cytotoxic response must be tested in parallel. Currently, the standard recommends a polyurethane film containing zinc diethyldithiocarbamate (ZDEC) or zinc dibutylthiocarbamate (ZDBC) as a positive control material; however, these materials have a limited availability. Previous work identified hypoallergenic silk tape as a suitable positive control material for use in the MEM Elution Cytotoxicity Test, a qualitative method. The purpose of this study was to evaluate whether Hypoallergenic Silk Tape can also serve as a suitable positive control material for the XTT and MTT Cytotoxicity Tests, which are quantitative in nature. **Methods:** Six independent runs of XTT cytotoxicity tests and six independent runs of MTT cytotoxicity tests were performed. Each run included six concentrations of the positive control, tested in sextuplicate. Extracts were prepared in accordance with ISO 10993-12: Biological Evaluation of Medical Devices Part 12: Sample Preparation and Reference Materials. The extraction medium consisted of Eagle Minimum Essential Medium (without phenol red) supplemented with 10% fetal bovine serum, 4mM Glutamax™, 100 IU/ml penicillin, and 100 µg/ml streptomycin (EMEM-PR10). Hypoallergenic Silk Tape was extracted at a surface area-to-volume ratio of 6 cm²/ml (for material thickness < 0.5 mm). Negative control (high-density polyethylene, to assess background cellular response) and vehicle control (extraction solvent only, to evaluate possible confounding effects due to the extraction vessel, vehicle, or extraction process) were prepared. All extraction vessels were incubated for 24 hours at 37°C under continuous agitation. This temperature allowed for maximal extraction, while remaining below the softening temperature of the test samples, and without adversely impacting the chemistry and/or stability of the serum and other constituents in the extraction solvent (culture medium). The outer 36 wells of each 96-well plate were each filled with 100 µl of EMEM PR10. The remaining 60 central wells were seeded with 100 µl of L929 mouse fibroblast cells at a density of 1 x 10⁵ cells/ml (1 x 10⁴ cells/well). Plates were incubated for approximately 24 hours at 37°C in a humidified atmosphere containing 5% CO₂ to allow formation of a sub-confluent monolayer. Within 24 hours of extraction preparation, the positive control extract was assessed at six concentrations, 100% (neat) and, 50%, 25%, 12.5%, 6.25%, and 3.13% (v/v) in EMEM-PR10. Each concentration was applied in sextuplicate and incubated for 48 hours at 37°C and 5% CO₂ for 48 hours. Following incubation, cell viability was assessed using XTT or MTT assay, according to standard procedures. **Results:** Viability reduced to < 70% of the blank is considered a cytotoxic effect. In the XTT cytotoxicity assay, cell viability decreased to below 70% following exposure to 100% and 50% concentrations of the positive control extract of Silk Tape. A clear dose-dependent response was observed. The average cell viability following treatment with the negative control and vehicle control was 96.8% and 97.9%, respectively, which is the expected response of these materials in the test system. In the MTT cytotoxicity assay, cell viability decreased to below 70% following exposure to 100%, 50%, and 25% concentrations of the positive control extract of Silk Tape. A clear dose-dependent response was observed. The average cell viability following treatment with the negative control and vehicle control was 98.2% and 105.3%, respectively, which is the expected response of these materials in the test system. **Conclusions:** Hypoallergenic Silk Tape was determined to be cytotoxic under both testing conditions and was shown to meet the positive control requirements of the XTT and MTT cytotoxicity tests as described in ISO 10993-5.

PS 3079 Passivation, Electropolishing and Anodization as Surface Residue Mitigation Steps for Biocompatibility Risk Management in Medical Device Manufacturing—A Retrospective Analysis

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Background and Purpose: Medical device biocompatibility evaluation per ISO 10993 is a risk management approach that involves a manufacturer evaluating existing relevant data from all sources, such as raw materials, process flow information, clinical history, etc., to identify any gaps in the data supporting biological safety. Reviewing all available data facilitates a comprehensive assessment for determining if new testing is required. This optimizes the manufacturer's resources, ensures the ethical use of animals per ISO 10993-2, and promotes timely, economic access of device technologies to healthcare providers and patients. Included in the hazards that can lead to biocompatibility risks are manufacturing material or processing aid residues that can remain on a device following manufacturing and become sources of clinical leachables. However, manufacturing processes often have built in steps that modify the surface of devices and intrinsically remove these residues which, in turn, can mitigate the risks stemming from surface residues. Passivation, electropolishing and anodization, which are chemical or electro-chemical processes employed on metallic devices to increase corrosion resistance (passivation, electropolishing, and anodization), reduce surface roughness (electropolishing), and impart a color (anodization), are examples of surface modifying processes that have the potential to mitigate manufacturing material residues on the device surface from previous manufacturing steps. However, there is a paucity of reports providing empirical evidence supporting that these processes can effectively mitigate medical device surface residues to toxicologically insignificant levels. **Methods:** A retrospective review of a medical device manufacturer's library of ISO 10993-18 extractables test data was performed to correlate the quantity and type of extractable identified on devices to the types of surface modification processes performed on the devices and the manufacturing materials used. The goal of this analysis was to determine if passivation, electropolishing, and anodization effectively remove manufacturing material residues from upstream manufacturing steps. It was also aimed at determining what, if any, limitations apply when considering these processes as biocompatibility risk mitigation steps. **Results:** Extractables test data from 112 devices with different surface modifications were reviewed. The passivation, electropolishing, and anodization subsets (18, 9, and 13 devices, respectively) were amongst the groups demonstrating the lowest total device residue burden, when considering both the per device surface area median residue amounts and range of residue amounts across all devices in each subgroup. When considered individually, all extractables identified on electropolished and anodized devices that were able to be plausibly traced to manufacturing materials were associated with manufacturing materials used in the surface modifying steps or operations thereafter. Most extractables identified on passivated devices were able to be plausibly traced to manufacturing materials from the passivation step or subsequent operations. Some extractables were linked to materials used before passivation, but these were associated with in-process cleaning prior to passivation and/or were present at toxicologically insignificant levels. As part of the review, factors impacting the effectiveness of passivation, electropolishing, and anodization as surface residue mitigation steps were also identified. **Conclusions:** Passivation, electropolishing, and anodization can be used within defined limitations to support the mitigation of upstream manufacturing material surface residues in biocompatibility risk management. When applied appropriately considering these limitations, biocompatibility risks from residues stemming from manufacturing materials used prior to these surface modification steps can be considered negligible. Importantly, taking advantage of these steps often already built into manufacturing processes can reduce and possibly eliminate the need for animal testing and optimize resource management and device time to market.

PS 3080 Worst-case Selection in Medical Device Toxicity Risk Assessment

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Background and Purpose: According to ISO 10993-17:2023 (Clauses 8 and 5.1.3), toxicological risk assessments for medical devices shall consider worst-case use scenarios. Factors relevant to worst-case exposure include the rate or extent of constituent release, the predictable maximum number or quantity of devices a patient may be exposed to, and the lowest body weight of the exposed population. Traditionally, worst-case devices are selected based on the largest surface area or mass. However, this method may not be appropriate when different device sizes are used across diverse patient populations. **Methods:** To address this complexity, we introduce the concept of the worst-case estimated exposure dose for medical devices (WCEED), derived from the maximum estimated exposure dose (EEDmax) of constituents for a given clinical scenario in ISO 10993-17. A formula is proposed to calculate WCEED, and three case examples are presented to demonstrate its application in toxicological risk assessment. **Results:** 1. Formula for worst-case medical devices selection in toxicity risk assessment $WCEED = N \times (\text{mass or surface area})$ of a device / BW_L Where: WCEED = worst-case estimated exposure dose for medical devices, the unit is g (or cm²)/kg body weight. The greatest value is the worst -case

scenario N = the maximum number of devices that may be used in a patient simultaneously. The number shall align with instructions for use in each patient's population. BW_L = lowest body weight of patients under the device use condition Note: patients < 6 months will be excluded from older patients due to special considerations. 2. Example A. Device A1 is identical to A2 (each device weight is w) and a maximum 2 devices can be applied in a patient. A1 can be applied in adults only (60 kg), A2 in adults and children (20 kg). $2w/60$ is less than $2w/20$, thus A2 used in children is the worst-case scenario. 3. Example B. same process aid changes in devices B1, B2, B3, and B4. They are long-term tissue-contacting implant devices. Maximum device quantity in a patient are 2 for all the devices. In Devices B1, B2, B3, and B4, patient-contacting mass of process aid is 0.4, 0.5, 1.2, and 1.4 g, respectively. Lowest patient body weight is 20, 20, 60, and 60 kg, respectively. WCEED is 0.04, 0.05, 0.04, and 0.047 g/kg body weight. Therefore, Device B2 in 20 kg patient is the worst-case scenario. 4. Example C. Same process aid changes in devices C1, C2, C3, and C4, all of which are long-term blood contacting devices. Patients are ≥ 6 months old. In devices C1, C2, C3, and C4, patient-contacting mass of process aid 15.6, 12.8, 4.8, and 7.7 g, maximum device quantity per patient is 1, 2, 1, and 2, and the lowest body weight is 60, 10, 6, and 10 kg, respectively. Therefore, WCEED is 0.26, 2.56, 0.8, 1.54 g/kg body weight, respectively, and device C2 in 10 kg patient is the worst case scenario. **Conclusions:** This quantitative approach to worst-case device selection is effective in diverse clinical scenarios and a scaling factor (SF) may be applied to approximate worst-case exposure when the worst-case device was not selected a priori. An advantage of that is that one could, if they so choose, derive distinct worst case exposure scenarios for different patient populations, all based off an E&L study. It provides a systematic and transparent method that complements ISO 10993-17 guidance, enhancing the rigor and clarity of toxicological risk assessments.

PS 3081 Toxicological Risk Based Particulate Acceptance Criteria for Medical Device Manufacturing

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Background and Purpose: FDA (Food and Drug Administration) and USP<790> require medical devices to be essentially free of visible particulates (VP, size $\geq 0.1\text{mm}$) and limit their occurrence. However, particulates are unavoidable in manufacturing environments, originating from intrinsic sources (e.g., manufacturing, assembly, packaging) and extrinsic sources (e.g., fibers, skin cells, atmospheric dust). While manufacturers employ cleanroom protocols, inspections, and quality controls, complete elimination of particulates remains difficult. This challenge is exacerbated by stringent acceptance criteria, often adapted from USP<788> for injectable products—without regard for device-specific factors like contact duration (short, prolonged), type (blood, tissue, skin), manufacturing processes, and materials. Consequently, unwarranted non-conformances and product rejections occur. This work proposes a toxicological risk-based approach to setting particulate acceptance criteria for medical device manufacturing. The approach guides manufacturers in establishing practical acceptance limits based on existing guidelines and toxicological safety thresholds, aiming to minimize undue rejections without compromising patient safety. **Methods:** We present a risk-based methodology, validated against safe toxicological limits for common chemicals, including systemic toxicants and carcinogens. Leveraging USP<788> our method extrapolates VP thresholds and introduces "Equivalence Surface Area" (ESA) to account for variability in particulate shape/size, focusing on total surface area (SA) versus particulate count. Exposure risk (dose) is estimated to be using the heaviest likely material (e.g., stainless steel) as a worst case, ensuring broad safety for all material types lighter than stainless steel. The calculated maximum dose is then compared against established Toxicity Screening Limits (TSL, ISO 10993-17) and mutagenicity guidelines (ICH M7 R1). If exposure is below these thresholds, the ESA is deemed acceptable, and can be used for setting visual inspection plan, reducing unnecessary non-conformances. **Results:** This proposed approach is demonstrated here using a real-world example. We extrapolated the acceptable VP count from USP<788> subvisible particulate (SVP) guidance, which limits (600 particulates $>0.025\text{mm}$). We compared SA of VP and SVP (SA of 0.1 mm VP $\div$ SA of 0.05 mm SVP = 4). This yields a VP count of 150 (600 $\div$ 4). The allowable surface area threshold for total VP on a device is thus 1.5 mm^2 ($150 \times 0.01\text{ mm}^2$). For the worst-case exposure (mass) calculation—stainless steel (density 8mg/mm^3), total SA of 1.5 mm^2 and thickness of 0.010 mm resulted in mass equal to $120\text{ }\mu\text{g/device}$ (Mass=Density*SA*Thickness). This amount meets TSL and ICH M7 R1 impurity criteria, indicating an acceptable risk from particulate exposure. Thus, adopting a total acceptable criterion of 1.5 mm^2 per device provides a protective threshold. Manufacturers may use this approach to derive their own acceptance criteria for particulates based on the nature of device contact, material types, and manufacturing complexity. However, it is also important to consider particulate shape and size for cardiovascular devices, as these factors may increase clinical risk and should be reflected in the acceptance criteria after clinical inputs. **Conclusions:** The proposed approach offers a robust, pragmatic solution to particulate challenges in medical device manufacturing. By integrating toxicological risk assessment, ESA-based thresholds, and established guidelines (USP<788>, ISO 10993-17, ICH M7 R1), manufacturers can implement acceptance criteria that balance real-world risk and patient safety. This approach addresses the limitations of particulate count metrics, considers material and device-specific variables, and reduces unnecessary non-conformances and supply interruptions, while also proactively incorporating toxicological principles into particulate evaluation rather than

addressing safety concerns only after issues arise. **The views, interpretations, and conclusions expressed in this abstract are solely those of the authors and do not necessarily reflect the official policies or opinions of their employer.**

PS 3082 Tailored E&L Strategies for Hyaluronic Acid Medical Devices: A Foundation for Toxicological Risk Assessment

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Background and Purpose: Hyaluronic acid (HA)-based products are widely used in medical applications such as intra-articular injections, ophthalmic solutions and dermal fillers. Although they differ significantly in form and function from traditional solid medical devices (e.g., implants or catheters), they are nonetheless classified as medical devices under applicable regulatory frameworks. This classification is based on their primary mode of action, which is physical rather than pharmacological for example, by providing lubrication, hydration or mechanical support. Due to their gel-like, non-solid nature, HA-based products pose unique challenges for chemical characterization and subsequent toxicological risk assessment. Standard extractables and leachables (E&L) methodologies, following ISO 10993-18 requirements, developed for solid polymeric or metallic devices are often not applicable. Therefore, tailored extraction and evaluation strategies were developed for the analysis of E&L compounds from HA-based products. **Methods:** Due to the varying textures of the HA-based products, different approaches were applied. For highly liquid gels, the medical device was dissolved in 0.9% saline and directly injected. For more viscous gels, the HA-based product was first enzymatically digested using hyaluronidase. Prior to analysis the calculation of the analytical evaluation threshold was performed (AET) for each method. The resulting solution was then directly injected into analytical systems. Furthermore an extraction of the HA-based products with isopropanol and/or n-hexane was performed. In addition, multiple analytical techniques were employed to characterize volatile, semi-volatile and non-volatile E&L compounds, as well as elemental impurities, including headspace GC-MS, GC-MS, UHPLC-HRMS, and ICP-MS. The identification of the E&L and HA-derived compounds was performed by using a customized multi-dimensional database combined with detailed analysis of fragmentation patterns for individual compounds. Complementarily, a toxicological risk assessment was performed in accordance with ISO 10993-17. **Results:** In total, seven different HA-based products were evaluated. One major challenge, besides the extraction procedure, in the analysis of the HA-based medical devices is that the product itself as well as the degradation products and additives are detectable by GC-MS and UHPLC-HRMS. A total of 113 compounds exceeded the applied AET in UHPLC-HRMS analysis, while 637 compounds were detected by GC-MS. Using a tailored in-house database combined with fragmentation pattern analysis, most detected compounds were assigned as product-derived or product-related. In addition, compounds such as acetyltributylcitrate (CAS 77-90-7), a common plasticizer, and 3-acetamidofuran (CAS 59445-85-1), identified as a manufacturing contaminant, were detected in some products. ICP-MS analysis revealed the presence of elements including phosphorus (P), silver (Ag), and tungsten (W) in certain samples. No compounds above the respective AET were detected in headspace analysis. All substances detected in amounts exceeding the applicable AET value were further subjected to the toxicological risk assessment. **Conclusions:** The chemical characterization and toxicological assessment of HA-based products is particularly challenging especially when focusing on the "standard" extraction-based procedures developed for medical device evaluation. However, using a combination of customized work-up procedures, including direct injection, enzymatic digestion and/or apolar solvent extraction, a complete E&L analysis can be achieved for these complex systems. The database-driven identification approach combined with the detailed analysis of fragmentation patterns enabled the assignment of most detected compounds across all analytical platforms and samples. This established approach ensures the analytical investigation and toxicological risk assessment of HA-based products, thereby strengthening the overall biological risk assessment of these medical devices.

PS 3083 Setting the Benchmark for Hemocompatibility: Advanced Testing for Transfusion Devices according to ISO 1135

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Background and Purpose: In addition to the horizontal standards for hemocompatibility testing like ISO standard 10993-4, there are several vertical standards addressing the need of individual types of devices. The ISO standard 1135-4/-5 provide a comprehensive framework for the hemocompatibility analysis of transfusion equipment used in medical settings. These standards ensure that the equipment is safe and effective for blood transfusions, minimizing the risk of adverse reactions in patients. While ISO 1135-4 focuses on transfusion equipment that operates with gravity feed, ISO 1135-5 addresses transfusion equipment used with pressure infusion apparatus. In 2025, these two guidelines were revised and the relevant sections

on the testing for hemocompatibility were replaced mentioning testing of hemolysis, coagulation and platelet activation and complete blood count in accordance with ISO 10993-4. However, our experience shows that regulatory bodies still prefer testing with separate blood components planned for administration mimicking clinical settings. Since the design of such a study has various pitfalls, we outline general considerations that should be taken into account. **Methods:** The design of this kind of study is tailored to the individual customer taking the real-world conditions into account. Thus, the following points should be taken into account: different flow rates, VTBI (volume to be infused), maximal time of use of the sets, etc. Further, the number of separate donors used should be determined based on the planned risk assessment and statistical analysis. The purely experimental procedure involves simulating clinical application of the test item. The corresponding blood product is connected to the transfusion device and the flow-through is performed based on the relevant parameters of the test design (VTBI, flow rate, etc.). Baseline values are taken prior to testing and are compared to samples taken after passage through the transfusion set. **Results:** When evaluating the hemocompatibility of transfusion equipment in accordance with ISO 1135-4 and -5, two critical assessment parameters are considered: retention and damage/activation of blood components in samples taken after passage through the transfusion set. Depending on the blood product used, different factors are considered for evaluation. For retention, the relevant constituents total hemoglobin, platelet count and Factor VIIIc are monitored. In parallel, the degree of hemolysis, platelet activation (e.g. β -Thromboglobulin release) and coagulation activation (e.g. formation of thrombin-antithrombin (TAT) complexes) are determined to assess the damage and activation. The different endpoints are determined using Cell counter, ELISA and/or chromogenic assays in samples taken prior to and after passage through the transfusion device allowing determination of the effects of tested test items. Our experience shows that the correct design of such a study strongly influence the regulatory acceptance and scientific weight. In general, it is recommended to discuss the planned design with regulatory bodies before execution of such studies because in the past, the analysis of effects on further plasma proteins such as Protein S and C, vWF protein and the Complement system was requested. **Conclusions:** The recent revisions of ISO 1135-4 and -5 align hemocompatibility testing requirements more closely with ISO 10993-4, emphasizing standardized endpoints such as hemolysis, coagulation, platelet activation, and complete blood count. However, regulatory expectations often extend beyond these guidelines, favoring study designs that replicate clinical conditions using separate blood components. This approach introduces significant complexity, requiring careful consideration of flow rates, VTBI, duration of use, and donor variability to ensure robust and scientifically meaningful results. Ultimately, a well-designed, clinically relevant study enhances both compliance and confidence in the safety and performance of transfusion equipment.

PS 3084 Refining Medical Device Chemical Safety Risk for Pediatric Patients: A Theoretical Case Study Considering Children Growth Trajectory

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Background and Purpose: Chemical toxicity is a critical component of device risk assessment. The ISO 10993-17:2023 introduces new tools to harmonize risk evaluation, including assumed exposure durations. It also provides specific guidance for pediatric population, acknowledging their unique physiological vulnerabilities with very conservative default body weight assumptions: 3.5 kg for infants (<1 yr) and 10 kg for children (>1 yr to ≤ 16 yrs), but not reflecting normal growth trajectory. Percentile charts published by WHO and US CDC, along with default values derived from survey data published by regulatory bodies for risk assessors (US EPA, Health Canada, EFSA, US ATSDR), offer a more representative basis for pediatric population. This case study explores how conservatism in ISO 10993-17 may overestimate risk and proposes a weight of evidence approach incorporating realistic pediatric growth to refine risk. **Methods:** Pebax, a thermoplastic elastomer composed of flexible polyether and rigid polyamide segments, is widely used in devices. The polyamide derived from lactam monomers forms the hard segment, while polyether (polytetrahydrofuran or PTHF) constitutes the soft segment. Barium sulfate is often added to materials to impart radiopacity. Lactams, PTHFs, and barium are published extractables from Pebax based devices in literature. A theoretical patient exposure scenario of these chemicals was analyzed, including 3 pediatric age groups: newborn to <6 months, 6-12 months, and ≥ 12 months. For each group, 3 assumed exposure durations within a 1-yr period were considered: ≤ 1 d, 2-30 d, and 31-365 d. The TRA followed ISO 10993-17 framework was conducted, involving a systematic review of adverse effects from chemical exposure. Key steps included the calculation of EED_{max} incorporating default body weight values from ISO 10993-17, US EPA, Health Canada, and EFSA, establishing TI for systemic harm-related toxicity endpoints, and determining and comparing MOS for each scenario. **Results:** Due to the lack of short term toxicity studies (≤ 1 d and 2-30 d), PODs for TIs were conservatively derived from longer duration subchronic toxicity studies. For infants aged ≤ 6 months, an additional UF of 3 was applied to chemicals primarily eliminated via hepatic metabolism or renal excretion, lactams and PTHFs, but not barium, which is not metabolized through those pathways. Newborn to <6 Months: For durations ≤ 1 d and 2-30 d, MOSs were only impacted by the additional UF of 3. For the 31-365 d duration, MOSs were not only inversely proportional to the default body weight values but also significantly impacted by the growth trajectory. The use of age specific default values (1-3

m, 3-6 m, and 6-11 m) by US EPA, Health Canada, or EFSA provided up to 8-fold higher MOS than by ISO 10993-17, reflecting the rapid growth trajectory in this age group. 6-12 Months: MOSs for ≤ 1 d and 2-30 d were only affected by the use of default body weight, due to less body weight variability within a month. For the 31-365 d duration, MOSs were again inversely related to default body weight. The inclusion of age specific subgroups (6-11 m and 1 to <2 yrs) refined to 2.6-fold higher MOS to account for continued growth during this period. ≥ 12 Months: Across all 3 exposure durations, MOSs were associated with body weight assumptions. In some subgroups, MOSs were up to 6-fold lower when using ISO 10993-17 defaults compared to defaults by agencies. **Conclusions:** This case study highlights the layered conservatism inherent in current practice, especially for pediatric population. Our findings demonstrated that the combination of conservative assumptions, e.g. reliance on subchronic toxicity data for short term exposure, additional UF to account for immature toxicokinetic, and assumed complete oligomer breakdown, collectively result in significantly overestimated risk. Especially, the default values specified in ISO 10993-17 are intentionally protective but do not reflect actual pediatric growth trajectories, potentially compounding overall conservatism as MOS is sensitive to body weight assumptions and exposure duration in pediatric. Incorporating age specific data from regulatory agencies enables more accurate pediatric exposure estimation by considering growth trajectories and age subgroups, supporting balanced, evidence based device safety decision and aligning with broader regulatory practices.

PS 3085 Defining the Chemical Space of Extractable and Leachables Found in Medical Devices

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Background and Purpose: The threshold of toxicological concern (TTC) is a risk assessment tool that establishes exposure levels for chemicals, below which there is a low probability of adverse health effects. It is especially useful for chemical constituents that have limited toxicity data available. Application of the TTC is not appropriate for Cohort of Concern (CoC) chemicals because CoCs are a class of highly potent, mutagens and carcinogens that pose a significant health risk at low exposures. The TTC has been applied to assess the safety of chemical constituents across multiple industries including food, pharmaceuticals, and medical devices, but to date there is no standardized list of CoCs specific to medical devices, which in turn leads to inconsistencies in assessing potential risk. To elucidate which chemical constituents, including cohorts of concern, are relevant to the chemical space of medical devices, extractable and leachables (E&L) profiles with the corresponding toxicological risk assessment from regulatory submissions were collected and curated into a single database. **Methods:** Data-mining and computational methods were utilized to compile extractable and leachable reports and corresponding toxicological risk assessments included in regulatory submissions from an internal FDA data repository. A combination of keyword searching and manual review of 4,000+ premarket notification (510(k)s) and premarket approval (PMAs) submissions was conducted to identify ones that included E&L testing and matching toxicological risk assessment. Extractable leachable testing included non-targeted and targeted analysis in the dataset. A document scraping Python script was applied to optimize data transfer from a multitude of layouts of chemical characterization and toxicological risk assessment reports to a consistent format. Subsequently, all outputs were reformatted and collated. **Results:** Extractable and leachable profiles and toxicological risk assessments from submissions were collated into a single database, referred to hereafter as ELTRA. The dataset contains 27,852 entries and consists of 3,227 unique compounds that are identified by name and at least one other identifier (i.e. CASRN). Where available, the following fields were populated: material of device, analytical technique (e.g. GC-MS), solvent of analytical technique (e.g. hexane), confidence level of identification (e.g. confident), chemical formula, retention time, mass, concentration, whether TTC was applied, whether the toxicological screening limit (TSL) was applied, tolerable intake (TI) calculation, and margin of safety (MoS) calculation. 30.5% of the entries have the MoS calculation field populated. ELTRA was compared to other lists of known toxic chemicals, or potential CoCs: Cheeseman et al. (1998) and WHO IARC Monographs on the identification of carcinogenic hazards to humans. Out of the 701 potential CoCs listed in the Cheeseman paper, 33 were found in ELTRA, out of the 621 Group 1, 2A, and 2B carcinogens identified by IARC, 49 were found in ELTRA, and 6 potential CoCs found in ELTRA were listed in both the Cheeseman paper and IARC Monographs. 68.5% of the submissions mined for data included at least one potential CoC. **Conclusions:** By leveraging the variety of medical devices that are reviewed by the FDA for chemical characterization, biocompatibility, and toxicology, this works establishes a foundational survey of the medical device chemical space and CoCs pertinent to medical devices specifically. This work can help inform the chemical space relevant to medical device.

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Background and Purpose: Medical devices contain various chemical components including polymer additives, processing aids, and manufacturing residues that may migrate and pose safety concerns to patients. Extractables and leachables (E&L) analysis serves as a basis for identifying these compounds and enabling comprehensive toxicological risk assessment (TRA) of medical devices. Conventional non-targeted liquid chromatography-mass spectrometry (LC-MS) approaches utilizing high-resolution mass spectrometry (HRMS) deliver precise mass measurements and detailed fragmentation patterns. However, these methods frequently encounter difficulties with spectral interpretation due to matrix complexity and inadequate chromatographic resolution of structurally related compounds. The integration of ion mobility spectrometry (IMS) with HRMS provides an additional dimension of separation based on molecular collision cross-section (CCS), which correlates with ion geometry, size, and charge distribution. This study proposes criteria for integrating IMS into E&L analyses and highlights its promising role in advancing future chemical identification methodologies. **Methods:** Twelve different polymer materials were extracted using water, isopropyl alcohol, and hexane as solvents to ensure wide range of compound recovery across a broad polarity spectrum. Extracts were analyzed using Agilent 6560 IM-QTOF LC-MS system in data-independent acquisition (DIA) mode to collect comprehensive MS/MS spectra across all precursor ions. This reduces the data-dependent acquisition (DDA) limitations that may miss low-abundance or co-eluting compounds. Using PNNL PreProcessor software, all ions IM/MS mobility-aligned fragmentation data were converted into a standard LC-MS DDA file format for downstream analysis in Agilent Mass Profiler and Qualitative Analysis software. **Results:** IMS provided clear improvements in resolving poorly separated LC peaks, particularly in complex polymer extracts where traditional LC-MS/MS often struggles to distinguish overlapping or co-eluting compounds. Our data demonstrated that in the case of separating isobaric and co-eluting species, IMS enhanced resolution and reduced the likelihood of false identifications. Drift-time alignment further improved spectral clarity by filtering out background ions and unrelated fragments, resulting in cleaner and more accurate MS/MS data. These translated into higher identification confidence where IMS-DIA identified more compounds than DDA, supported by increased fragmentation coverage and the ability to confirm assignments using CCS values. **Conclusions:** IMS-enhanced HRMS combined with DIA improves E&L chemical identification by increasing sensitivity, selectivity, and reliability through enhanced spectral clarity, fewer misassignments, and broader ion coverage. Incorporation of CCS measurements provides a robust structural descriptor that further increases specificity and confidence in identifying compounds within complex extract matrices. Collectively, these capabilities deliver a more comprehensive and dependable approach for non-targeted chemical identification.

PS 3087 **Solvent properties and the effect on extraction kinetics from silicone rubber**

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Background and Purpose: The properties of silicone have led to its broad usage as a material of construction in medical devices. The safety of these devices is evaluated based on the extractable chemicals collected and analyzed according to ISO 10993-18:2020, and assessed for their toxicity based on ISO10993-17. ; However, the selection of extraction solvent and conditions can present challenges for silicones since as these materials swell to more than 200% of their initial size and experience fast extraction kinetics. The excessive swelling may result in device damage which is not indicative of the real-world performance. Furthermore, the expedited extraction kinetics may offer an opportunity to use data-driven selection of shorter extraction intervals. This work addresses data-driven extraction intervals for silicone devices while addressing how solvent properties affect the kinetic of extraction by comparing extraction kinetics of several solvents with varying characteristics. The extraction kinetics are analyzed in the context of multiple solvent metrics such as polarity, dielectric constant, and Hansen solubility parameter. **Methods:** This study used extractions from silicone rubber and assesses siloxane species at 3, 6, 10, 24, and 72 hours as well as extractions at 6 and 7 days with solvents selected for their polarity, solubility parameters, and dielectric constant. The extracts were analyzed using gas chromatography mass spectrometry (GC-MS) at each time interval to assess the depletion of extractable levels. **Results:** Extractions were completed in 10 hours regardless of the solvent strength. Polarity, Hansen solubility parameter (HSP), and the dielectric constant were all used to assess the relationship between the extraction power of the solvent and the kinetics. While the total extracted quantity of high abundance siloxanes varied with solvent choice, the extraction kinetics were constant after the initial 3-hour extraction cycle. **Conclusions:** The extraction of silicone rubber may be more affected by the number of extraction cycles rather than longer extraction times. Solvent selection may affect the total amount of extractable siloxanes observed but the results suggest the extraction kinetics are similar for different solvents.

PS 3088 **Developmental Lead Exposure and Sex-Specific Glutamatergic Pathways Underlying Convergent Anxiety-Like Phenotypes in Adult Zebrafish**

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Background and Purpose: Lead (Pb) is a pervasive environmental neurotoxicant and a recognized developmental origin of health and disease (DOHaD) toxicant, capable of reprogramming neural circuitry and behavior at concentrations well below current regulatory thresholds. Early life represents a uniquely sensitive window in which perturbations to neurotransmitter homeostasis may produce long-term neurobehavioral vulnerabilities. This study investigated the hypothesis that developmental Pb exposure alters anxiety-like behavior and glutamatergic signaling in a sex-dependent manner late in life in adult zebrafish. **Methods:** Wild-type (5D) zebrafish (*Danio rerio*) embryos were exposed to 0, 0.01, 0.1, or 1 ppb ($\mu\text{g/l}$) Pb acetate from 1 to 72 hours post fertilization (hpf) (embryogenesis) in groups of 50 larvae per plate. At 72 hpf, zebrafish were rinsed of the Pb exposure solution and allowed to mature with no additional Pb exposure in system water. At 1 year of age, behavioral assessment was conducted using the Light and Dark Preference Test (LDT). After behavioral testing, adult brains were dissected for molecular analysis of key regulators of glutamatergic signaling. Targets quantified included gamma glutamate cysteine ligase (GCL), phosphate-activated glutaminase (PAG), glutamine synthetase (GS), vesicular glutamate transporter 1 (VGLUT1), excitatory amino acid transporter 1 (EAAT1), and glutamate dehydrogenase (GDH). All outcome analyses were completed by sex. **Results:** In the behavior assessment, Pb exposure reduced anxiety-like responses across sexes, including prolonged permanence in the clear zone and reduced exploratory movement. Pb exposed zebrafish also displayed disrupted decision-making behaviors, characterized by fewer cautious exploratory transitions between light and dark zones, indicating altered evaluation of environmental threat. These phenotypes were most pronounced in 1 ppb treatment group for both sexes but was more prominent in females. At the biochemical level, distinct sex-specific patterns emerged despite convergent behavioral outcomes. In females, Pb exposure reduced VGLUT1 while elevating PAG, GS, and EAAT1. These changes suggest weakened antioxidant buffering, impaired synaptic packaging, and a maladaptive recycling loop in which excess glutamate is inefficiently cleared and packed but continually generated. In contrast, males exhibited increased VGLUT1 and EAAT1 consistent with enhanced synaptic glutamate packaging and shunting into glutamine as a compensatory detoxification mechanism. GDH and GCL activity was not affected in either sex. **Conclusions:** Functionally, these disruptions implicate multiple nodes of glutamatergic regulation. PAG drives conversion of glutamine to glutamate with upregulation potentially exacerbating excitotoxic potential. GS catalyzes the reverse reaction, detoxifying glutamate by conversion back to glutamine. In this study sex-specific elevation suggests differing compensatory pressures. EAAT1 mediates glial uptake of synaptic glutamate. Increase of EAAT1 in both sexes may reflect an adaptive change to compensate for the excessive amount of extracellular glutamate. Finally, VGLUT1 governs vesicular packaging of glutamate with downregulation in females compared to elevation in males highlighting fundamental sex differences in synaptic protection. Taken together, these findings demonstrate Pb acts as a DOHaD neurotoxicant disrupting glutamatergic homeostasis in the developing brain which results in sex-specific biochemical mechanisms. Although the biochemical pathways diverge with impaired buffering and recycling in females compared to enhanced packaging and shunting in males, behavioral impacts are similar in both sexes with reduced anxiety-like phenotypes and impaired decision-making observed. This work underscores the importance of considering sex as a biological variable in developmental neurotoxicology and provides mechanistic insight into how environmentally relevant Pb exposures may establish lasting vulnerability to neurological diseases and disorders.

PS 3089 **Developmental Pb exposure affects neurotransmitters distributions in the brain exerting late-in-life neurotoxic effects**

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Background and Purpose: The Developmental Origin of Health and Disease (DOHaD) paradigm emphasizes that early-life environmental exposures shape long-term health outcomes, particularly in the nervous system where critical windows of vulnerability define susceptibility to neurotoxicants. Lead (Pb) is a pervasive environmental contaminant with no safe exposure threshold, and increasing evidence suggests that even parts-per-billion concentrations can disrupt neurodevelopment and predispose organisms to late-life neurological impairments, including cognitive decline and neurodegenerative disease-like phenotypes. **Methods:** In this study, we applied a combination of behavioral and neurochemical approaches to evaluate the persistent effects of developmental Pb exposure in zebrafish (*Danio rerio*). Embryos were exposed from 1 to 75 hours post-fertilization (hpf) to 0, 0.01, 0.1, or 1 ppb Pb, reflecting environmentally relevant concentrations. At one year of age, adults were subjected to a battery of validated behavioral tests including the Y-maze (spatial memory), novel object recognition test (NORT; recognition memory), novel tank test (NTT; anxiety-like behavior), social preference test (SPT; sociability), and shoaling test (group interaction). After behavioral assessments, brains were collected, cryosectioned, and analyzed using desorption electrospray ionization mass spectrometry imaging (DESI-MSI) to investigate the spatial distribution of key neurotransmitters and metabolites, with a particular focus on the glutamatergic system. **Results:**

Results revealed striking and sex-dependent impairments. Pb-exposed zebrafish displayed altered distributions of glutamate, glutamine, acetylcholine (ACh), and dopamine (DOP), indicating dysregulation of excitatory, cholinergic, and dopaminergic signaling. Females exhibited an anxiogenic-like phenotype in the NTT, whereas males showed a clear antisocial phenotype in both the SPT and shoaling test, suggesting sex-specific vulnerabilities in socio-emotional processing. In cognitive domains, both sexes demonstrated impaired recognition memory in the NORT, while spatial memory deficits were consistently observed in the Y-maze across exposure groups. Notably, glutamatergic neurotransmission appeared particularly disrupted, aligning with the hypothesis that Pb interferes with excitatory pathways critical for learning, memory, and long-term synaptic plasticity. **Conclusions:** These findings support the concept that low-dose Pb exposure during early embryogenesis produces persistent, multilevel alterations detectable in adulthood, long after the exposure period has ended. By integrating high-resolution neurotransmitter imaging with behavioral phenotyping, our study reveals that Pb disrupts the spatial organization of key neuromodulators in the brain, which may underlie the observed impairments in cognition, emotional regulation, and social behavior. The sex-dependent effects further highlight the importance of considering biological sex as a determinant of neurotoxic outcomes. Developmental Pb exposure, even at parts-per-billion levels, has profound and enduring effects on brain chemistry and function. Altered glutamatergic, cholinergic, and dopaminergic distributions detected by DESI-MSI strongly suggest mechanistic links between neurotransmitter dysregulation and behavioral phenotypes consistent with early-onset cognitive decline and neurodegenerative-like conditions. These results expand the DOHaD framework by demonstrating how trace-level Pb exposures during critical windows of development can reprogram neural circuits and predispose organisms to long-term neurological dysfunction. Our findings underscore the urgent need for stricter environmental Pb regulation and highlight the utility of zebrafish as a translational model for identifying molecular and behavioral biomarkers of neurodegenerative risk associated with early-life toxicant exposure.

PS 3090 Inflammation-induced metal dyshomeostasis increased susceptibility to manganese toxicity in HMC-3 microglia cells

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Background and Purpose: Manganese (Mn) is a trace metal that plays an important role in antioxidant defense and glutamate synthesis in the brain. Over exposure to Mn can result in accumulation within the Central Nervous System (CNS), resulting in neurotoxicity. It is well established that Mn exposure results in increased neuroinflammation, predominantly driven by microglia, the first responders of the CNS. In contrast, the role of inflammatory stimuli in altering Mn transport and Mn-induced neurotoxicity is less known. This is important to understand because changes in metal transport and increased neurotoxicity may play a detrimental role in contributing to neurodegeneration. Therefore, the purpose of this study is to determine how inflammatory stimuli influence the neurotoxic effect of Mn on microglia, while providing a mechanism to explain the potentiated effects. **Methods:** The HMC-3 cell line is a human-derived *in vitro* model for microglia. Cells were used between passage 9-19 in this experiment. HMC-3 cells were exposed to Mn (50 μ M MnCl₂), Lipopolysaccharide (LPS) (100 ng/ml), or a co-exposure (Mn and LPS) for 4-HR or 24-HR. RNA was collected at both timepoints for synthesis to cDNA and use in RT-qPCR experiments. RT-qPCR housekeeping genes were GAPDH and 18S. RT-qPCR experiments assessed changes in inflammatory markers (NF- κ B, Casp1, NLRP3, IL-1 β , IL-6, TNF α), metal importers (DMT1, Zip8, Zip14), metal exporters (Fpn1, Znt10), and metal storage (H-FTN, L-FTN). At both timepoints, live cell assays were conducted to look at metabolic activity (MTS), oxidative stress (ROS-glo), and viability (LDH-glo). At the 24-HR timepoint, protein was collected for mass spectrometry analysis (ICP-MS). 2-Way ANOVA and Fisher's LSD test were used to determine statistical significance. **Results:** Inflammatory marker gene expression was increased following the LPS exposure, with a more pronounced effect seen in the co-exposure with LPS and Mn. There was no effect of Mn on metal importer expression at either timepoint. At 4-HR, LPS treatment was associated with a transient decrease in expression of genes related to metal import, while at 24-HR there was a robust increase. In contrast, in the 4-HR co-exposure metal importer gene expression generally increased, while at 24-HR there was a decrease in expression. Additionally, While Mn single exposure caused an increase in metal exporter expression, LPS and the co-exposure resulted in a decrease in expression at 4 and 24-HR. Genes related to storage increased with the single exposure to Mn and LPS, and the co-exposure at both timepoints. ICP-MS analysis at the 24-HR timepoint showed that the co-exposure resulted in a greater increase in intracellular Mn compared to the single exposure to Mn and LPS. Moreover, increased neurotoxicity was observed with the co-exposure at both timepoints as seen with MTS, ROS-glo, and LDH-glo assays. **Conclusions:** LPS single exposure impacts metal transport dynamics by increasing expression in genes related to metal import and storage at 24-HR. Moreover, there is a greater effect of the co-exposure on changes in metal transport compared to either single exposure scenario indicating a potential for metal dyshomeostasis to increase microglial susceptibility to Mn toxicity. The changes in metal transport, within the co-exposure, work together to ultimately increase metal accumulation in cells. Changes in metal transport and increased metal accumulation were associated with cytotoxicity in the HMC-3 cells as seen through increases in oxidative stress, inflammation, and decreased metabolic activity. This data indicates that LPS

increases susceptibility to Mn exposure ultimately leading to increased cellular toxicity. Moreover, this study shows that inflammation can drive metal accumulation in the CNS. This new perspective could further explain the link between metals, inflammation, and neurodegeneration.

PS 3091 Regional Kinetics of Human Brain Manganese Clearance Measured by Quantitative Magnetic Resonance Imaging Relaxometry

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Background and Purpose: Excessive environmental Manganese (Mn) exposure can cause chronic manganese poisoning, a neurotoxic condition resembling Parkinsonian disorders, with symptoms including motor deficits, dystonia, rigidity, speech, and mood disturbances. Understanding not only the accumulation but also the clearance kinetics of Mn in the human brain is critical for assessing neurotoxic risk and recovery potential. Magnetic resonance imaging (MRI) is currently the only modality capable of providing *in vivo* noninvasive measurement of brain Mn burden. Due to the paramagnetic properties of Mn, the longitudinal relaxation rate ($R_1 = 1/T_1$) increases proportionally with Mn concentrations. Previously, we developed a whole-brain Mn-mapping approach to detect subtle but significant changes in individual welders by comparing their MRI contrast data against the normative map from controls. In this study, we followed welders who adopted powered air-purifying respirators (PAPRs), enabling a longitudinal assessment of Mn clearance. By further extracting regions of interest (ROIs) from major brain structures, we quantified regional washout rates to characterize the spatial dynamics of Mn clearance across the brain. **Methods:** As a part of a large imaging study on welders, 6 welders who had adopted PAPRs and 37 non-welding factory controls from a U.S. truck manufacturer were recruited, along with 12 university controls with no Mn exposure history. All participants provided written informed consent to participate in the study. MRI data were acquired using a 3T Siemens Prisma scanner. Welders were scanned prior to initiating PAPR use and again at 1-2, 4, 6, 8, and 12 months after PAPR use. Controls were scanned once. Quantitative brain T1 relaxometry maps were reconstructed to assess Mn-related relaxation changes and then converted to R_1 ($=1/T_1$) maps. ROIs were extracted from major brain structures, including the globus pallidus, putamen (anterior and posterior), caudate nucleus, and frontal white matter. For each ROI, mean z-scores relative to the control cohort were computed across time points. Slopes of z-score change were estimated to quantify regional washout rates, with values indicating the rate of movement toward normative values over time. Statistical significance was assessed using linear mixed-effects models with false discovery rate (FDR) correction for multiple comparisons. **Results:** The longitudinal analysis of R_1 revealed a significant effect of time on Mn deposition reduction across ROIs ($p < 0.0001$), and a significant ROI $\times$ time interaction ($p = 0.047$), indicating regional variability in clearance dynamics. Variance-standardized slopes ($SD \cdot month^{-1}$) showed that the globus pallidus exhibited the fastest recovery, with z-scores decreasing at 0.30 SD per month toward normative values. Frontal white matter and anterior putamen also demonstrated significant washout after FDR correction, though at more modest levels. In contrast, the posterior and caudate nucleus showed slower and less consistent recovery. **Conclusions:** Mn clearance in the human brain is regionally heterogeneous following reduced exposure. The globus pallidus showed the fastest and most robust Mn clearance, with frontal white matter and anterior putamen also demonstrating significant recovery, while the caudate nucleus and posterior putamen exhibited slower, non-significant changes. These findings are consistent with prior nonhuman primate data, which also reported preferential Mn accumulation and regionally variable clearance within the basal ganglia. Future work will use subject-specific prediction maps, derived from controls with each welder's age and sex, to generate voxel-wise z-scores. Using a regional brain atlas, this approach will enable more precise characterization of Mn clearance kinetics across brain structures. Overall, this study provides the first *in vivo* evidence of regional Mn clearance dynamics in the human brain and underscores their potential relevance for understanding and monitoring metal-related neurotoxic disorders.

PS 3092 Manganese Exposure Reveals Sex Differences in Stress-Induced Drug Seeking

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Background and Purpose: Manganese (Mn) is an essential trace element that serves as a cofactor for several biological pathways. Optimal Mn exposure follows a hormetic curve, where both deficiency and overexposure cause neurological dysfunction. Overexposure may occur through occupational sources, drinking water, or air pollution. Chronic Mn exposure, even at subtoxic levels, leads to accumulation in dopaminergic brain regions, disrupting dopamine homeostasis. Dopamine, a key neurotransmitter released by dopaminergic neurons, is central to reward-seeking behavior through the mesolimbic pathway. When this pathway is dysregulated, the brain's ability to process and respond to rewards is compromised, forming the underlying mechanism of addiction. The mesolimbic pathway, which projects dopamine

from the ventral tegmental area (VTA) to the nucleus accumbens, hippocampus, amygdala, and cortex, is central to the brain's reward circuitry. However, its susceptibility to Mn exposure and role in drug-seeking behavior are poorly understood. This study investigates how chronic dietary Mn exposure affects dopaminergic activity and drug-seeking behavior. **Methods:** Six-week-old C57BL/6J mice were placed on either a control diet (70 ppm Mn, n=36; 18 males, 18 females) or a high-Mn diet (2400 ppm Mn, n=36; 18 males, 18 females) for six weeks. After dietary exposure, mice completed a behavioral battery including conditioned place preference (CPP), forced swim, and open field activity. CPP was conducted in a two-compartment chamber with restricted access between sides. During the pretrial, each mouse's chamber preference was recorded. Mice then received alternating IP injections of cocaine (10 mg/kg) on the nonpreferred side (afternoon) and saline on the preferred side (morning) for four days. On the CPP test day, mice had open access to both compartments without drug administration to assess conditioned preference. Daily testing continued until extinction, followed by a 10-minute forced swim test to induce stress, after which CPP was reassessed to measure reinstatement of drug-seeking behavior. After CPP, all mice underwent open-field testing to measure cocaine-induced hyperactivity. Each mouse received an IP saline injection, followed 30 minutes later by cocaine at 5 mg/kg and 10 mg/kg to assess Mn effects on cocaine-induced hyperactivity. Following behavioral testing, cortical and striatal tissues were collected for molecular analysis. RNA from striatal tissue was converted to cDNA for RT-qPCR using GAPDH and 18S as housekeeping genes. Genes of interest included metal ion importers (Zip8, Zip14, DMT1), exporters (Fpn1, Znt10), and the dopamine transporter (DAT). HPLC quantified cortical dopamine metabolites, and ICP-MS validated Mn accumulation in high-Mn versus control mice. **Results:** During CPP extinction, no significant behavioral differences were observed between high-Mn and control mice of either sex. Similarly, open field testing revealed no group differences in cocaine-induced hyperactivity. However, during stress-induced reinstatement, females exhibited stronger reinstatement of preference for the cocaine-paired environment than males, regardless of diet. This suggests Mn exposure did not broadly alter CPP behavior but highlighted increased female vulnerability to stress-induced relapse. Gene expression analyses showed a general trend toward higher Mn importer expression (Zip8, Zip14, DMT1) in high-Mn males, while exporter expression (Fpn1, Znt10) remained stable across groups. ICP-MS confirmed increased Mn accumulation in high-Mn mice, and HPLC revealed elevated dopaminergic metabolites in high-Mn males. **Conclusions:** These findings refine our understanding of how chronic, subtoxic Mn exposure influences reward circuitry and relapse-related behaviors. While Mn did not cause broad changes in cocaine-conditioned preference or locomotor activity, the pronounced female-specific reinstatement effect indicates potential sex-dependent vulnerability to relapse. The molecular profile, with increased importer but unchanged exporter expression, suggests Mn accumulation may sensitize striatal neurons to altered homeostasis even without major behavioral changes. Together, these results indicate that chronic Mn exposure interacts with sex and stress to reveal hidden vulnerabilities, with implications for environmental neurotoxicology and addiction biology.

PS 3093 Mixed Metal Oxide Exposure Drives Astrocytic Activation *In Vitro* and *In Vivo*

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Background and Purpose: Approximately 50 million people worldwide are affected by neurodegenerative diseases (NDD), with this number predicted to double in the next 50 years. Metal imbalance is a key characteristic of many of these NDDs, which may potentially be driven by environmental metal exposures. Epidemiological studies have implicated metals such as iron (Fe) and manganese (Mn) in NDD pathophysiology. However, human exposures to metals are rarely singular; rather, people are exposed to complex metal mixtures. The role of metal mixture in driving NDDs is underexplored. Astrocytes have been repeatedly associated with metal exposure-associated NDDs. These cells play a key role in metal homeostasis in the brain, but metal overexposure can push astrocytes from homeostatic to a pathological state. In this pathological state, astrocytes can directly or indirectly damage surrounding neurons, leading to cell death and further promotion of pro-inflammatory activity in the brain, both key features of many NDDs. This astrocytic reactivity has been highlighted in traditional models of metal exposure, which utilize individual soluble metal exposure, such as MnCl₂ and FeCl₂ (i.e., metal chlorides). However, occupational human metal exposure studies done in welding sites have characterized the metal particles in the air, which workers are exposed to, as metal oxides. These are insoluble and potentially participate in different interactions between astrocytes and biochemical processes. Additionally, these studies have rarely considered the implications of metal mixtures, further limiting our understanding of the role of mixed metal oxide exposures in neurodegeneration. **Methods:** First, to understand the astrocytic response to mixed metal exposure, we conducted both *in vitro* and *in vivo* studies utilizing occupationally relevant metal exposures. We were able to show that the characteristics of the astrocytic response depended on oxidation state of the metal - metal oxides versus metal chloride exposures. Exposure to metal oxide mixtures resulted in increased mitochondrial deficits and inflammatory markers in comparison to the metal chloride mixture exposure. This highlights the importance of replicating human exposures by matching both the chemical species and mixture composition, as these factors seem to modify astrocytic responses. Second, we explored the

effects of mixed metal oxide inhalation on neurodegeneration. We developed a novel sub-acute exposure model that was capable of recapitulating the characteristics of welding inhalation exposures. Exposure to mixed metal oxides in our rodent model led to astrocytic activation in a region-specific manner, which was demonstrated in both small animal MRI and immunohistochemistry (IHC). Furthermore, these regions can be functionally associated with the behavioral alterations we have characterized. **Results:** This work has demonstrated a cell-specific vulnerability of astrocytes to mixed metal oxide exposure and the regional sensitivity in inhalation exposures. **Conclusions:** This project is an important step in promoting the study of the neurodegenerative consequences of complex metal mixtures, as well as the consideration of chemical speciation in astrocytic metal handling and senescence induction. These are essential questions in developing translationally relevant models and furthering our understanding of the role of environmental metals in neurodegenerative diseases and aging.

PS 3094 Low Concentrations of Hexavalent Chromium in Drinking Water Induce Oxidative Damage in Endothelial Cells: Using a Toxic Aging Coin to Assess Cr(VI) Impacts on the Blood-Brain Barrier

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Background and Purpose: Hexavalent chromium [Cr(VI)] is a widespread environmental pollutant that was recently identified as the #1 chemical hazard in drinking water in the U.S. Cr(VI) contaminates drinking water from geogenic sources (e.g. ultramafic bedrock), industrial effluents, and aging cast iron or steel water pipes. The WHO and EPA have established maximum contaminant levels (MCLs) for total Cr at 0.05 and 0.1 mg/L, respectively. While Cr(VI)'s health effects have been identified in other peripheral tissues, significant knowledge gaps exist regarding its neurotoxicity. Compounding this, we are facing an aging crisis, with 20% of the U.S. population projected to be geriatric (65+) by 2030, yet we have a poor understanding of how geriatric exposures contribute to health outcomes. To combine aging and toxicology research, we use a Toxic Aging Coin approach to investigate: 1) how age impacts toxicity (*heads*), and 2) how chemicals accelerate aging (*tails*). We recently reported Cr selectively accumulated in the hippocampus of rats exposed to levels of Cr(VI), especially middle-aged and geriatric females, and induces essential metal dyshomeostasis with distinct age and sex differences. Here, we investigate how Cr(VI) damages the blood-brain barrier (BBB), a critical structure to protect the brain parenchyma. Within the BBB, endothelial cells create tight junctions that permit selective transport of key materials into the brain while preventing penetration of noxious materials. Astrocyte end-feet provide further structural and functional support. The BBB naturally becomes leakier with age and chronic inflammation and/or oxidative damage can accelerate BBB deterioration. We hypothesize that Cr(VI) damages the BBB by inducing oxidative damage in endothelial cells. **Methods:** We exposed Sprague-Dawley rats (both sexes) from three ages (3-, 7-, and 18-months-old) to low concentrations of Cr(VI) in drinking water (0.05 and 0.1 mg/L) for 90 days. After 90 days we harvested the brains and one hemisphere was preserved to assess BBB integrity and protein expression in the dorsal hippocampus by immunofluorescence (i.e., Tissue Plasminogen Activator [tPA], and glial fibrillary acidic protein [GFAP]). GFAP marks astrocytes and can indicate astrocyte activation. tPA converts plasminogen to plasmin, a serum protease linked to inflammation, and decreased tPA expression is linked to aging. 8-hydroxy 2'-deoxyguanosine (8-OHdG) was used to assess oxidative damage in endothelial and astrocytic nuclei. Female rats were selected for tPA analyses and male rats were selected for 8-OHdG expression analysis. Protein expression and 8-OHdG levels were quantified via ROI analyses and data were reported as fold-change relative to control. **Results:** tPA expression was significantly decreased in the hippocampus of young female rats, middle-aged female rats exposed to 0.1 mg/L, and geriatric female rats. GFAP expression around capillaries was significantly increased in middle-aged female rats exposed to 0.1 mg/L but significantly decreased in geriatric female rats. Preliminary data indicate that Cr(VI) induces a concentration-associated increase in 8-OHdG levels in endothelial cell nuclei of young and geriatric male rats. **Conclusions:** Cr(VI) likely damages the BBB in the process of entering the brain. Cr(VI) induces changes in GFAP expression at the capillary level with distinct age differences, with increased expression in middle-aged females and decreased expression in geriatric females. Decreased tPA expression suggest BBB dysfunction and may indicate an accelerated aging. Additionally, increased 8-OHdG levels in endothelial nuclei of male rats indicate Cr(VI) induced oxidative DNA damage, which can contribute to genomic instability if left unrepaired. Together, these results indicate accelerated BBB aging. Future directions will further examine and clarify Cr(VI)'s effects on the BBB, including assessment of 8-OHdG levels in both endothelial cells and astrocytes in female rats. Support from R21ES033327 (JPWJr), T32ES011564 (STV), University of Louisville Summer Research Opportunity Program (WJB).

PS 3095 *In vivo* repressor screen identifies dopaminergic and glutamatergic neurons as targets of manganese toxicity

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Background and Purpose: Manganese (Mn) is an essential micronutrient, but in excess, it is neurotoxic. Under conditions of over-exposure, Mn builds up in the basal ganglia of the brain and induces motor deficits, but the specific neuronal targets of Mn are unclear. Knockout studies have revealed that selective loss of the Mn efflux transporter SLC30A10 in dopaminergic or glutamatergic, but not GABAergic, neurons leads to increased Mn in targeted neurons and early-life motor deficits in mice. These findings indicate that the activity of SLC30A10 in dopaminergic or glutamatergic neurons is required to protect against Mn toxicity. **Methods:** Knockout studies raised the hypothesis that Mn targets dopaminergic and glutamatergic, but not GABAergic, neurons of the basal ganglia. To definitively determine the neuronal targets of Mn, we generated dopaminergic-, GABAergic-, or glutamatergic-specific Slc30a10 knockin mice to selectively increase Slc30a10 in targeted neurons. Knockin of Slc30a10 was driven by Cre recombination and targeted to the mROSA 26 locus. Knockin mice and control littermates received Mn treatment from birth and were run through neurobehavioral assays at early-life time-points. Metal level analyses were performed using ICP-MS and laser-ablation ICP-MS. **Results:** Results showed that neuron-specific Slc30a10 knockin mice have increased expression of Slc30a10 in targeted neurons and have attenuated Mn levels in targeted neurons after Mn exposure. Behavioral assays revealed that dopaminergic- and glutamatergic-, but not GABAergic-specific, Slc30a10 knockin mice are protected from early-life Mn-induced motor deficits after exposure compared to control littermates. **Conclusions:** These findings show that Mn targets dopaminergic and glutamatergic neurons of the basal ganglia to induce motor disease.

PS 3096 Arsenic Exposure Increases Anxiety-Like Phenotypes in Female Mice and Decreases Microglial Responsiveness to Pathogens

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Background and Purpose: Anxiety afflicts more than 300 million people worldwide and is associated with a 3-fold higher risk of dementia. Until recently, efforts to clarify disease etiology have often neglected environmental drivers that are increasingly linked to neuropsychiatric and neurocognitive disorders. Arsenic (As) is a common environmental toxicant that is strongly associated with anxiety and cognitive impairment. However, the mechanisms by which As contributes to neurobehavioral dysfunction remain incompletely understood. **Methods:** Using a battery of behavioral tests and hormonal assessments, our studies examine As-induced behavioral changes in female mice exposed from adolescence to early adulthood. This included open field, light/dark box, fear conditioning, and acute restraint. We also examined how As disrupts microglial function in SIM-A9 cells while in the presence or absence of inflammatory challenges. **Results:** As increased anxiety-like behavior during cued fear conditioning; moreover, As increased corticosterone secretion during acute restraint, indicating a hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis. In addition, As attenuated pro-inflammatory cytokine expression in microglia challenged with an inflammatory stimulus. This effect was consistent whether the stimulus was lipopolysaccharide, interferon-gamma, or tumor necrosis factor-alpha, though the magnitude of change differed. **Conclusions:** As exposure induces an anxiety-like phenotype in female mice through disruption of the HPA axis. As also causes inhibition of normal microglial responses to pro-inflammatory stimuli. Studies are underway to decipher the mechanisms of As-induced neurotoxicity using brain slice electrophysiology and other approaches in order to empower interventions to address the deleterious impacts of this common threat to human health.

PS 3097 A Quantitative Cell Painting Assay to Evaluate Organoid Viability and Morphodynamics During Acute Metal Exposure

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Background and Purpose: Developmental neurotoxicity from exposure to heavy metals is a significant public health concern, and there is still much to be learned about their full neurological risk profiles and mechanisms of action. Microphysiological systems, such as organoids, are powerful *in vitro* models that recapture organ cytoarchitecture and higher-order functions. As such, they are highly valuable for toxicological screening and mechanistic studies. However, their increased cost and technical complexity often limit throughput compared to traditional cellular assays. *MortaLIVE* is a commercially available, biologically inert fluorescent dye that simultaneously detects both live and dead cells in live cell cultures, enabling viability monitoring over time. Our goal was to optimize the use of this cell

painting dye for organoids and assess whether *MortaLIVE* could provide not only an efficient measure of organoid viability over time but also be integrated with other functional assays to increase experimental output from a limited number of samples. We therefore conducted an acute exposure study using neural organoids treated with cadmium (Cd), hexavalent chromium (Cr), a Cd+Cr mixture, and lead (Pb) to assess the utility of *MortaLIVE* in complex *in vitro* models. **Methods:** Following 8 weeks of culture, human iPSC-derived neural organoids were attached to a glass-bottom black plate and exposed for 72 hours to a concentration range (from 0.32 μ M to 50 μ M) of each metal (n=4 per condition). Viability was assessed using the Yokogawa high-content live-imaging platform following treatment with *MortaLIVE*. In parallel, neural functionality in the form of neurite and glial outgrowth from the organoids was quantified by live-tracking high-contrast cell projections. Both viability and outgrowth results were validated using previous *in vitro* assays established at our lab: resazurin reduction for cytotoxicity and immunostaining with neuronal and glial markers for neurite and glial migration. **Results:** Concentration-response curves were generated for all metals with *MortaLIVE*, and the results were consistent with the resazurin assay. Acute metal exposure produced a dose-dependent increase in cytotoxicity. The relative neurotoxicity of the metals followed the trend Cd+Cr > Cd > Cr > Pb. Use of this *MortaLIVE* cell painting assay presents an advantage over classical viability/cytotoxicity assays such as a resazurin reduction assay or LDH release as: (a) it is not terminal, cells can be washed and further cultured and (b) one organoid per replicate is needed, while at least 10 organoids per replicate would be necessary to reach sufficient sensitivity in a resazurin-based assay. In addition, labeling of the organoids allowed neurite and glia outgrowth to be tracked in real time and quantified from the same samples. Interestingly, the Cd+Cr mixture appeared to exhibit a less-than-additive effect on both cytotoxicity and outgrowth impairment. The interaction between these two metals in neurological systems merits further exploration. **Conclusions:** We developed and validated a *MortaLIVE*-based assay that enables rapid assessment of cell viability and functionality in organoid models. Because the dye is biologically inert and preserves sample integrity, it also permits quantification of morphological features and subsequent use of samples in additional functional assays. Although demonstrated here in neural organoids, this approach is broadly applicable to other microphysiological systems.

PS 3098 Metabolic disruptions in the cerebellum of welders co-exposed to Manganese and Iron

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Background and Purpose: Exposure to welding fumes, containing Manganese (Mn) as well as Iron (Fe), is a well-established neurotoxic risk. Mn neurotoxicity has been associated with motor dysfunction, with higher exposure relating to worse Unified Parkinson's Disease Rating Scale (UPDRS) scores. While most studies have focused on the basal ganglia, the cerebellum, central to motor control and coordination, may also play a role in motor impairment. Using short-TE semi-LASER (sLASER) magnetic resonance spectroscopy (MRS), we examined associations between metal exposure and cerebellar metabolites: N-acetylaspartate (NAA), myo-inositol (mIns), glutamate (Glu), total creatine (tCr), and choline (Cho) as biomarkers of neuronal integrity and energy metabolism. Glu was of particular interest, as Mn disrupts astrocytic glutamate transport and glutamate-glutamine cycling, leading to excitotoxic dysregulation. Given Mn and Fe's shared transport and regulatory pathways in the brain, we hypothesized that cumulative Mn/Fe exposure alters cerebellar metabolism and contributes to motor dysfunction in welders. **Methods:** As part of a larger imaging study, 45 welders and 22 factory controls were recruited from a U.S. truck manufacturing facility. ¹H MRS was acquired on a 3 T Siemens MAGNETOM Prisma using sLASER (TE/TR = 35/2000 ms; 64 averages) with a 35x25x25 mm³ volume of interest (VOI) in the right cerebellar lobe. Spectra were processed and quantified using Osprey and LCModel; metabolite fits with SD% > 15% were excluded. Motor function was assessed with UPDRS-part III, from which rigidity and tremor composite scores were derived. Personal air sampling informed cumulative metal exposure indices (CEI) for lifetime (since age 18; Mn_{cumul}, Fe_{cumul}). Group differences were tested with Wilcoxon rank-sum tests; multivariable linear regressions examined correlations between exposure, metabolites, and motor outcomes. **Results:** Cumulative Mn and Fe exposures were significantly higher in welders than controls (Mn_{cumul} (mg/m³-yr): 1.12 ± 1.80 vs 0.10 ± 0.10, p < 0.001; Fe_{cumul} (mg/m³-yr): 7.52 ± 11.60 vs 0.80 ± 0.80, p < 0.001). Despite these exposure differences, cerebellar metabolite levels differed only in mIns between groups (p < 0.05). **Metabolite-Exposure Relationships:** Using each metabolite as the dependent variable, we modeled age, sex, Mn_{cumul}, and Fe_{cumul} as predictors. In controls, cerebellar Glu was negatively correlated with age (p < 0.05) and positively correlated with Mn_{cumul} (adj.R² = 0.60, p < 0.01), consistent with preserved glutamatergic regulation at low exposure levels. Fe_{cumul} was positively correlated with tCr (adj.R² = 0.40, p < 0.05) and Cho (adj.R² = 0.31, p < 0.05). In welders and in the combined cohort, no metabolite-exposure correlations were significant. **Metabolite-Motor Relationships:** Using UPDRS-III as the dependent variable, we modeled age, sex, and all cerebellar metabolites as predictors. In welders, this model explained 44% of the variance (adj.R² = 0.44, p < 0.001); older age (p < 0.001) and lower tCr (p < 0.05) predicted worse motor function. In controls, no metabolites significantly predicted UPDRS scores. In the combined cohort, the model remained significant (adj.R² = 0.30, p < 0.001), with age (p < 0.001), sex (p = 0.05), and lower tCr (p < 0.001) predicting worse performance. For rigidity, welders showed moderate negative correlation with NAA and moderate positive correlation with age and mIns.

In the combined cohort, rigidity increased with age, sex, and mIns, and decreased with tCr. For tremor, only age was a significant positive predictor. **Conclusions:** Cumulative Mn/Fe exposure predicted cerebellar metabolites only in controls: Mn_{cumul} predicted Glu and Fe_{cumul} predicted tCr and Cho. The absence of a Glu exposure relationship in welders may reflect disrupted glutamatergic regulation under chronic Mn/Fe exposure; by contrast, controls showed the expected age-related Glu decline, suggesting reduced glutamatergic turnover with aging. No other metabolites showed significant exposure relations. Motor impairment in welders appeared primarily age-related, with lower tCr (reduced energy reserve), lower NAA (neuronal integrity), and higher mIns (gliosis) associated with worse performance. Overall, cerebellar energy and glial alterations, rather than direct glutamatergic changes, may represent a secondary pathway for Mn/Fe-related motor effects.

3099 Comparison of welding fume exposure metrics in relation to motor function

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Background and Purpose: Particles released from the welding process often contain metals such as manganese (Mn) and iron (Fe). Continuous exposure to Mn in welders has been linked to deficits in motor function, and specifically to increased scores in the Unified Parkinson's Disease Rating Scale (UPDRS) - Part III. Yet, literature uses many different forms of exposure metrics, ranging from years of welding to complex cumulative exposure indices (CEIs). For a study on Mn neurotoxicity in welders, we have recently developed refined short-term and long-term CEIs that are based on personal air sampling during a work shift and a work history questionnaire and take into account changes in department, as well as past jobs that included welding. In this study, seven different exposure metrics, years-of-welding (YW), Mn and Fe air levels, CEI for past three months (CEI_{3m}) and cumulative CEI (CEI_{cumul}) for both Mn and Fe were compared 1) to determine group differences between welders (W), non-welding factory controls (FC), and non-exposed university controls (UC) and 2) to explore relations with UPDRS scores for each of these exposure metrics. **Methods:** A total of 78 subjects (37 welders, 28 FCs - age matched to the welders, and 12 UCs) was recruited from a trailer manufacturing company in the USA. Years of welding were based on months of welding, but did not take part-time jobs or the type of welding into account. Personal air sampling was conducted following NIOSH Method 0600 and the metals collected on the filters were analyzed using inductively coupled plasma-optical emission spectroscopy (ICP-OES). Mn_{air} and Fe_{air} were determined and used to calculate CEI_{3m}(Mn), CEI_{3m}(Fe), CEI_{cumul}(Mn), and CEI_{cumul}(Fe). Specifically, CEI calculations incorporated welding hours and days per week, respirator use, as well as prior jobs using weighting factors. The UPDRS-III was assessed by a trained specialist. Independent t-tests were used for group comparisons, and linear regression models including age as covariates were run to predict UPDRS-III as a dependent variable for each of the exposure metrics as predictor. **Results:** The three groups did not differ significantly in age, but had different variances in all variables. UPDRS-III scores were 6.7±4.3 (W), 5.1±3.6 (FC), and 1.9±1.6 (UC) and were only significantly different between W and UC, as well as FC and UC (p<0.0001), but not between W and FC. Mean Mn air levels for the three groups were 220±232 µg/m³ (W), 8.0±10.3 µg/m³ (FC), and 0.07±0.06 µg/m³ (UC). All 7 exposure metrics, YW, air levels, and CEIs, found a significantly higher exposure to both Mn and Fe in welders compared to either control group (all p<0.005). Noteworthy, all exposure metrics but YW also found a significantly increased exposure in FC compared to UC (all p<0.01). In the linear mixed models for UPDRS-III, age was always a significant predictor for all models. In welders, YW was also a significant predictor (p<0.05) of UPDRS. Air levels as well as CEI_{3m} were only significant predictors of the UPDRS across all subjects, not within welders or FC alone, for both Mn and Fe. In contrast, CEI_{cumul} were more significant predictors of UPDRS-III than age both in welders alone (p<0.04 for Mn and <0.02 for Fe) as well as across all subjects (p<0.00005), but not in FC alone. **Conclusions:** These results confirm once again that chronic exposure to welding fumes, particularly Mn and Fe, contributes to measurable motor function impairment, in addition to age. Moreover, these findings demonstrate that the group of "factory controls", mostly assembly workers working in the same large factory hall as the welders, experience a significant co-exposure to welding fumes, which would not be picked up when using only years of welding as an exposure metric. This explains that the difference in motor function, while still worse in welders than FC, does not reach significance. This furthermore suggests that the parent study would benefit from a larger and age-matched "university control group" of truly non-exposed subjects. Finally, UPDRS-III scores are not predicted by short-term exposure metrics, such as air levels or past-3-months CEIs, but only by long-term CEIs, such as CEI_{cumul}. This is true for both Mn and Fe. However, Mn and Fe exposure are highly correlated and thus the role of Fe exposure through welding fumes will need to be further explored.

3100 Role of brain HIF signaling in motor function and manganese neurotoxicity

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Background and Purpose: Excess Mn accumulates in the basal ganglia of the brain, which leads to neurological disease. Our prior work identified SLC30A10 as a Mn-specific efflux transporter that protects against Mn toxicity. We discovered a fundamental role for the HIF signaling pathway in regulating Mn homeostasis via transcriptional activation of SLC30A10. Recent data from our group further suggests that there are organ-specific effects of Mn-induced HIF activation. However, the definitive role of HIF activation in the brain is unclear. **Methods:** In this study, we sought to determine the role of neuronal HIF signaling in response to elevated Mn. We generated several cell-specific HIF1α and HIF2α knockout mice lines targeting key neuron types in the basal ganglia: dopaminergic, GABAergic, or glutamatergic neurons. Knockout mice and littermate controls were treated with Mn modeling human environmental exposure. Mn-induced neuromotor dysfunction was measured using balance beam and open field behavioral assays at four weeks of age. **Results:** Dopaminergic knockout of HIF1α did not impact Mn-induced sensorimotor dysfunction as measured by the balance beam assay, whereas in the open field assay, Mn-treated knockout mice moved more and rested less than Mn-treated littermate controls. Glutamatergic knockout of HIF1α did not impact Mn-induced motor dysfunction. However, untreated knockout mice moved more slowly to cross the balance beam, and moved less and rested more in the open field assay compared to untreated littermate controls, suggesting a glutamatergic role of HIF1α in basal motor function. Pan neuronal and glial knockout of HIF2α also impacted basal motor function; untreated knockout mice moved less and rested more in the open field assay compared to untreated littermate controls. In addition, whereas Mn treatment caused decreased movement in control mice, Mn treatment did not cause further motor deficits in HIF2α knockout mice. **Conclusions:** Our results suggest that both HIF1α and HIF2α play a role in basal motor function, while their role in Mn-induced motor deficits is uncertain.

3101 Sex-Specific Hippocampal Metabolomic Perturbations Following Chronic Hexavalent Chromium Exposure in Guinea Pigs

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Background and Purpose: Hexavalent chromium [Cr(VI)] is a ubiquitous environmental pollutant and potent toxicant, currently ranked as the number one chemical hazard in US public drinking water. To date, most mechanistic data describing Cr(VI) toxicity come from lung, liver, and kidney tissues. While these studies have yielded extensive datasets, there remains comparatively little information on neurotoxicity. Even less is known about how pathways of Cr(VI) neurotoxicity may vary by sex. This study assessed how chronic Cr(VI) exposure induces distinct metabolic signatures in the hippocampus that reflect oxidative stress, altered energy metabolism, and neurotransmitter disruption, and how these signatures differ between male and female guinea pigs. **Methods:** Hartley guinea pigs were given either tap water or 5 mg/L Cr(VI) in drinking water for 90 days. Hippocampal tissues were collected and processed for untargeted metabolomics and metalomics. Inductively coupled plasma-mass spectrometry was conducted to determine hippocampal Cr accumulation. Statistical analyses used the Mann-Whitney test with significance set at p < 0.05. **Results:** Hippocampal metabolomics from Hartley guinea pigs exposed to 5 mg/L Cr(VI) in drinking water for 90 days revealed distinct metabolite profiles that differed markedly between males and females. In males, Cr(VI) exposure was associated with a broad loss of metabolic reserve capacity: creatine levels were lower and glyceric acid levels were also reduced, suggesting a diminished ability to buffer energy demand. There were reductions in several amino acids that support neurotransmitter synthesis, including lower GABA, glutamate, and histidine, suggesting a weakened ability to sustain synaptic signaling. Males displayed decreased membrane-building precursor molecules such as O-phosphorylethanolamine and neuraminic acid, implying stress on membrane synthesis. Levels of antioxidant-linked intermediates such as γ-glutamyl-2-aminobutyric acid and phenyl-lactate were lower, consistent with a reduced redox response. N1-acetylsermidine decreased, indicating reduced polyamine turnover, which is important for stress resilience and synaptic plasticity. Cr(VI) exposure in females was accompanied by increased dipeptides and increased glutamate, lysine, O-phosphorylethanolamine, and neuraminic acid, indicating amino acid and membrane-related support pools are increased in response to Cr(VI) exposure. Females displayed coordinated decreases in major myelin-associated lipid classes, including lower levels of ceramides, sphingomyelin, and galactocerebrosides. This pattern of lipid loss is consistent with vulnerability of white-matter-related lipid structures and may indicate stress to myelin integrity. **Conclusions:** Chronic Cr(VI) exposure induced distinct hippocampal metabolomic fingerprints between sexes. Male guinea pigs exhibited broad depletion of metabolic substrates that normally support energy buffering, neurotransmitter cycling, antioxidant defense, and membrane synthesis. Meanwhile, females exhibited increases in amino acid metabolites but coordinated losses in myelin-associated lipids. Together, these results demonstrate chronic Cr(VI) exposure produced distinct metabolic response patterns in the hippo-

campus between sexes. The hippocampus is a key structure for learning and memory, making these metabolic shifts relevant to functional neurotoxicity. These metabolic shifts align with prior Cr(VI) findings, including disrupted GABA/glutamate pathways and evidence of myelin-related vulnerability. Further, these results underscore the importance of evaluating sex as a biological variable for brain susceptibility to environmental toxicants. Support from R35ES032876 (JPWSr.), "la Caixa" Foundation LCF/BQ/EU22/11930060 (IM), T32ES011564 (STV, MD, GCG), R21ES033327 (JPW), and USDA Multistate Research Project W5045 (JTFW).

PS 3102 From Exposure to Emotion in Welders: Does Metabolism in the Anterior Cingulate Cortex Play a Role?

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Background and Purpose: Despite the function of manganese (Mn) and iron (Fe) as critical nutrients, chronic exposure to these metals can lead to their accumulation in the brain, disrupting normal metabolite levels and metal metabolism. This has been linked to increased psychological distress, including depression and anxiety, particularly among individuals with higher cumulative manganese exposure. Mood and emotional regulation are closely tied to metabolism, particularly in the Anterior Cingulate Cortex (ACC), the brain region that plays a pivotal role in cognition and mood. This study sought to evaluate how exposure to metals found in welding fumes, particularly Mn and Fe, affect metabolite concentrations in the ACC. The study further investigated how these neurometabolic changes mediate the relationship between metal exposure and psychological domains, examining correlations between exposure and metabolites as well as metabolites and mood. **Methods:** We studied 35 career welders and 29 non-welding factory controls. All participants obtained a single-voxel ¹H Magnetic Resonance Spectroscopy (MRS) exam on a 3T Siemens Prisma scanner of the Anterior Cingulate Cortex (ACC) using short-TE semi-LASER (sLASER) to quantify metabolites: N-acetylaspartate (NAA), total creatine (tCr), choline (Cho), myo-inositol (mlns), and glutamate (Glu). Participants completed the Brief Symptom Inventory (BSI; 9 symptom domains) yielding summary scores for each domain, as well as the Global Severity Index (GSI). Personal air sampling during one work shift and a work history questionnaire were used to calculate cumulative exposure indices (CEIs) for Mn and Fe. Group differences in exposure levels, ACC metabolite concentrations, and BSI outcomes were tested with two-tailed Student's t-tests. Associations were evaluated using Spearman rank correlations. Statistical significance was defined as $\alpha = 0.05$. **Results:** Welders had significantly higher exposure indices for Mn and Fe than controls. However, no significant differences were observed for any of the ACC metabolites between the groups. Both groups reported high levels of mood symptoms, indicating comparable levels of psychological distress on self-reported rating scales. In welders, a moderate negative correlation was identified between NAA and Fe cumulative exposure index (FeCEI) ($R = -0.355$, $p = 0.036$) as well as MnCEI ($R = -0.373$, $p = 0.027$). Cho presented a moderate positive correlation with FeCEI ($R = 0.337$, $p = 0.048$). Correlations between MnCEI and Cho trended in the positive direction, while MnCEI vs. Glu, and FeCEI vs. Glu trended in the negative direction. In FC, no correlation between exposure and metabolites was found. However, moderate positive correlations were observed between NAA and the domains of Obsession-Compulsion ($R = 0.436$, $p = 0.042$), Hostility ($R = 0.467$, $p = 0.029$), and Psychoticism ($R = 0.467$, $p = 0.028$). Similarly, Glu showed moderate positive correlations with Obsession-Compulsion ($R = 0.437$, $p = 0.042$) and Depression ($R = 0.440$, $p = 0.041$). Strong positive correlations were found between Cho and Psychoticism ($R = 0.664$, $p < 0.001$) as well as between Glu and Anxiety ($R = 0.657$, $p < 0.001$). Correlations between metabolites and GSI showed significance only in Glu ($R = 0.464$, $p = 0.03$). **Conclusions:** Cumulative Mn and Fe exposure indices showed a negative correlation to NAA levels in welders, but not factory controls, indicating that higher exposure to these metals is linked to decreased NAA levels in the ACC, possibly due to damage or loss of healthy neurons. In welders, increased Fe levels are linked to elevated Cho. This may reflect increased cell membrane turnover or activation of glial cells in response to metal exposure. Study results suggest that higher metabolite levels in the ACC are linked to greater psychological distress across several symptom domains. However, the strong positive correlation between Glu and anxiety is not consistent with typical findings. Overall, this data does not suggest that any of the metabolites investigated in the ACC in this study play a mediating role between welding fume exposure and mood. Future studies may need to include whole brain MRS approaches to study the role of brain metabolism in mood changes associated with welding fume exposure.

PS 3103 Symmetry of Quantitative MRI Indicators of Brain Manganese and Iron and Relation to Welding Fume Exposure, Mood and Motor Function

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Background and Purpose: Chronic occupational manganese (Mn) exposure can cause adverse health effects such as deficits in motor and cognitive function, as well as mood disturbances. Welders exposed to Mn through welding fumes, even at low levels, have been shown to accumulate Mn in the brain and perform worse

than controls on tests of motor function. Quantitative Magnetic Resonance Imaging (qMRI) quantifies the relaxation rates R1 and R2*, altered by paramagnetic Mn and iron (Fe), respectively. As part of a larger neuroimaging study in welders, this analysis investigated whether Mn and Fe deposition was symmetric in the human brain, and if qMRI changes in specific brain regions related to exposure metrics of Mn and Fe, mood or motor function. **Methods:** A total of 104 subjects, consisting of 53 career welders and 51 non-exposed, age-matched controls, participated in this imaging study after giving written informed consent. qMRI data was acquired on a Siemens 3T Prisma scanner and reconstructed to R1 and R2* maps, allowing us to explore regions-of-interest (ROIs) from major brain structures, including the caudate nucleus (CN), globus pallidus (GP), and putamen (Put). Each ROI value was compared between left or right brain regions using a two-tailed t-test with two-sample equal variances to test for symmetry. Motor function was assessed through the Unified Parkinson's Disease Rating Scale (UPDRS) Part III. The Brief Symptom Inventory test is a self-administered survey on 9 mood domains, summarized by the Global Severity Index (GSI) score. Cumulative and short-term (past 3 months) exposure indices (CEIs) for Mn and Fe were estimated using personal air sampling and a work history questionnaire. Group differences for mean ROI values, CEIs, GSI data, and UPDRS scores were explored using two-tailed t-tests with unequal variances. Spearman correlations were used to test correlations of qMRI values with exposure, UPDRS and GSI scores. **Results:** All ROIs were found to be symmetric. All exposure metrics showed a significant group difference for welders versus controls. Consistent with higher Mn deposition, welders showed lower T1 ($=1/R1$) than controls across all basal ganglia: CN ($p = 0.007$), GP ($p < 0.0001$), and Put ($p < 0.05$). R2* showed no group differences. When considering all subjects, R1 did not correlate with R2* in any ROI, whereas R2* correlated strongly across regions. In all ROIs, R2* related to age ($p < 0.001$) and to cumulative CEIs (all $p < 0.04$). In the GP, R1 was not correlated to age in the pooled analysis but showed a positive correlation with both short- and long-term exposure metrics ($p > 0.4$, $p < 0.001$ for all). In welders alone, this correlation was only a trend for short-term CEI, but still significant for Mn and Fe air levels and cumulative CEIs. Interestingly, in welders, R1 was also positively correlated with age. Significant symptom associations were found for R1 in the GP with GSI ($p = 0.024$), which vanished in the pooled analysis, and for R1 and R2* in all ROIs with UPDRS ($p < 0.01$) both in all subjects and welders alone. **Conclusions:** Mn and Fe brain deposition across all ROIs were found to be symmetric, allowing us to use bilateral means of each ROI for analysis. In the analysis of welders versus controls, T1 was significantly lower across all basal ganglia ROIs for the welding group, consistent with increased Mn brain deposition in welders. The lack of group differences in R2*, a proxy for brain Fe, indicates that exposure to welding fumes does not alter brain Fe, whereas its relation to cumulative CEI even in non-welders is consistent with age-linked Fe effects. In contrast, R1, a proxy for brain Mn, was not related to age, except in welders alone, likely only due to increased cumulative Mn deposition with age in welders. Instead, R1 is associated with short- and long-term Mn exposure and thus is a better function of exposure. Increased UPDRS scores indicating worsening motor function were positively associated with both Mn and Fe brain concentrations, in line with a well-described decline both with cumulative exposure to Mn as well as the known decline with age, which in turn is linked to increased brain iron. The association with mood scores in welders only is also in line with previous literature on mood - exposure associations in Mn exposed subjects. A whole-brain analysis, exploring many more brain regions for such associations is in progress.

PS 3104 Chromium (VI) and Manganese (III) Exposure Induces Lifespan Reduction and Endothelial Barrier Disruption

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Background and Purpose: Chromium (Cr) and manganese (Mn) are trace metals that pose significant risk at elevated levels. Naturally occurring Cr(III) can be converted to a more toxic form, Cr(VI) in the presence of manganese oxides (MnOx), especially Mn(VI), resulting in soluble Mn(II) or (III) oxides. Co-occurrence of Cr(VI) and MnOx commonly occurs in geogenic sources (e.g. ultramafic bedrock) and cast iron pipes. Both Cr(VI) and Mn(III) are independently associated with oxidative stress, mitochondrial dysfunction, and disruption of neurovascular integrity. While Cr(VI) exposure is linked to motor neuron diseases, the underlying mechanism is unknown. Comparative evaluation of metal toxicity at both the organismal and cellular levels is essential to understand the mechanisms underlying metal-induced neurovascular impairment. In this study, we employed *Caenorhabditis elegans* as an *in vivo* model and human umbilical vein endothelial cells (HUVECs) as an *in vitro* system to assess the biological effects of Cr(VI) and MnOx exposures across different levels of biological organization. **Methods:** Synchronized *C. elegans* at the L1 larval stage were exposed to sodium chromate ($\text{Na}_2\text{Cr}_2\text{O}_7$; 5, 50, and 100 mg/L) and manganese (III) oxide (Mn_2O_3 ; 5, 50, and 100 mg/L), either individually or in combination. Combination treatments included 5 mg/L Cr(VI) + 5 mg/L Mn(III), 5 mg/L Cr(VI) + 50 mg/L Mn(III), 50 mg/L Cr(VI) + 5 mg/L Mn(III), and 50 mg/L Cr(VI) + 50 mg/L Mn(III). Exposures were conducted for 24 h at 22 °C. Lifespan assays were performed, and survival distributions were analyzed using the Kaplan-Meier method to evaluate treatment-related effects on organismal longevity. For the cellular assessment, HUVEC monolayers were exposed to sodium chromate at concentrations of 0.5, 1, and 2 μM . Trans endothelial electrical resistance (TEER) was measured for up to five days to monitor time- and concentration-dependent changes in endothelial barrier integrity.

Results: Lifespan analysis of *C. elegans* revealed a statistically significant reduction in survival following metal exposures. Control worms exhibited a median lifespan of approximately 12 days, whereas exposure to sodium chromate and manganese (III) oxide resulted in median lifespans of 11-13 days and 9-11 days, respectively. Worms co-exposed to both metals exhibited overall shorter lifespans (8-10 days), suggesting potential additive toxicity between chromium and manganese. Kaplan-Meier survival analysis confirmed significant differences among treatment groups (log-rank test, $p < 0.0001$), and an apparent dose-dependent decline in lifespan was observed in single-metal exposure groups. TEER measurements showed minimal changes at 0.5 μM chromium, with resistance remaining close to baseline levels (0.95-1.09 relative to control). In contrast, both 1 μM and 2 μM exposures exhibited a gradual and sustained reduction in resistance through day 5, reaching approximately 0.76-0.82 of baseline levels (18-24 % decrease). This pattern suggests a progressive weakening of endothelial barrier stability and intercellular cohesion over time. **Conclusions:** Chromium (VI) and manganese (III) exposures led to significant reductions in *C. elegans* lifespan, with combined treatments producing additive effects. TEER analysis revealed a gradual decline in endothelial resistance following 1-2 μM chromium exposure, suggesting progressive weakening of barrier stability over time. Collectively, these results demonstrate that heavy metal exposures disrupt physiological homeostasis in a concentration-dependent manner and may contribute to cellular and tissue-level dysfunction. Consistent with previously reported mechanisms involving oxidative stress, mitochondrial dysfunction, and weakening of intercellular junctions, these observations suggest that metal exposure may trigger early alterations that compromise vascular and physiological integrity, warranting further molecular investigation.

PS 3105 Non-monotonic NMDA receptor localization alterations resulting from low-dose, early developmental lead (Pb) exposure in excitatory hiPSC-neurons

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Background and Purpose: Developmental lead (Pb) exposure remains a major public health concern due to its lasting impact on brain function through disrupted excitatory neurotransmission—a feature commonly shared by many neuropsychiatric disorders. The CDC's blood lead reference level is 3.5 $\mu\text{g}/\text{dL}$, a threshold exceeded by 2.5% of U.S. children. Excitatory neurotransmission is heavily regulated by N-methyl-D-aspartate receptor (NMDAR) synaptic localization and composition. In the hippocampus and prefrontal cortex, NMDARs contain two obligatory NR1 subunits and two modulatory NR2A or NR2B subunits. NR2A-containing receptors are predominantly synaptic, whereas NR2B-containing receptors localize to both synaptic and extrasynaptic membranes. Synaptic NMDAR activation promotes synaptic strengthening, while extrasynaptic activation drives synaptic weakening and can induce autophagy. Thus, developmental shifts in NMDAR subunit composition and localization are critical for cortical maturation, and perturbations to this process in early life have been implicated in psychiatric disorders such as ADHD, schizophrenia, and bipolar disorder in adulthood. Previous research has indicated an increase in synaptic NMDARs resulting from early developmental exposure to 1 μM Pb in hippocampal rat cultures (Neal et al., 2010). However, the impact of Pb exposure on NMDAR localization in human-neuronal synapses resulting from low dose Pb exposure is unknown. **Methods:** Neurotypical patient-derived hiPSCs were differentiated into excitatory neurons via NGN2 overexpression and co-cultured with rat astrocytes. Cells were exposed to 0, 0.16, 0.625, or 2.5 μM Pb via culture media between day-in-vitro (DIV) 8-DIV28, corresponding to concentrations of blood Pb of approximately 0, 3.32, 12.95, and 51.80 $\mu\text{g}/\text{dL}$, respectively. Neurons were fixed in paraformaldehyde (PFA) and stained for axon initial segment marker (ankyrin-G), post-synaptic density protein (PSD95), obligatory NMDAR subunit (NR1), and dendritic marker (MAP2), and imaged via confocal microscopy ($n = 3$ per condition). IMARIS was used to quantify NR1 colocalization with PSD95. Synaptic NMDARs were calculated as the proportion of NR1-PSD95 colocalized spots relative to total NR1 puncta, and analyzed using two-way ANOVA with Tukey's multiple comparisons. **Results:** NR1 colocalization with PSD95 was significantly increased following exposure to 0.16 μM Pb relative to control ($p = 0.0084$), but was significantly lower in the 2.5 μM Pb group relative to the 0.16 μM Pb group ($p = 0.0033$). No significant differences were observed between 0.625 μM Pb and control ($p = 0.473$), or in NR1 and PSD95 values relative to MAP2 at 0.16 μM ($p = 0.213$, 0.205) or 0.625 μM ($p = 0.425$, 0.930). **Conclusions:** To our knowledge, this is the first study examining the impact of Pb exposure on NMDAR synaptic localization in human-iPSC neurons. Experimental results show that low-dose Pb exposure increases synaptic NMDAR localization in hiPSC-excitatory neurons relative to control, and decreases synaptic NMDAR localization at intermediate doses. These findings indicate a non-monotonic effect of early neurodevelopmental Pb exposure, and that neurotoxicological effects of Pb exposure may begin occurring at concentrations below both current public health thresholds and those observed in previous non-human animal model research. Further research is in progress to explore how Pb exposure affects NMDAR subunit composition and associated epigenetic mechanisms in both control cell lines and bipolar disorder patient-derived hiPSCs. This work contributes to understanding the relationship between developmental Pb exposure in excitatory synapses and potential compensatory mechanisms.

PS 3106 Lead(Pb)-induced neuroinflammation in the developing zebrafish brain

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Background and Purpose: Despite global efforts to decrease lead (Pb) exposure and contamination, the challenge remains substantial, and Pb still poses a significant threat to human health globally, being classified by the World Health Organization (WHO) as one of the top 10 chemicals of major public health concerns. Infants and children are particularly vulnerable to the neurotoxic effects of Pb, due to the complexity of the developmental stage of the Central Nervous System (CNS). Pb has been associated with reduced IQ, attention deficits, impaired brain development, and the onset of neurodegenerative conditions later in life. Dysregulation of neuroinflammation, or inflammation within the CNS, is one of the main mechanisms by which contamination with Pb exerts its effects. This study aims to elucidate how Pb exposure dysregulates neuroinflammation during development, which pathways and cell types are involved, and how these disruptions are associated with certain neurological conditions. The central hypothesis is that Pb developmental exposures in zebrafish, exacerbate neuroinflammation through microglial activation and dysregulate anti- and pro-inflammatory and complement cascade genes. **Methods:** Due to its many advantages, such as the high-throughput tractability of various developmental processes, fast and external development and high similarities with the human genome, the zebrafish has become an ideal animal model for toxicological studies and analyses of neurodevelopmental aspects. To study the effects of Pb on dysregulation of neuroinflammation, zebrafish embryos were exposed starting at 1-2 hours post fertilization (hpf) until 72 hpf to different concentrations of Pb [1, 10, or 100 parts per billion (ppb), $\mu\text{g}/\text{L}$], which represent values below (1 ppb) and above (10 and 100 ppb) the limit established as safe by Center for Disease Control and Prevention (CDC). This exposure period encompasses important developmental windows when the zebrafish brain is formed and is comparable to critical windows of exposures in humans. First, microglia dysregulation was assessed through live-imaging surface staining and immunostaining of microglia cells within the brain of zebrafish. Next, through RNA and protein analyses, dysregulated genes and protein expression of markers for neuroinflammation, including complement cascade-related genes, microglia activation, and genes implicated in synapse elimination, were evaluated. **Results:** Following Pb exposure, a concentration-dependent increase was identified in the number of microglia cells throughout the brain in zebrafish through live-imaging with neutral red staining at 96 hpf. This increase was significant in the 10 and 100 ppb Pb exposed groups, with more microglia cells visible in the forebrain region of the zebrafish when compared to the negative control. Analysis of RNA expression through qPCR indicated a possible immediate neuroinflammatory response due to Pb toxicity, with an increase in *mpeg* (macrophage expressed 1 gene) and *c3a.1* (Complement Component 3 gene) expression in some of the Pb-exposed groups at 120 hpf. **Conclusions:** In this study, analyses of neuroinflammatory responses following Pb exposure provides important mechanistic understanding of the neurotoxic effects of Pb and its impacts on neurodevelopment, particularly involving neuroinflammatory processes. This understanding can guide needed identification of biomarkers for early detection of the neurotoxic effects of Pb and of neuroinflammation. Ultimately, these findings can be used to improve interventions to mitigate the effects of Pb exposures, which continue to be devastating not only for development but also having consequences throughout different life stages.

PS 3107 Developmental Methylmercury Exposure Causes Persistent Proteomic Perturbations in Cortically Differentiated Human Induced Pluripotent Stem Cells

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Background and Purpose: Developmental exposure to the environmental toxicant Methylmercury (MeHg) is linked to long-lasting neurotoxic outcomes such as cognitive decline, but molecular mechanisms behind these persistent effects remain unclear. Our prior work (published and unpublished) identified persistent transcriptomic alterations following transient developmental MeHg exposure; here, we further assessed whether such persistent changes are also observed/translated at the protein level and across different MeHg concentrations using global proteomics. We hypothesized that developmental exposure of different environmentally relevant MeHg concentrations can induce persistent proteomic changes that manifest across different stages of brain cortical development. **Methods:** Three neurotypical human induced pluripotent stem cell (hiPSC) lines—two female (AD2-2, CC3) and one male (CD10)—were differentiated into cortical lineages via dual SMAD inhibition. Developing cultures were exposed to 1, 10, or 100 nM MeHg from days 7-13, when neuroepithelial and radial glial cells predominate, followed by differentiation and collection in form of cell pellets at days 43 and 120. Cell pellets were processed for LC-MS/MS-based global proteome profiling using data-independent acquisition (DIA). Normalized protein matrices were analyzed using RStudio and Qiagen Ingenuity Pathway Analysis (IPA) to identify significantly altered proteins and pathways. Autophagy, one key perturbed pathway, was validated by immunofluorescence (IF) using p62, LC3, and ubiquitin markers, and autophagic flux was examined after Bafilomycin (10 nM) treatment. **Results:** DIA proteomics quantified over ~8,000 proteins across MeHg concentrations and time-points. Principal component analysis

(PCA) at D43 revealed distinct separation of 100 nM MeHg from vehicle control in all lines, with AD2-2 showing a clear dose-response. Volcano plots indicated lower doses (10, 1 nM) mainly downregulated proteins, while 100 nM induced widespread upregulation. Only one protein/pathway was shared across all doses between all lines (Cell-cycle check points), whereas 100 and 10 nM shared nine. Pathway analysis for top 20 pathways from D43 100 nM overlapped strongly with our prior scRNA-seq data top pathways (e.g. mitochondrial dysfunctions, oxidative phosphorylation, sirtuin signaling etc.). At D120, even 10 nM exposure caused PCA separation from vehicle control indicating persistent proteomic effects. Additionally, 10 and 100 nM MeHg shared 209 altered proteins (compared to 9 for D43) indicating lower dose of MeHg development exposure is causing similar protein changes like higher MeHg dose at later-time points. Comparison between D43 and D120 (100 nM) identified 28 common proteins and 11 pathways across all lines. Common pathways involved proteostasis (autophagy), mitochondrial bioenergetics, and epigenetic regulation. IF confirmed increased p62, LC3, and ubiquitin puncta, indicating lower autophagy flux. Common protein analysis helps in identification of protein biomarkers for early detection of MeHg persistent toxicity. **Conclusions:** Developmental MeHg exposure caused persistent proteomic alterations across all hiPSC lines, with the strongest effects being observed at 100 nM. AD2-2 exhibited concentration-dependent effects, suggesting genetic variation influences MeHg sensitivity. Analysis across both male and female hiPSC lines suggests that the persistent effects of MeHg developmental exposure is sex-independent. High MeHg dose induced protein upregulation, whereas low dose promoted downregulation suggesting different effects of MeHg dose on proteome. Overlap between D43 proteomic and scRNA-seq pathways indicates transcript-to-protein concordance. Shared pathways and proteins between D43 and D120 reveal long-term proteome remodeling after transient developmental MeHg exposure. Protein biomarker was identified to understand the MeHg persistent toxicity and the same is validated using different techniques of IF, and western blotting. Disrupted autophagy suggests loss of proteostasis as a possible mechanism linking developmental MeHg toxicity to later-life neurodegenerative diseases (e.g. Alzheimer's, Parkinson etc.) vulnerability. The work was funded by NIH [R01 ES07331 and AG080917].

PS 3108 Gut Microbe *Akkermansia muciniphila* Mitigates Cadmium-Induced Cognitive and Hippocampal Dysfunction in Mice

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Background and Purpose: Cadmium (Cd) is a widespread environmental contaminant and neurotoxicant associated with learning and memory impairments in humans. We and others showed that Cd-induced gut dysbiosis contributes to neurotoxicity through disrupting the gut-brain axis, a bidirectional signaling pathway between the gastrointestinal (GI) tract and the central nervous system (CNS). Given the critical role of the gut microbiome in regulating brain function, modulating the gut microbiome has gained increasing attention as a potential strategy to mitigate cognitive deficits. *Akkermansia (A) muciniphila*, a mucin-degrading bacterium residing in the intestinal mucus layer, has been proposed as a next-generation probiotic with neuroprotective potential. This study investigated whether *A. muciniphila* pretreatment could prevent or mitigate Cd-induced hippocampus-dependent cognitive deficits and explored the underlying mechanisms. **Methods:** Adult male C57BL/6J mice (n = 10/group) were pretreated with live *Akkermansia muciniphila* (1×10⁹ CFU/day, oral gavage, once daily) for 5 days prior to and throughout cadmium (Cd) exposure (3 ppm Cd in drinking water) for nine weeks. Cognitive performance was assessed using a battery of behavioral tests evaluating learning and memory. Molecular changes in hippocampus were analyzed by RNA sequencing to identify differentially expressed genes associated with cognitive function by Cd and *A. muciniphila*. Intestinal tissues were examined by qPCR for genes related to intestinal integrity and inflammation. Short-chain fatty acids (SCFAs), bile acids, and inflammatory cytokines were quantified using targeted biochemical assays. Brain Cd levels were also quantified using ICP-MS. **Results:** Cadmium exposure impaired hippocampus-dependent spatial learning and memory in adult mice. Pretreatment with *A. muciniphila* effectively prevented these behavioral deficits. Short-chain fatty acid or bile acid levels in brain or serum and Cd levels in brain were unchanged by *A. muciniphila*, suggesting other biochemical mechanisms leading to the mitigation of Cd-induced learning and memory deficits. RNA sequencing of the hippocampus revealed two major gene clusters altered by Cd exposure and reversed by *A. muciniphila* treatment. Cd downregulated genes involved in neuronal signaling and synaptic plasticity (e.g., *Otof*, *Lypd1*, *Penk*, and *Trh*) while upregulating stress- and inflammation-related genes (*Hp*, *Alb*, *Serpina1e*, and *Uox*). Consistent with these findings, qPCR and cytokine analyses showed that *A. muciniphila* restored Cd-suppressed tight junction gene expression (*Cldn*, *Tjp*, *Jam*) and reduced circulating inflammatory cytokines (*IL-10*, *IL-1α*). **Conclusions:** *Akkermansia muciniphila* treatment may mitigate Cd-induced cognitive impairments by preserving intestinal barrier integrity and modulating neuroinflammation and synaptic signaling. These results underscore the preventive and therapeutic potential of microbiome-targeted strategies for reducing environmental neurotoxicity.

PS 3109 Quantitative MRI and Light-Sheet Microscopy Reveal Subchronic Manganese-Induced Neuronal Activation in a Neurodevelopmental Mouse Model

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Background and Purpose: Manganese (Mn) is an essential trace element for dopaminergic function; however, even moderate, sustained Mn exposure through drinking water can disrupt dopamine neurotransmission. Dysregulation of the dopamine transporter (DAT) produces anomalous dopamine efflux and impaired reuptake, resulting in behavioral deficits observed with autism spectrum disorders (ASD) and attention-deficit/hyperactivity disorder (ADHD). To investigate an interplay between gene and environment, we leveraged the DAT^{T356M/+} knock-in mouse model carrying the *de novo* ASD-associated T356M variant originally identified in an individual with restricted and stereotyped behaviors. Although the neurotoxic effects of acute Mn toxicity are well known, the *in vivo* effects of prolonged, lower-level exposure remain unclear. We tested whether subchronic Mn exposure alters DAT-dependent circuit responsiveness to amphetamine (AMP) and methylphenidate (MPH) and used whole-brain neuroimaging modalities to visualize, map, and quantify Mn distribution. We hypothesized that subchronic, low-dose Mn in drinking water would induce region-specific T1/T2 shortening on quantitative magnetic resonance imaging (MRI) without volumetric loss and that these relaxometry changes would align with altered *c-Fos* and tyrosine hydroxylase (TH) activity within the basal ganglia-thalamocortical circuits using light-sheet fluorescence microscopy (LSFM). **Methods:** Male and female DAT^{T356M/+} mice and WT littermates were pseudorandomly assigned at weaning to control water (2.5% stevia) or Mn water (2.5 mg/mL Mn + 2.5% stevia) *ad libitum* for 5 weeks. Locomotor activity was recorded for three 60-minute habituations and a 3-hour drug session on separate days (MPH 5 mg/kg or AMP 3 mg/kg; 1-week washout). *Ex vivo* brains were imaged at 7 Tesla to generate quantitative T1/T2 parametric maps; voxel-wise mono-exponential fits were registered to the Allen Brain Atlas and processed in Slicer 5.4.0 and MATLAB R2024a to quantify means and volumes. Mice received AMP 1 hour pre-euthanasia; brains were fixed in 4% paraformaldehyde, embedded in SHIELD/CLARITY hydrogel, optically cleared, and imaged with a SmartSPIM LSFM at 3.6x/0.2 NA; 488 nm autofluorescence, 561 nm *c-Fos*, 647 nm TH. Stitched volumes were analyzed with SmartAnalytics v2.0 software, a machine-learning pipeline to enable automated, region-wise quantification of TH+ and *c-Fos* + cell counts, volumes, and densities. **Results:** Male DAT^{T356M/+} mice exposed to Mn exhibited increased hyperactivity compared to littermate controls, travelling further than wild-type (WT) littermates during initial habituation sessions. Mn potentiated the effect of both AMP and MPH in males although the effect differed according to genotype. These differences were not observed in females for either drug according to Mn treatment. Mn exposure produced robust T1 shortening across seven brain regions (two-way ANOVA, p<0.0001) in both cohorts, with the largest effects in the globus pallidus, substantia nigra, thalamus, and striatum. This pattern reflects region-selective susceptibility to Mn accumulation and early basal ganglia-thalamocortical disruption before atrophy. Volumetric analyses showed no differences in regional brain volumes across groups, indicating Mn exposure altered relaxation properties in the absence of structural changes. Optically cleared brains showed greater TH+/*c-Fos*+ co-labeling in female DAT^{T356M/+} mice than in WT females and males, indicating enhanced dopaminergic activation. Co-localization was most pronounced in the ventral tegmental area, while the isocortex exhibited the highest overall *c-Fos*+ cell counts. **Conclusions:** By linking drinking-water Mn exposure to alterations in catecholaminergic circuits, these data provide a translational basis for differences in how patients from different environmental contexts respond to AMP, MPH, and associated drugs. This multimodal workflow offers exposure-sensitive, whole-brain biomarkers for preclinical testing and future patient stratification, advancing personalized therapeutics for ADHD, ASD, and related neurological disorders.

PS 3111 Cadmium exposure alters mitochondrial function and promotes *in vitro* aggregation of the tau protein, a neuropathological hallmark of Alzheimer's disease

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Background and Purpose: Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by the pathological accumulation of tau protein and amyloid beta (Aβ). Around 90% of the AD cases are sporadic with no clear genetic cause. Cadmium (Cd) is a heavy metal used in a variety of industrial processes. Typical routes of human exposure include contaminated food, tobacco smoke, and occupational exposures. Cd is known to bioaccumulate, leading to elevated concentrations in humans from chronic exposure, even exceeding safe limits. Up to 20% of Americans have elevated levels beyond what is considered safe. Increased Cd levels are associated with increased risk of AD, lower levels of cognition, and increased levels of AD biomarkers. However, little is known about how Cd might increase risk of AD and what effect it might have on progression of tau pathology. **Methods:** In this study, we use extracted mitochondria, *Caenorhabditis elegans*, and rodent models

of tauopathy to identify how Cd exposure affects disease mechanisms and the characteristic pathological hallmarks of AD. Mitochondria were acutely exposed to CdCl₂ and evaluated for enzyme activity. Mitochondria isolated from sub-chronically exposed mice-brain were tested for enzyme activity (normalized to citrate synthase). *Caenorhabditis elegans* nematodes were exposed to CdCl₂ for two days and stained with reduced Mitotracker red to test for mitochondrial viability. Nematodes expressing anti-aggregation and pro-aggregation tau were tested for Ach-associated paralysis through Aldicarb assay. Using Quantitative Elemental Mapping techniques, we measured Cd accumulation in the nematodes. *In vitro* tau aggregation studies were conducted in Hek 293 cells expressing fluorescently tagged truncated P301S tau (RD cells). The cells were exogenously treated with combinations of 150 nM recombinant tau and CdCl₂, then assessed for tau aggregation compared to controls. **Results:** Exposing nematodes to cadmium induced a dose-dependent decrease in mitochondrial viability and Ach-associated paralysis (Aldicarb assay). Notably, the effect on Ach-associated behavior was higher in anti-aggregation tau strains and highest in pro-aggregation tau strains. Mitochondrial complex II was significantly inhibited in both acute exposure studies and in brain mitochondria isolated from mice exposed to Cd subchronically (21 days). Notably, sex differences were observed as we found that the mitochondria isolated from male mice were more vulnerable. *In vitro* tau aggregation assays in RD cells revealed a significant increase in tau aggregation upon exposure to Cd. **Conclusions:** These results highlight mitochondrial dysfunction as a potential synergistic mechanism of tau- and Cd-induced toxicity. They also indicate that Cd may promote aggregation of tau, a neuropathological hallmark of AD. Together this supports further studies exploring these potentially toxic mechanisms using *in vitro*, transgenic *C. elegans*, and rodent models to understand the interplay between Cd exposure and AD.

PS 3112 The Gut Microbial Metabolite S-equal Partially Protected against Cadmium-induced Neuronal Cell Death Associated with Transcriptomic Signatures involved in Neurogenesis

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Background and Purpose: Cadmium (Cd) is an emerging environmental neurotoxicant and blood Cd levels positively associate with increased risk of Alzheimer's disease mortality in older U.S. adults. Neural progenitor cells in the subventricular zone (SVZ), one of only two adult neurogenesis sites, are particularly vulnerable to cadmium exposure due to high metabolic activity and preferential toxicant accumulation. S-equal, a gut microbiome-derived metabolite of the soy isoflavone daidzein and selective estrogen receptor beta (ERβ) agonist, has been suggested to be neuro-protective, yet no studies have tested its effect against Cd neurotoxicity. Importantly, only about 20-30% of individuals in Western populations naturally produce S-equal, compared with 50-70% in Asian populations. This disparity underscores the need to evaluate S-equal supplementation in high-risk groups within the United States. Such efforts could advance precision medicine approaches targeting at-risk populations to Cd exposure. As an initial step towards this goal, the present study tested our hypothesis that S-equal protects against Cd neurotoxicity associated with transcriptomic remodeling. **Methods:** Primary mouse SVZ neural progenitor cells from 8-week-old male C57BL/6 mice were exposed to CdCl₂ and S-equal at various concentrations for 24 hours. Cell viability was assessed using WST-8 colorimetric assay (n=8-20 per group). RNA-sequencing was conducted at selected concentrations and differential expression analysis was performed using DESeq2. Pathway and co-expression network analyses were also performed to identify core genes coordinately responding to Cd toxicity and S-equal intervention. **Results:** Cd induced neuronal cell death in a concentration dependent manner, with the first observed cytotoxicity at 5 μM (17% cell death) and the highest cytotoxicity at 10 μM (32% cell death). S-equal displayed a safe toxicological profile with no observed cytotoxicity at any concentrations tested. S-equal at both 100 and 200 μM partially protected against Cd (5 μM) neurotoxicity. Interestingly, even at lower S-equal concentration (25 μM) and higher Cd concentration (7.5 μM), S-equal still offered partial protection against Cd neurotoxicity. Bulk RNA-Seq was performed to determine the effect of S-equal (25 μM) on Cd (7.5 μM)-induced transcriptomic response. Cd dysregulated 10,320 genes, with the top up-regulated pathways involved in neurodegeneration, amyotrophic lateral sclerosis, and Alzheimer's disease; whereas the top down-regulated pathways were involved in neurogenesis and neuronal differentiation, neuronal survival and synaptic signaling, as well as cell polarity and synaptic architecture. S-equal altered the expression of 1950 Cd-dysregulated genes, among those 655 genes were normalized towards baseline. The top pathways of the S-equal remediated gene battery were synaptic transmission and plasticity, neuronal differentiation, axon guidance and circuit organization, as well as myelination and neuronal membrane integrity. Co-expression network analysis identified 20 core genes coordinately dysregulated across all treatment comparisons (correlation threshold 0.7), revealing key mechanistic pathways including upregulated stress response and neuroprotective genes (Hspa5/GRP78, Sv2c, Elovl4, Nab2). Examples of the S-equal remediated genes include: Vstm2b (neural circuit formation), Necdin/Ndn (Prader-Willi syndrome gene essential for neuronal survival), and Slc22a29 (organic cation transporter). Notably, S-equal also restored the expression of Prox1, a master regulator of hippocampal neurogenesis critical for learning and memory, and Sv2c, a synaptic vesicle protein associated with epilepsy, highlighting preservation of both neurogenic capacity and synaptic function. **Conclusions:** Our study demonstrated for the first time that the gut microbial metabolite S-equal protects neural progenitor cells from Cd-induced neurotoxicity associated with restoring transcriptomic networks involved in synaptic plasticity,

neuronal differentiation, and myelination. Follow up *in vivo* studies are needed to further explore the therapeutic potential of S-equal as a precision neuroprotective intervention for populations at elevated Cd exposure risk.

PS 3113 Evaluation of Neurotoxicity in Guinea Pigs Exposed to Hexavalent Chromium in Drinking Water for 90 Days

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Background and Purpose: Hexavalent chromium [Cr(VI)] is a ubiquitous environmental pollutant and potent toxicant, currently ranked as the #1 chemical hazard in U.S. public drinking water. Cr(VI) toxicity is well-characterized for inhalation exposures with known links to lung cancer; however, there is minimal research available for drinking water exposures. Similarly, the neurotoxic effects of Cr(VI) are poorly characterized in animal models or human studies. There are a few epidemiology studies that established links for motor neuron disease and attention/learning disorders in children, but no clear mechanistic details. These limitations diminish our ability to adequately characterize health risks associated with Cr(VI) exposure from drinking water, presenting a significant knowledge gap given its prevalence. We previously reported Cr(VI) drinking water exposures result in neurotoxicity that appears to target the hippocampus and related behaviors. Here, we present new results from a similar study using Hartley guinea pigs given tap water or 5.0 mg/L Cr(VI) in drinking water for 90 days. We chose guinea pigs, as they are more homologous to humans for vitamin C synthesis and Alzheimer's disease risk, as they require vitamin C supplementation in their diet and their amyloid β sequence is 100% identical to humans. We performed serine/threonine (STK) and phosphotyrosine (PTK) kinase array profiling to determine which protein kinase signaling pathways were impacted by Cr(VI) exposure in the dorsal hippocampus, considering both sexes. The integrated pathway analysis suggested risks for neurodegenerative diseases, with notable signaling effects in neuroinflammation, oxidative stress, myelin damage, synaptic dysfunction, and metabolic stress. **Methods:** Forty Hartley guinea pigs aged 10.5-11.5 months (both sexes) were exposed to Cr(VI) (0 or 5 mg/L) in drinking water for 90 days (N = 10/group). After sacrifice, brains were bisected with one half preserved for histology and the other microdissected into 12 regions for metallomic or proteomic analyses. Metallomic samples from 11 different brain regions were analyzed on an Agilent 8300 (N = 4-5). Kinome array samples were analyzed with STK and PTK chips on the PamStation 12 (PamGene Inc) followed by a bioinformatics workflow including the KRSA (Kinome Random Sampling Analyzer) R package for the dorsal hippocampus (N = 4-5). **Results:** Metallomic analyses revealed significant Cr accumulation in the dorsal hippocampus and pituitary gland of females, but little to no accumulation in males or other brain regions. Kinomic data revealed distinct clustering of results for control vs. Cr(VI) exposed, and between sexes. Dominant effects in males were mitochondrial and genotoxic stress driven by MAPK-GSK3-CHK2 activation and suppression of ATM/ATR and PDGFR/SRC pathways. Dominant effects in females were neuroinflammation and cytoskeletal stress driven by FAK, JAK/STAT, and DAPK/AKT pathways. Effects in both sexes converged on neuroinflammation and cognitive impairment, but by distinct mechanisms. **Conclusions:** We conducted a pilot study with guinea pigs exposed to Cr(VI) through drinking water to better model what may happen in human physiology. Guinea pigs are more similar than traditional rodent to humans for Alzheimer's disease contexts. Our data suggest Cr(VI) may be a risk factor for Alzheimer's disease and other neurodegenerative diseases based on brain regions that are targeted and protein kinase pathways that are activated. Our STK/PTK profiling predicts cognitive impairment, memory loss, neuroinflammation, demyelination, and vulnerability to several neurodegenerative diseases. Funding support from "la Caixa" Foundation LCF/BQ/EU22/11930060 (IM), NIEHS R35ES032876 (JPWSr.), and NIEHS T32ES011564 (MD, STV).

PS 3114 Transcriptional mechanisms of Manganese-induced dysregulation of 14-3-3γ via YY1 in CAD neuronal cells

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Background and Purpose: Chronic overexposure to manganese (Mn) via occupational and environmental sources causes a neurological condition known as manganism, sharing similar symptoms with Parkinson's Disease (PD). Although several mechanisms of Mn toxicity have been reported, the precise underlying mechanism is yet to be fully delineated. 14-3-3s are a class of regulatory proteins that are abundant in the brain and play a crucial role in various cellular processes by interacting with diverse target proteins. There are seven isoforms of 14-3-3s, regulated by different genes, but dysregulation of the 14-3-3γ isoform has been implicated in neurodegenerative diseases, including Alzheimer's Disease and PD. Moreover, Parkinsonian neurotoxins such as MPTP and rotenone are known to decrease the expression of 14-3-3γ, whereas overexpression of 14-3-3γ protects against neurotoxicity induced by these toxins. However, it is not known if Mn dysregulates the expression of 14-3-3γ, particularly at the transcriptional level. Notably, the promoter sequence of 14-3-

3y contains a putative binding site for transcription factor Yin Yang 1 (YY1), which is well established to be activated by Mn, collaborating with the epigenetic modifier histone deacetylases (HDACs) in other neural cell types, including astrocytes. Therefore, the present study investigated if Mn dysregulates 14-3-3y expression in neuronal cells. **Methods:** Cath-a-differentiated (CAD) neuronal cells were employed in this study. A luciferase assay was performed to measure the promoter activity of 14-3-3y. Quantitative polymerase chain reaction (qPCR) was used to measure mRNA levels/ Western blotting was employed to measure protein levels. Transient transfections were also used to overexpress 14-3-3y, YY1, and HDACs in CAD cells. Co-immunoprecipitation (Co-IP) was performed to determine the interaction between YY1 and HDACs in the nuclear fraction of CAD cells. **Results:** Our results showed that Mn treatment (250 μ M, for up to 12 hours) decreased 14-3-3y promoter activity and mRNA/protein levels in CAD cells. Mn also increased YY1 protein levels and its nuclear translocation at 12h, while overexpression of YY1 decreased 14-3-3y promoter activity, mRNA/protein levels. Moreover, YY1 also recruited several HDAC isoforms, such as HDACs 1, 2, 5, and 6, to repress 14-3-3y transcription. Accordingly, histone deacetylase inhibitors, such as Suberoylanilide hydroxamic acid (SAHA) and Valproic acid (VPA), attenuated Mn-induced decrease in 14-3-3y promoter activity and protein levels. **Conclusions:** Taken together, these results suggest that the Mn-activated YY1-HDAC pathway may mediate Mn-induced decrease in 14-3-3y expression in neuronal cells.

PS 3115 Psychological Symptomatology in Welders and Non-Welders Exposed to Manganese and Iron

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Background and Purpose: Chronic occupational exposure to manganese (Mn) has been shown to lead to psychiatric problems. Mood disorders and changes in mood are among the first symptoms reported in those with chronic Mn exposure. Welding fumes also contain a high percentage of iron (Fe). Disruptions in Fe homeostasis have been linked to the progression of neurodegenerative disorders, which are often accompanied by psychiatric diseases. Furthermore, both Fe deficiency and overload have been shown to relate to psychopathology. Both Mn and Fe exposure are being tested in this study as they are known to share transport and regulatory pathways in the brain. In a past cohort, our lab found that both welders and non-welders at the same factory reported high levels of psychological distress. In a new cohort, we sought to reassess whether reports of psychological distress differed across welders and non-welders and if symptomatology related to Mn and Fe exposure levels. **Methods:** The two groups consisted of 35 welders and 26 non-welding employees at the same factory. Exposure to Mn and Fe was assessed using personal air sampling and a work history interview. The Brief Symptom Inventory (BSI) was administered to evaluate psychological symptoms across nine domains. Domains assessed include somatization, obsessive-compulsive, interpersonal sensitivity, depression, anxiety, hostility, phobic anxiety, paranoid ideation, and psychoticism. A Global Severity Index (GSI) measuring psychological distress across all domains was also calculated. Furthermore, T-scores that compare the raw scores of participants to a normative non-patient sample were calculated. T-tests were conducted to compare exposure levels and psychological domains across the two groups. Then, Spearman's rho correlations were calculated to assess the relation between exposure levels and psychological distress. **Results:** Welders had significantly higher Mn and Fe exposure than non-welders ($p < 0.001$ for all tests). Welders and non-welders at the same factory displayed similar reports of psychological symptoms across all domains and similar levels of overall psychological distress. 34.3% of welders and 26.9% of non-welders had a clinically significant total level of psychological distress as indexed by the GSI. T-scores above 62 are considered clinically significant for each domain. When plotted against normative data, both groups demonstrated a clinical elevation on the paranoid ideation subscale (W: $T=64$, NW: $T=64$) and the psychoticism dimension subscale (W: $T=63$, NW: $T=64$) compared to a non-patient normative sample. This indicates elevated suspicion of others and increased withdrawal and social isolation. Non-welders at the factory reported a clinical elevation on the obsessive-compulsive subscale ($T=69$) reflecting an increase in subjective cognitive complaints. Notably, Mn and Fe exposure did not significantly correlate with overall psychological distress in both welders and non-welders. **Conclusions:** While welders are exposed to significantly higher levels of Mn and Fe, there is no difference in reports of psychological distress between welder and non-welder factory workers. These findings suggest that elevated psychological distress in factory workers may not be driven only by Mn or Fe exposure and highlight the need to consider workplace, social, and environmental factors when developing interventions to address mental health in occupational settings. Next, it will be important to assess how exposure impacts mood symptoms in individuals over time as well as what other factors may be explaining elevated psychological symptoms in workers at this factory. We plan to collect matched office controls to compare with both welder and non-welder factory workers. This will allow us to assess if other aspects of factory work or socio-cultural factors may better explain this distress.

PS 3116 APOE3/4 Heavy Metal Exposure: Synaptic Dysfunction and Alzheimer's Disease Progression

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Background and Purpose: Alzheimer's disease (AD) is a progressive neurodegenerative disorder affecting an estimated 6.9 million Americans aged 65 and older in 2024. Although the exact causes of AD remain unclear, both genetic and environmental factors play crucial roles in its onset and progression. The *APOE4* allele of the apolipoprotein E (*APOE*) gene is widely recognized as the most significant genetic risk factor for AD, and metals such as cadmium (Cd) and chromium (Cr) have been shown to induce neurotoxicity. However, little is known about how these risk factors interact and contribute to AD pathogenesis. This study aims to investigate the interaction between environmental exposure to Cd and Cr and *APOE4* genotype (GxE) in the context of AD, focusing on neuroinflammation-mediated synaptic alterations that may precede the development of the main pathological hallmarks of the disease. **Methods:** In this study, we sought to determine GxE effects on synaptic functions in AD. For this purpose, neural organoid derived from induced pluripotent stem cells (iPSCs) with *APOE3/3* and *APOE4/4* genetic background were used. As additional controls, organoids from age-matched healthy donor and donor with ideopathic AD were processed in parallel. Microglia-like cells (MGLs) were introduced into neural organoids in a stepwise manner through iPSCs differentiation to hematopoietic progenitor cells (HPC) and subsequent integration with NPCs. Neural organoids with and without microglia were exposed to 320 nM Cd, 320 nM Cr, and 320 nM Cd-320 nM Cr mixture for one week at 8 and 12 weeks of differentiation. Immunostaining was used to visualize and quantify synaptic integrity and density, microglia morphology, neurite outgrowth, and astrocyte migration. AD hallmarks such as Amyloid beta plugs and Tau tangles are assessed at 12 week time point with immunostaining and western blot. **Results:** Preliminary results showed distinct effects of Cd, Cr, and their mixture on neurite outgrowth and astrocyte migration in *APOE3/3* and *APOE4/4* neural organoids. Cd exposure consistently inhibited both processes in the *APOE3/3* neural organoid at both time points, whereas its effects were less pronounced in the *APOE4/4* neural organoids. Cr exposure produced variable effects across lines and time points. Mixture exposure did not have a synergistic effect on neurite outgrowth. It significantly increased neurite outgrowth in the *APOE3/3* neural organoid at 8 weeks and in both lines at 12 weeks. At the same time, a significant decrease in astrocyte migration was observed in both lines at both time points. SynapseJ analysis in ImageJ further revealed that metal exposure reduced synapse count in both cell lines at 8 weeks. In contrast, the preliminary 12-week data showed less evidence of perturbed synapsis in both lines. Currently experiments are ongoing to verify the microglia morphology upon exposure in both cell lines as well as spontaneous and evoked electrical activity of these organoids upon exposure to the metals. We are optimizing the experiments to quantify AD hallmark via western blotting and immunostaining. **Conclusions:** These preliminary results indicate that Cd and Cr exposure can differentially affect neuronal and astrocyte development in an *APOE* genotype-dependent manner. While Cd exerted stronger inhibitory effects on neurite and astrocyte migration in *APOE3/3* neural organoids, both *APOE3/3* and *APOE4/4* neural organoids exhibited marked synaptic loss at 8 weeks following metal exposure. This early synaptic vulnerability may reflect disrupted synaptic integrity and neuron-astrocyte interactions. More data is needed to further characterize to synaptic and glial dysfunction, potentially contributing to early neurodegenerative change underlying AD. With the ongoing introduction of MGL-neural organoid, this neural organoid model will provide a baseline system lacking neuroinflammatory components, enabling subsequent studies to investigate how metal-induced early synaptic dysfunctions interacts with inflammation-driven AD mechanisms. These findings may offer critical insights into gene-environment interactions driving AD progression and support the early identification of individuals at risk before the appearance of established pathological biomarkers.

PS 3117 Yin Yang 1 (YY1) Mediates Manganese-Induced Downregulation of REST in CAD Neurons

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Background and Purpose: Prolonged manganese (Mn) exposure leads to a neurological condition known as manganism, which shares similar symptoms with Parkinson's disease (PD). RE1-Silencing Transcription Factor (REST) has been shown to exert neuroprotection in experimental models and is dysregulated in the brains of several neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease. At the transcriptional level, REST increased the expression of antioxidant genes and the dopamine (DA) synthesis gene tyrosine hydroxylase (TH). Notably, our previous studies have shown that Mn decreased REST expression in *in vivo/vitro* experimental models, yet the underlying mechanism of Mn-induced decrease in REST expression is yet to be elucidated. Prior studies have revealed that Mn enhanced a transcription factor Yin Yang 1 (YY1) expression to induce Mn toxicity in neurons and astrocytes, but it is unknown if YY1 is involved in Mn-decreased REST expression. Therefore, the present study investigated whether Mn downregulates REST expression via YY1 in CAD neuronal cells. **Methods:** Cath-a-differentiated (CAD) neuronal cells were used, and REST promoter activity was measured by luciferase assay. Quantitative

PCR (qPCR) and western blot (WB) were employed to assess mRNA and protein levels, respectively. We also overexpressed YY1 and histone deacetylases (HDACs) in CAD cells by transient transfection of YY1 and histone deacetylases (HDACs) plasmid vectors to determine their regulatory effects on REST expression. Chromatin Immunoprecipitation (ChIP) assay was performed to determine the effects of Mn on YY1 binding to the REST promoter. **Results:** Mn decreased REST promoter activity, mRNA, and protein levels in CAD cells. Mn increased YY1 expression, and YY1 overexpression decreased REST and TH transcription. Three YY1 binding sites were identified in the REST promoter, and Mn enhanced YY1 binding to these sites within the REST promoter region. Mn also enhanced YY1's interaction with epigenetic modifiers HDACs. **Conclusions:** Our findings demonstrate that YY1 plays an important role in Mn-induced decrease in REST expression in CAD neurons.

PS 3118 The Behavioral and Neurotoxic Effects of Arsenic and Alcohol Co-Exposure

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Background and Purpose: Arsenic (As) exposure through contaminated drinking water remains a major global health concern. Chronic As exposure has been associated with a range of neurobiological and behavioral abnormalities, including anxiety-like behaviors, impaired learning, and disruptions in dopaminergic (DA) signaling. Similarly, excessive alcohol consumption is a major public health issue that also alters DA transmission, stress pathways, and neurocircuitry related to reward and anxiety. While both As and alcohol independently induce adverse neurobehavioral outcomes, the potential for co-exposure to exacerbate these effects remains poorly understood. Given the overlap in their neural targets, combined exposure to As and ethanol (EtOH) may produce additive or synergistic effects on behavior and neurochemistry. The current study aimed to (1) determine whether chronic low-dose As exposure increases binge-like EtOH intake, (2) assess whether As and EtOH co-exposure exacerbates anxiety-like behaviors, and (3) identify exposure-related changes in behavioral and neural biomarkers associated with DA function and stress regulation. **Methods:** Male and female C57BL/6J mice ($n = 39$) were randomly assigned to one of four exposure conditions in a 2×2 design: arsenic (in the form of arsenite, AsIII) exposure (0 ppm or 1 ppm) $\times$ EtOH exposure (tap water or 20% EtOH). AsIII exposure was delivered continuously in drinking water, while EtOH exposure was administered using a Drinking-in-the-Dark (DID) paradigm to model binge-like self-administration. During daily, 3-week DID procedure, mice had 2-hour access to 20% EtOH or tap water beginning 3 hours into the dark cycle. Behavioral assays were conducted to assess locomotor activity and anxiety-like behavior following exposure. Locomotor activity was quantified by total distance traveled in the open field test (OFT), while anxiety-like behavior was assessed by time spent in the center zone of the OFT. Various tissues, including the brain, were collected for ongoing analyses of As accumulation and neurochemical biomarkers related to DA and stress systems. **Results:** Significant sex-dependent effects of AsIII exposure were observed in locomotor behavior. AsIII exposure decreased locomotor activity in males and increased activity in females ($p < .05$), suggesting a sexually dimorphic response to chronic low-dose arsenic. However, co-exposure to AsIII and EtOH did not produce additional alterations in locomotor behavior. Additionally, there were no significant differences in ethanol or water consumption between groups ($p > .05$), indicating that AsIII exposure did not enhance binge-like drinking behavior under the conditions tested. Anxiety-like behavior was not significantly affected by co-exposure; however, a main effect of sex was detected, with males spending more time in the center zone than females ($p < .05$). This pattern suggests greater anxiety-like behavior in females across exposure conditions. **Conclusions:** Co-exposure to AsIII and EtOH did not exacerbate behavioral phenotypes relative to individual exposures. Nevertheless, the observed sex-specific effects of AsIII on locomotor activity highlight the potential for differential vulnerability to environmental neurotoxins across sexes. Ongoing analyses are quantifying As accumulation in peripheral and neural tissues and assessing alterations in DA and stress-related markers within the striatum and hippocampus. These data will provide critical mechanistic insight into how chronic low-dose arsenic exposure interacts with alcohol to influence brain function and behavior. Collectively, this work advances understanding of combined environmental and substance-based neurotoxicity.

PS 3119 Perinatal Lead Exposure Associated with Increased Diffuse and Vascular β -Amyloid in Aged Murine Brain

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Background and Purpose: Exposure to neurotoxicants during development can have long-lasting implications for cognition. Exposures such as lead (Pb) are suspected contributors to dementia. Several types of dementia, particularly Alzheimer's disease (AD), are associated with abnormal accumulations of β -amyloid (A β). Vascular accumulation of A β can contribute to AD pathology, vascular dementia, or general cognitive decline. A β accumulations contribute to neural toxicity via amyloid angiopathy and neuroinflammation. We explored the impact of developmental Pb exposure on

brain A β accumulation in a mouse model. **Methods:** Female mice (6-8 weeks old) were exposed to 32ppm Pb or control water for two weeks prior to mating through offspring weaning at postnatal day 21. Offspring were aged to 18 months. The final sample size included 11 female and 10 male control and 10 female and 10 male Pb-exposed. The left brain hemisphere was formalin fixed and paraffin embedded. Standard hematoxylin and eosin (H&E) and Luxol Fast blue (LFB) stained slides were used to assess general morphology and myelin, respectively. Immunohistochemical staining with an antibody targeting monomeric, oligomeric, and fibrillar forms of A β 1-42 peptide was performed. Descriptive and quantitative analysis was performed by a board-certified veterinary pathologist blinded to experimental conditions. For immunostaining, slides were digitized and staining intensity/distribution was assessed using pixel classifiers using QuPath (v0.6.0). Controls included an archival mouse AD brain slide (positive), and a non-immune serum in place of primary antibody (negative). The percent of non-staining, lightly staining, and darkly staining area was assessed across the entire tissue. A separate threshold was used to quantify the highest intensity staining, which corresponded to capillaries and foci of mineralization. Differences in all outcomes were compared between sex-stratified exposure groups using two-sided t-tests and significance set to $p \leq 0.05$. **Results:** There was no evidence of neuronal necrosis, endothelial cell/vascular necrosis, decreases in neural number/density, or changes in myelin in any of the samples as assessed. Focal mineralization in the thalamus was noted in 9 of 41 samples but was not specific to exposure group. Distinct A β plaques were absent but there was low to moderate A β immunolabeling diffusely across the grey matter of the cerebrum and thalamus. Mean A β intensity across the entire tissue was higher in exposed female samples relative to controls, with a fold change (FC) of 1.26 ($p = 0.004$). This trend was also present in male tissues (FC = 1.12) but did not reach statistical significance ($p = 0.095$). The highest intensity A β labeling was located in capillary walls throughout the brain and in the mineralized thalamic foci described above. There were significantly more A β positive cells in the exposed samples versus control for both males and females. Exposed males had, on average, 469 A β positive cells per tissue compared to 258 in control (FC = 1.82, $p = 0.032$). Exposed females had, on average, 320 A β positive cells per tissue compared to 166 in control (FC = 1.92, $p = 0.011$). **Conclusions:** Perinatal Pb exposure led to an increase in diffuse A β and A β -positive cells in 18-month murine brain. Although A β plaques were not present, this was not an AD-specific model, and C57BL/6 mice are known to be resistant to AD-like pathology. Diffuse and vascular distribution of A β may be more reflective of the general increase in A β deposition associated with general cognitive impairment or amyloid angiopathies. Ongoing work is quantifying the presence of other dementia hallmarks, including phosphorylated tau and neuroinflammation in the same tissues, as well as perinatal Pb exposure in the 5xFAD mouse model, which robustly presents dementia hallmarks.

PS 3120 RNA-sequencing reveals dose- and sex-specific transcriptomic disruptions in the adolescent rat hippocampus following developmental lead exposure.

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Background and Purpose: One in three children worldwide are susceptible to the detrimental health effects of lead (Pb²⁺), a toxic heavy metal ubiquitous in the environment. Although the Center for Disease Control (CDC) has set the level of concern to 3.5 $\mu\text{g}/\text{dL}$, they recognize that any amount of Pb²⁺ exposure has detrimental effects to the developing brain. Infants and young children are particularly susceptible to Pb²⁺ neurotoxicity during brain development. Children exposed to Pb²⁺ are at risk of neurodevelopmental delays, cognitive impairment, memory and learning disabilities, and behavioral abnormalities. Epidemiological studies indicate that early life Pb²⁺ exposure is associated with neurodevelopmental disorders (ND) such as Autism Spectrum Disorder (ASD), attention-deficit hyperactivity disorder (ADHD), and Schizophrenia (SZ). A significant number of similarities in the neurobiological pathways affected provoked us to investigate if shared molecular mechanisms are observed across multiple Pb²⁺ exposure levels and how they relate to ND. This study explores the effect of exposure to different levels of Pb²⁺ exposure on gene expression changes in the hippocampus of adolescent animals. **Methods:** We chronically exposed Long-Evans rats to Pb²⁺ acetate (PbAc) using chow containing low (180 ppm PbAc), medium (650 ppm PbAc), and high (1500 ppm PbAc) concentrations, as well as a non-exposed control group. Exposure occurred throughout gestation and after birth, when the pups were chronically exposed to the same diet as the dams until they were used at a specific age. RNA-sequencing (RNAseq) was utilized to explore the differential effects of chronic early life lead exposure (CELLE) at all four doses in the hippocampus of male and female rats. Briefly, RNA was isolated using TRIzol from the hippocampus of male and female postnatal day 28 (PN28) rats at each exposure level ($n=4-5$). RNA pellets were then collected, resuspended in TAE buffer, and read using the BioTek Cytation5 Take3. Purity was assessed and recorded. Finally, isolated RNA was sent to Novogene for sequencing and analysis. **Results:** Blood Pb²⁺ levels of non-exposed controls, low, medium, and high exposure groups were as follows: 1.9 $\mu\text{g}/\text{dL}$ (limit of detection; male $n=12$; female $n=12$), 5.2 $\pm$ 0.5 $\mu\text{g}/\text{dL}$ (male $n=9$; female $n=9$), 15.6 $\pm$ 0.9 $\mu\text{g}/\text{dL}$ (male $n=10$; female $n=10$), and 22.4 $\pm$ 0.8 $\mu\text{g}/\text{dL}$ (male $n=10$; female $n=10$), respectively. RNAseq results indicate that there were significant sex- and dose-dependent differences in male and female PN28 hippocampus-isolated RNA. Males had significantly more differentially expressed genes (DEG) compared to females at low (168:0), medium (13:1), and high Pb²⁺ concentra-

tions (442:4) [significant factors = $-\log_{10}(\text{padj}) > 1.3$; $\log_2\text{FoldChange} < -0.5$ and > 0.5]. Additionally, female DEG follow a dose-dependent increase correlating to blood Pb²⁺ levels. However, DEG in the male cohort were greatest at the high and low concentration, with less DEG in the medium exposure group. Finally, the most enriched GO pathways for both sexes and exposure levels were related to signal transduction, mitochondrial metabolism, and ribosomal structural pathways, while enriched KEGG pathways included signal transduction pathways, mitochondrial metabolism, and neurodegenerative diseases. **Conclusions:** Our hippocampal RNAseq analysis reveals sex- and dose-specific transcriptional responses to Pb²⁺ exposure in adolescent rats, with males exhibiting a markedly higher number of DEG and enriched GO/KEGG pathways. Disruption of signal transduction, mitochondrial metabolism, and ribosomal function in CELLE rats mirror pathophysiological features of ND such as ADHD, ASD, and SZ. Given epidemiological studies indicating that boys are 2-4 times more likely to be diagnosed with ND than girls (Ferri, Abel, & Brodtkin, 2018; Dimitri et al., 2025), our data in CELLE rats suggests environmental exposure to Pb²⁺ may influence ND through convergent neurotoxic mechanisms. To our knowledge, this is the first study to conduct RNA-sequencing of isolated hippocampal RNA in male and female adolescent rats ranging from non-exposed to high-exposure levels with significant implications to children's health.

PS 3121 Akkermansia muciniphila Prevents Cadmium-Induced Alzheimer's Disease-Like Dementia Linked to Epitranscriptomic Modulation in the Mouse Hippocampus

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Background and Purpose: Cadmium (Cd) is an emerging environmental neurotoxicant associated with Alzheimer's disease (AD) mortality in older adults in the United States. Emerging evidence suggests that the gut-brain axis contributes to neurotoxicity and neuroprotection. Our recent findings unveiled that Cd produced dysbiosis of the gut microbiome associated with memory deficits in mice. For example, *Akkermansia muciniphila*, a mucin-degrading bacterium in the intestine, was decreased by Cd over a time course. Epitranscriptomic N⁶-methyladenosine (m⁶A) modifications in the brain have emerged as novel regulators of AD. Yet, very little is known regarding how Cd or the gut-brain axis contributes to the brain epitranscriptome. This study tested our hypothesis that *A. muciniphila* prevents Cd-induced AD-like dementia associated with epitranscriptomic modulation of the hippocampus. **Methods:** Eight-week-old male C57BL/6J mice (n = 9-10 per group) were orally gavaged *A. muciniphila* (ATCC BAA-835, 10⁹ CFU per mouse/day, 5 days a week) or vehicle for 11 weeks, beginning one week prior to 10-week exposure to CdCl₂ (3 mg/L) via drinking water. Learning and spatial memory was assessed using novel object location (NOL) test at weeks 0, 5, 7, and 9 of Cd exposure. At week 10 of Cd exposure, hippocampal tissue was collected for bulk RNA sequencing and epitranscriptomic analysis. *A. muciniphila* abundance was quantified using qPCR. Cd concentrations in brain tissue were determined using inductively coupled plasma mass spectrometry (ICP-MS). Molecular analyses identified differentially expressed (DESeq2) and methylated hippocampal genes (ArrayStar m⁶A array; FDR < 0.05). **Results:** *A. muciniphila* co-exposure resulted in increased *A. muciniphila* abundance in the gut microbiome, and it protected against Cd-induced memory deficits at all behavioral time points sampled. ICP-MS analysis unexpectedly revealed moderately elevated hippocampal Cd levels in mice co-exposed to Cd and *A. muciniphila* compared with mice exposed to Cd alone, suggesting that neuroprotection occurred despite increased metal burden. *A. muciniphila* supplementation up-regulated hippocampal genes involved in m⁶A modification, whereas Cd downregulated all of them. Interestingly, *A. muciniphila* co-exposure reduced the mRNA levels of most differentially regulated hippocampal genes involved in m⁶A modification, with the exception of *Ythdf1*, a key mediator of methylation-guided translation, whose expression was restored under co-exposure conditions as compared to Cd exposure alone. Minimal hypermethylation patterns were observed in any exposure groups. Interestingly, *A. muciniphila* administration, either alone or in combination with Cd, led to a widespread hypomethylation of hippocampal transcripts, with the top pathway involved in alternative splicing. An overlay between RNA-Seq and epitranscriptomic data unveiled that the majority of the co-regulated genes were co-down-regulated with decreased mRNAs and hypomethylation patterns. This *A. muciniphila*-remediated gene battery against Cd insult was mainly involved in amino acid transport, lipid metabolism, as well as RUNX-mediated inflammatory response. In addition, many of these genes have been recognized to have plausible protective roles in AD-relevant processes, including synaptic signaling, neuroinflammation and innate immune activation, lipid homeostasis, calcium handling, and proteostasis or cellular stress responses. **Conclusions:** These findings support the hypothesis that *A. muciniphila* mitigates Cd-induced AD-like cognitive impairment associated with epitranscriptomic remodeling in the hippocampus rather than through reductions in brain metal burden. Together, these analyses point to m⁶A-dependent regulatory mechanisms as a potential interface through which gut microbial interventions buffer hippocampal function against environmental toxicants.

PS 3122 m6A mRNA Epitranscriptomic Dysregulation Drives Microglia-Induced Neuroinflammation in Models of Environmentally Linked Parkinsonism

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Background and Purpose: Parkinson's disease (PD) and related disorders, collectively known as Parkinsonisms, can be characterized by the accumulation of pathological α -synuclein (α Syn) in the basal ganglia (BG), including the substantia nigra (SN). The highest non-genetic risk factor for PD is aging, followed by exposure to certain environmental toxins, including the heavy metal manganese (Mn). These factors directly activate glial cell populations, leading to chronic neuroinflammation and progressive neurodegeneration. Emerging evidence suggests that epitranscriptomic mRNA modification represents a top-tier regulatory mechanism governing essential biological processes in both health and disease. Our recent studies suggest the m⁶A epitranscriptomic pathway, specifically the METTL3 writer complex and the downstream reader YTHDF2, is critical in Mn and α Syn-induced glial activation. To this end, we premise that dysregulation in the m⁶A reader YTHDF1 complex is critical in promoting neuroinflammatory processes observed in environmentally induced Parkinsonism. **Methods:** We employed C20 human microglial or U373 human astrocytic cells and exposed them to a non-cytotoxic dose of Mn (100 μ M) conditions for *in vitro* characterization. YTHDF1-2 knockdown (KD) was achieved utilizing siRNA against YTHDF1-2. Overexpression (OE) studies were accomplished via PEI-mediated delivery of YTHDF1/2 or YTHDF1/2-FLAG plasmids. For *in vivo* studies, mice were given saline or Mn (0.4 g/L)-containing drinking water for 30 d or orally gavaged with Mn (30 mg/kg) dissolved in ultrapure water daily for 30 d. **Results:** We observed that Mn significantly increased the expression of the m⁶A readers YTHDF1 and YTHDF3 in microglia as well as astrocytes 24 h post exposure. Mn exposure in astrocytes and microglia significantly increased the levels of proinflammatory chemokines (Cxcl12, Cxcl10, Cxcl8, Ccl2, and Ccl3) and cytokines (IL-1 β , IL-1 α , and IL-6). YTHDF1 KD attenuated Mn-induced proinflammatory factors while YTHDF1 OE exacerbated Mn-neuroinflammation. In contrast, YTHDF2 KD stabilized these transcripts, thus exacerbating Mn-induced neurotoxicity, and YTHDF2 OE attenuated the Mn-induced increase following Mn-induced activation in both immune cells. Together, this work suggests opposing functionality of YTHDF1 and YTHDF2 may modulate neurotoxic signaling processes. Interestingly, we also noted that YTHDF1 KD alleviated and YTHDF2 KD exacerbated activation of Fyn and PKC δ , a tyrosine and serine kinase, respectively, both being strongly implicated in neuroinflammatory processes. This work confirmed our RIP-seq dataset, showing that YTHDF2 regulated the stability of Fyn transcripts. Corroborating *in vitro* results, striatal YTHDF1 and YTHDF3, along with Fyn and PKC δ , significantly increased in IBA1⁺ microglia as well as GFAP⁺ astrocytic cells in Mn-exposed mice compared to controls, with Fyn and PKC δ protein levels being rescued upon YTHDF1 KO and further stabilized with YTHDF2 KO. **Conclusions:** In conclusion, we demonstrate that the YTHDF1 paralogs are dynamically dysregulated in Mn-induced models of glial activation. Our data also reveal that increases in YTHDF1 and YTHDF3 correlate with increased proinflammatory signaling in microglia and astrocytes, while YTHDF2 negatively regulates proinflammatory events. Interestingly, the opposing regulation of the YTHDF1 and YTHDF3 reader translational machinery complex vs the YTHDF2 decay machinery appears to differentially modulate the Fyn-PKC δ axis in neurotoxicant-induced glial activation. Collectively, our study advances our understanding of m⁶A-mediated epitranscriptomic dynamics in models of environmentally induced PD and highlights the YTHDF paralogs as major upstream mRNA regulators of neuroinflammatory processes preceding the onset of neurodegenerative diseases.

PS 3123 Characterizing brain region-specific gene expression changes following perinatal lead (Pb) exposure in the mouse brain

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Background and Purpose: Lead (Pb) exposure is associated in epidemiological studies with both childhood neurological disorders and dementias like Alzheimer's disease (AD) later in life. Since AD is characterized, in part, by neuroinflammation and altered gene expression patterns in the brain, it is pertinent to assess whether early-life Pb exposure results in changes consistent with those observed in AD. The purpose of this study is to assess immediate and long-term gene expression changes across brain regions in response to perinatal Pb exposure, to address the question of how Pb exposure contributes to neuroinflammation, neurotoxicity, and AD development later in life. **Methods: Sample Size, Exposure, and Tissue Collection** C57BL/6 females between 6-8 weeks of age were randomly assigned to control or Pb water exposure two weeks prior to mating with C57BL/6 males. Females were exposed to a 32-ppm solution of drinking water mixed with Pb-acetate (pH 4.5). Exposure continued throughout gestation and weaning, until the offspring reached post-natal day 21 (PND21), at which time offspring were either sacrificed (PND21, N = 39) or

switched to control water and housed until 18 months of age (N = 41). At sacrifice, three brain regions - hippocampus, cortex, and cerebellum - were collected from each mouse. Tissues were flash frozen and stored at -80°C prior to use. **RNA Isolation, Sequencing, and Analysis** RNA was isolated from each brain region using the Qiagen Allprep Kit. RNA samples were used for library preparation and sequencing using the plexWell method (SeqWell). Libraries were sequenced on the Illumina NovaSeq X Shared Flow Cell at the UM Advanced Genomics Core, and reads were aligned to the mouse genome via STAR and counted using FeatureCounts. The counts-per-sample matrices generated were filtered to remove genes with low counts using the edgeR filterByExpr() function with default settings. Differential gene expression analysis (adj. P-val < 0.05) of PND21 and 18-month RNA transcript count data, stratified by timepoint, brain region, and sex, was performed using edgeR. Pathway enrichment analysis to identify enriched biological ontologies was performed using the fgsea package, in which a ranked list of genes is used to perform enrichment analysis. **Results:** Analysis of PND21 brain region-specific RNA-sequencing data identified 7 genes (cerebellum), 0 genes (hippocampus), and 6 genes (cortex) that differed in expression between Pb-exposed and control samples. Interestingly, controlling for brain region resulted in 71 differentially expressed genes across all three brain regions in PND21 mice (adj. P-val < 0.05). Example genes of interest found to be differentially expressed include *Rsad2* (adj. P-val = 1.53e-10, log2FC = -0.889), *Mt1* (adj. P-val = 5.26e-06, log2FC = 0.247), and *Ube2l6* (adj. P-val = 2.00e-06, log2FC = -0.386). Genes differentially expressed at PND21 were found to be enriched in hallmark pathways including TNF- α signaling, interferon gamma response, and interferon alpha response (adj. P-val < 0.05). Analysis of 18-month brain region-specific RNA-sequencing data identified 0 genes (cerebellum, cortex, and hippocampus) that differed in expression between Pb-exposed and control samples. Likewise, controlling for brain region resulted in 0 genes differentially expressed across brain regions (adj. P-val < 0.05). Ranked gene expression results from the 18-month-old mice were found to be enriched only in the interferon alpha hallmark pathway, with increased expression of genes enriched in this pathway (adj. P-val < 0.05). **Conclusions:** PND21 mice exposed to Pb during gestation showed differential gene expression changes, while no genes reached genome-wide significance in 18-month-old mice. Pb-exposed mice exhibiting differential expression of genes associated with immune signaling suggests a potential mechanism by which perinatal Pb exposure contributes to AD via neuroinflammation. Future analysis will involve deconvolution of bulk RNA-seq data to determine if cell proportions are affected by Pb-exposure and aging.

PS 3124 Neuroinflammation in Rats after 90 Day Drinking Water Exposure to Low Concentration Hexavalent Chromium

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Background and Purpose: We are living in an era of unprecedented aging and extended lifespan. The global geriatric population (65+) is expected to reach 20% by 2050 and at the same time, the centenarian population (100+) will increase 7-fold. These dramatic demographic shifts are set to bring a host of new challenges, in particular the health impacts of environmental pollution on an aging population. Hexavalent chromium [Cr(VI)] is a ubiquitous environmental pollutant and highly potent toxicant - recently identified as the #1 chemical hazard in U.S. public drinking water. However, few studies have considered the interactions between Cr(VI) and brain aging. Reports suggest Cr(VI) induces neuroinflammation, a complex immunological reaction involving activation of microglia and astrocytes. Astrocytes are crucial for brain health as they maintain neurotransmitter homeostasis, provide structural support, help form the blood-brain barrier, among many other roles, while microglia act as resident immune cells. **Methods:** We investigated the intersection of toxicology and aging using the Toxic Aging Coin approach; the heads side considers how different ages are impacted by chemical toxicity, while the tails side focuses on how chemicals accelerate biological aging. We investigated Cr(VI) neurotoxicity by exposing young (3-m.o.), middle-aged (7-m.o.), and geriatric (18-m.o.) male Sprague-Dawley rats to Cr(VI) in drinking water consistent with the World Health Organization (WHO) and United States Environmental Protection Agency (U.S. EPA) maximum contaminant levels (0.05 and 0.1 mg/L, respectively). Effects of Cr(VI) on astrocyte (GFAP) and microglia (IBA-1) activation in the dorsal and ventral hippocampus were assessed as markers of neuroinflammation, using immunohistochemistry and Sholl analysis. Sholl analysis was performed using open source software Fiji and custom python code. We hypothesized that Cr(VI) induces neuroinflammation. **Results:** Initial results from the ventral hippocampus show microglial activation with increasing Cr(VI) concentration seen by decreases in both cell processes length and distance from cell body. Astrocyte activation was also noted with higher average intersection maxima and shifts in intersections further from the nucleus with increasing concentration. **Conclusions:** Our data begins to characterize a mechanism for which Cr(VI) exposure through drinking water induces neurotoxicity. Future work will investigate mechanisms driving microglial and astrocyte activation and better define sex-, age-, and brain region-specific differences. These data will be critical for determining how low concentrations of Cr(VI) in drinking water contribute to neurological diseases. Funding provided by T32-ES011564 (STV) and R21-ES033327 (JPW).

PS 3125 Nickel dysregulates Hox-dependent spinal motor neuron differentiation

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Background and Purpose: Nickel (Ni) is a naturally occurring ubiquitous heavy metal. However, anthropogenic activities such as industrial processes and widespread use of Ni-containing products have elevated its levels in the atmosphere. Furthermore, fossil fuel combustion, including diesel exhaust, has significantly contributed to its increase in the environment. Humans are exposed to Ni through inhalation, ingestion and dermal contact. Ni is well-known to be toxic to multiple organ systems, including lungs, liver and kidneys. Recently, developmental neurotoxicity has emerged as an important health concern associated with Ni exposure. Studies show that Ni can cross the placental barrier in mice, causing neural tube defects, and early Ni exposure in zebrafish and *C. elegans* leads to impaired neurodevelopment and neuronal damage. In infants, higher Ni levels are linked to lower neurocognitive scores. However, the mechanisms underlying Ni's developmental neurotoxicity remain unclear. In this study, we investigated Ni-induced neurotoxicity during spinal motor neuron development using mouse embryonic stem cells (mESCs) to model early neurodevelopment. **Methods:** Mouse embryonic E14Tg2a stem cells were differentiated into cervical motor neurons using well-established protocols employing retinoic acid and smoothened receptor agonist (SAG). For Ni exposure, the cells were treated with non-cytotoxic doses of 1-150 μ M NiCl₂. Gene expression changes were examined by RT-qPCR. H3K27me3 enrichment was assessed by chromatin immunoprecipitation (ChIP) assay using ChIP-grade antibodies. **Results:** The activity of *Hox* transcription factors patterns every developing organ and tissue along the main body axis. The *Hox* genes are organized into four chromosomal clusters (*HoxA, B, C, D*) each harboring a subset of 13 *Hox* gene paralogs (*Hox1* to *Hox13*). During development, the spinal motor neuron (MN) subtype identity along the spinal cord is controlled by *Hox* activity. While the rostral *Hox1-Hox5* genes are activated in the cervical MNs, the caudal *Hox6-Hox13* are activated at the brachial, thoracic, and lumbar MNs. Our results show that exposure to Ni during cervical MN differentiation resulted in the activation of *Hox10-13*, indicating potential disruption of *Hox* gene regulation and spinal MN subtype switching. Investigation of the underlying mechanisms revealed that Ni exposure caused a loss of the repressive histone modification H3K27me3 at the promoters of *Hox10-13*. Furthermore, we found an upregulation of *Cdx* genes (*Cdx1, Cdx2*, and *Cdx4*), which play a critical role in caudal *Hox* gene activation by binding the regulatory elements within the caudal *Hox* clusters. Moreover, we found upregulation of lncRNA HOTTIP, which also plays an important role in *Hox10-13* activation. **Conclusions:** Ni-induced activation of *Hox10-13*, which are normally not expressed in cervical motor neurons, suggests a loss of motor neuron subtype identity. This disruption may contribute to neural dysfunction and represent a potential mechanism underlying Ni-induced neural toxicity. Furthermore, since *Hox* transcription factors pattern every developing organ and tissue along the main body axis, our findings raise the possibility that Ni exposure may cause broader developmental abnormalities.

PS 3126 Peripheral HIF-2 Deficiency Attenuates Manganese-Induced Motor Deficits Independent of Brain Mn Load

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Background and Purpose: Manganese (Mn), an essential metal, but induces incurable parkinsonism at elevated levels by unknown mechanisms. Our previous work showed that transcriptional activation of HIF-1/HIF-2 by elevated Mn is required and sufficient for upregulation of SLC30A10 (a critical Mn efflux transporter) within the hepatocyte, thereby contributing considerably to provide protection against Mn induced toxicity. The overall aim of study is to define the role of HIF-1/HIF-2 signaling from liver and intestine in regulating the response of SLC30A10 and motor dysfunction following Mn exposure. As part of this, the current study focused on isolating the role of peripheral HIF-2 in Mn neurotoxicity. **Methods:** We generated endoderm specific HIF2a knockout mice lacking expression of the specific HIF2a isoform in the liver and intestine, exposed mice to a human relevant Mn regimen, and analyzed motor function, metal levels, and gene expression. **Results:** We unexpectedly observed that *HIF2a* knockout mice were protected against Mn-induced motor deficits. But, brain Mn levels of the *HIF2a* knockout mice were comparable to controls. Thus, rescue of the motor phenotype of the *HIF2a* knockouts is independent of a change in brain Mn. Interestingly, blood Fe levels of the knockout mice were significantly lower than controls after Mn exposure, suggesting that HIF2 may impact Mn neurotoxicity via effects on Fe metabolism. **Conclusions:** These findings indicate that peripheral HIF-2 signaling is a critical regulator of Mn induced motor deficits, likely through effects on Fe metabolism.

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Background and Purpose: Some methylene tetrahydrofolate reductase (MTHFR) polymorphisms can result in the deficiency of the enzyme MTHFR, which is crucial for the conversion of homocysteine to methionine, an integral precursor for the synthesis of monoamine neurotransmitters. The C677T polymorphism is the most common and clinically relevant genotype, with approximately 30-40% of the global population carrying at least one allele. Individuals with MTHFR enzyme deficiency are prone to hyperhomocysteinemia-induced oxidative stress, which may deplete the body's endogenous glutathione. As a result, toxins like mercury, which depend on glutathione conjugation for excretion, would bioaccumulate, increasing their toxicity. Mercury is considered the most toxic heavy metal that can cause serious neurological and psychiatric health effects. A common source of mercury exposure is through dietary consumption of seafood, which makes mercury poisoning particularly prevalent in populations where fish is a part of the staple diet. Although such correlations have been somewhat identified, the exact toxicological and biochemical processes/pathways involved are still an area of research. Here, we attempt to map such correlations using the existing literature. **Methods:** A structured literature search was conducted using PubMed and Google Scholar. Search terms included 'mercury,' 'Hg,' 'MTHFR,' and 'methylene tetrahydrofolate reductase.' Articles were screened for relevance to biochemical mechanisms, toxicokinetics, or clinical interactions between MTHFR polymorphisms and mercury exposure. **Results:** MTHFR polymorphisms and mercury exposure share several overlapping pathophysiological pathways, particularly those involving oxidative stress and glutathione exhaustion, which may contribute to similar neurological and psychiatric manifestations. **Conclusions:** MTHFR polymorphism may play an important role in some individuals with prolonged and persistently elevated mercury levels, despite taking precautionary measures. Testing for MTHFR polymorphism and/or homocysteine levels may help to identify metabolic factors that could influence the excretion of mercury. Additionally, it has been proposed that supplementation with methylfolate, methylcobalamin, and glutathione may help to overcome some of these issues associated with MTHFR polymorphism.

PS 3130 Developmental lead (Pb) exposure alters noradrenergic regulation of stress behavior in zebrafish (*Danio rerio*)

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Background and Purpose: Developmental Pb exposure disrupts neurophysiological systems that govern stress and emotional regulation. Prior work from our laboratory demonstrated that Pb impairs behavioral stress responses and alters biomarkers of noradrenergic (NA) signaling. Using *in vivo* fluorescence brain imaging, we observed disruptions in the locus coeruleus (LC) NA neuronal morphology—increasing neuron number, disrupting spatial organization, and suppressing spontaneous neuronal activity—as well as dose-dependent reductions in volume and axonal complexity of NA innervation to the dorsal telencephalon (zebrafish amygdala homolog). These findings drove our hypothesis that developmental Pb exposure perturbs LC NA neurons, thereby altering NA signaling mediated through adrenergic receptors that regulate stress responses. **Methods:** To evaluate this, we combined behavioral pharmacology, calcium imaging, and transcriptomic approaches. Zebrafish larvae were exposed to prazosin (α_1 -antagonist) or yohimbine (α_2 -antagonist) at 0.5, 2.3, 11.0, 56.5, and 280 μ M for 20 min or 12–16 hr. Stress responses were quantified using automated locomotor tracking during acoustic startle response (100 Hz, 100 ms; AMR) and visual motor response (1200 lux, VMR) assays. RNA-seq and calcium imaging were performed on larvae exposed to 75 and 750 μ g/L Pb to assess transcriptomic and LC NA neuronal activity changes. **Results:** Transcriptomic profiling revealed upregulation of oxidative phosphorylation genes alongside downregulation of proteolytic and ion-channel functions, indicating compensatory metabolic activation and impaired calcium signaling. Calcium imaging confirmed reduced spontaneous LC activity despite increased neuron number, suggesting enhanced neuronal survival but diminished functional integration. Acute yohimbine exposure produced significant hyperreactivity during AMR at 56.5 and 280 μ M and during VMR at 11.0 and 56.5 μ M, whereas prolonged exposure (12–16 hr) caused hyporeactivity at 56.5 and 280 μ M. Acute prazosin exposure produced significant increases in AMR endpoints and in VMR maximum distance while VMR total distance was significantly decreased at all but the highest exposure concentration (280 μ M). Similar to the effects of Pb exposure observed in VMR assays, 12–16 hr exposures to Prazosin produced a biphasic response, with significant increases in stress response at moderate concentrations and significant decreases in stress response at higher concentrations. **Conclusions:** Together, these findings indicate that α_1 - and α_2 -adrenergic receptor antagonists differentially modulate stress-response behaviors in zebrafish and that Pb exposure induces a LC phenotype characterized by neuronal hyperplasia coupled with reduced functional output; a phenotype possibly mediated by compensatory metabolic upregulation and disrupted calcium-dependent excitability. Ongoing studies are assessing the role of other adrenergic modulators in stress response and whether they can restore behavioral and neurophysiological changes mediated by Pb exposure. Identifying these mechanisms will advance understanding of NA dysfunction in developmental Pb neurotoxicity and support the development of targeted therapeutic strategies.

PS 3131 The effect of exercise on behavior and motor function following developmental benzo[a]pyrene exposure

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Background and Purpose: Pregnant women and their fetuses can be exposed to neurotoxic polycyclic aromatic hydrocarbons (PAHs) through air pollution and by eating foods cooked at high temperatures. Exercise is known to improve brain function, but little is known about how exercise during pregnancy and early life can affect the developing brain. In this study, we wanted to find out if exercise could protect against exposure to the model PAH, benzo[a]pyrene. **Methods:** To determine if exercise could mitigate the adverse effects, we compared *Cyp1b1* (-/-) knockout and *Cyp1b1* (+/+) wildtype mice. Dams were exercised two weeks prior to mating and until gestational day 10 (G10). From G10 to postnatal day 25 (P25), dams were dosed with either corn oil or 10mg/kg/day of BaP dissolved in corn oil. Offspring ran from P30-P60 when neurobehavioral testing began. Exercise consisted of 1h/day of voluntary running on wheels. We tested anxiety-like behaviors using marble burying and elevated zero maze. We tested mice in open field locomotor activity and used rotarod to assess motor coordination and balance. **Results:** Our results represent the findings from the first seven cohorts of mice tested. There was a significant main effect of genotype for locomotor activity with *Cyp1b1* (+/+) wildtype mice having higher activity levels compared with *Cyp1b1* (-/-) knockout mice. There was a main effect of exercise with offspring that exercised buried significantly fewer marbles ($P < 0.05$). There was a significant gene $\times$ treatment interaction with BaP exposure increasing anxiety-like behavior in wild type mice while decreasing it in the knockouts. There were no significant differences in zero maze. In rotarod, exercise significantly improved rotarod performance on Days 2-5 of testing ($P < 0.01$). **Conclusions:** Our preliminary results suggest that offspring exercise improved both motor function and motor memory in both genotypes of mice, regardless of treatment. We will continue testing until we have n=15-20 litters per group at which point we will also be able to test for sex differences.

PS 3132 Can Exercise Improve Learning and Memory Following Developmental Exposure to Benzo[a]pyrene?

C. Gogzheyana, M. Feltner, D. Fox, S. Lillard, L. Bishop, E. Lallai, T. Easybuck, R. Spigelmyer, and C. P. Curran. Northern Kentucky University, Highland Heights, KY.

Background and Purpose: Normal 0 false false false EN-US ZH-CN X-NONE /* Style Definitions */ table.MsoNormalTable {mso-style-name:"Table Normal"; mso-tstyle-rowband-size:0; mso-tstyle-colband-size:0; mso-style-noshadow:yes; mso-style-priority:99; mso-style-parent:""; mso-padding-alt:0in 5.4pt 0in 5.4pt; mso-para-margin:0in; mso-pagination:widow-orphan; font-size:10.0pt; font-family:"Times New Roman",serif;} Benzo[a]pyrene (BaP) is a neurotoxic polycyclic aromatic hydrocarbon that can be found in grilled foods, air pollution, car exhaust, and more. Pregnant women can be exposed to this carcinogen, which has been shown to cause major health deficits in their offspring. Exercise is known to improve brain function, but little is known about how exercise during pregnancy and early life can affect the developing brain. In this study, we wanted to find out if exercise could protect against exposure to BaP and its potential effect on motor function. **Methods:** Normal 0 false false false EN-US ZH-CN X-NONE /* Style Definitions */ table.MsoNormalTable {mso-style-name:"Table Normal"; mso-tstyle-rowband-size:0; mso-tstyle-colband-size:0; mso-style-noshadow:yes; mso-style-priority:99; mso-style-parent:""; mso-padding-alt:0in 5.4pt 0in 5.4pt; mso-para-margin:0in; mso-pagination:widow-orphan; font-size:10.0pt; font-family:"Times New Roman",serif;} We compared *Cyp1b1* (-/-) knockout and *Cyp1b1* (+/+) wild type mice, because our previous studies showed the knockouts were more susceptible to developmental BaP exposure. We treated the dams with either 10mg/kg/day BaP in corn oil-soaked cereal or corn oil-soaked cereal alone from gestational day 10 (G10) to postnatal day 25 (P25). Dams were exercised for two weeks prior to mating through G10. Offspring exercised from P30-P60 when behavioral testing began. Both groups received 1hr daily of voluntary exercise on running wheels. We used Novel Object Recognition and Morris Water Maze to test hippocampal-dependent learning and memory. **Results:** Normal 0 false false false EN-US ZH-CN X-NONE /* Style Definitions */ table.MsoNormalTable {mso-style-name:"Table Normal"; mso-tstyle-rowband-size:0; mso-tstyle-colband-size:0; mso-style-noshadow:yes; mso-style-priority:99; mso-style-parent:""; mso-padding-alt:0in 5.4pt 0in 5.4pt; mso-para-margin:0in; mso-pagination:widow-orphan; font-size:10.0pt; font-family:"Times New Roman",serif;} Our preliminary findings are based on seven cohorts of mice. *Cyp1b1* (-/-) knockout mice took significantly longer to complete the Novel Object Recognition test compared with *Cyp1b1* (+/+) wild type mice, but there was no significant difference in visual recognition memory. Exercise appeared to benefit BaP-exposed *Cyp1b1* (+/+) wild type mice more than *Cyp1b1* (-/-) knockout mice in the hidden platform phases of Morris water maze. BaP-exposed offspring that exercised had shorter average distances to the platform in the Reverse Phase. However, none of the differences weren't statistically significant. **Conclusions:** Normal 0 false false false EN-US ZH-CN X-NONE /* Style Definitions */ table.MsoNormalTable {mso-style-name:"Table Normal"; mso-tstyle-rowband-size:0; mso-tstyle-colband-size:0; mso-style-noshadow:yes; mso-style-priority:99; mso-style-parent:""; mso-padding-alt:0in 5.4pt 0in 5.4pt; mso-para-margin:0in; mso-pagination:widow-orphan; font-size:10.0pt; font-family:"Times New Roman",serif;} We will continue testing until we have 15-20 litters per group.

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Background and Purpose: Lead (Pb), a World Health Organization-identified toxicant of major public health concern, can cross placental and blood brain barriers during early development, resulting in heritable, life-long neurotoxicity. The aging brain is also uniquely susceptible to neurotoxic insult, triggering more rapid onset of cognitive decline and associated pathology. However, the contributions of lead exposure during these sensitive windows to neuromotor and neurodegenerative disease outcomes are currently not well understood. Thus, our overall goals are to characterize the molecular mechanisms driving Pb-induced persistent neurotoxicity and to identify the roles of individual and "multi-hit" exposures in disease progression using the zebrafish model (*Danio rerio*). **Methods:** We exposed fish cohorts to environmental levels of waterborne lead acetate (1-10,000 µg/L) at two neurosensitive windows: 2-26 hours post fertilization (hpf) in early neurodevelopment, and an acute five-day exposure in aged, 2-year-old fish. Larval fish were evaluated for mortality and neurodevelopmental phenotypes at 5 days post fertilization (dpf), including systemic abnormalities, a light:dark neuromotor assay, and an acoustic stimuli battery measuring habituation and learning. Pooled larvae tissues were collected. Fish were raised and spawned at 9 months post fertilization to produce F1 offspring. Fertility (% egg survival) and fecundity (eggs/female) were assessed. F1 offspring were raised to 5 dpf and evaluated as above. Separately, exposed aged fish were evaluated in a five-minute novel tank test, then euthanized for brain tissue collection. Genomic DNA and RNA were extracted from tissue samples. RNA libraries were generated using the Lexogen QuantSeq 3' mRNA-Seq kit, and DNA was processed for whole-genome bisulfite sequencing using an Illumina NEB Enzymatic-based Methylation library kit; both were sequenced with NovaSeq 6000. Differentially expressed genes (DEGs) were analyzed with DESeq2. DEG cut-offs were set at $|\log_2 FC| \geq 0.75$ and $p < 0.05$. DEGs were entered into IPA software to determine pathway enrichment. Methylation calling was performed using cscall v1.0 and the "mcomp" program. Differentially methylated CpG sites (DMCs) were defined as CpG with p -value < 0.01 . Average genome-wide methylation was compared and differentially methylated genes (DMGs; 1+ DMC) were identified ($p < 0.01$). DMGs were analyzed for gene ontology enrichment using DAVID (FDR < 0.05). **Results:** Early (2-26 hpf) exposure resulted in hyperactivity ($p < 0.001$) and increased rate of yolk sac edema ($p < 0.05$) at low-intermediate lead levels (10 µg/L). This behavioral phenotype persisted into larval offspring, where effects were more pronounced: 1 and 10 µg/L lead exposure induced hyperactivity in F1 larvae ($p < 0.001$), with 100 µg/L F1 displaying novel hypoactivity ($p < 0.05$). Acutely exposed aged fish were less sensitive, demonstrating non-monotonic effects on neuromotor activity at doses ≥ 10 µg/L ($p < 0.01$). Overall, neurotransmission and oxidative stress response transcriptomic pathways were disrupted across doses, with the most DEGs reported at 100 µg/L. We determined that average genome-wide methylation was increased at doses ≥ 100 µg/L, with ion transportation and neuronal signaling pathways most affected. A small subset of cytoskeletal, synaptic, and transcription-regulating genes were both differentially methylated and differentially expressed at the highest doses (100 - 10,000 µg/L). **Conclusions:** Larval lead exposure for 24 hours causes heritable disruptions to zebrafish behavior and development at low-medium doses. Aged fish also demonstrate non-monotonic impacts of lead exposure to behavior, transcriptome, and methylome at higher doses, particularly at 100 and 10,000 µg/L. While specific overlap was minimal, similar neurotransmission pathways were implicated in both the transcriptome and methylome, with a small subset of overlapping potential biomarkers identified. Future work will compare -omics and phenotypic profiles across neurosensitive windows and explore the role of multiple lifespan exposures in contributing to heritable neurobehavioral disease.

PS 3134 Gestational Exposure to Ultrafine Iron-Oxide Particles Produce Sex-Dependent Changes in Behavior and Neurotransmitters in Cortico-Striatal Brain Regions

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Background and Purpose: Air pollution exposure during gestation has been associated in epidemiological studies with neurodevelopmental disorders (NDDs). Gestational exposures to real-world, complex ultrafine particulate matter (UFP) air pollution have revealed neuropathological changes (neurotransmitter disruption) and behavioral alterations (alterations in sociality and ability to inhibit responding) in mice consistent with symptoms seen in these NDDs. Compositional analyses of ambient UFP mixtures in our experiments have consistently identified iron (Fe) as a major metallic component. In the early postnatal phase, inhalational exposure to Fe nanoparticles alone has reproduced male biased effects seen with ambient postnatal UFP exposure. However, it remains unclear whether gestational exposure to Fe UFP alone contribute to the adverse effects produced by ambient UFP exposures during gestation. Addressing this question is critical as previous UFP exposures result in differential sex-specific susceptibilities between postnatal and gestational exposures. This study hypothesized that exposure to Fe alone during gestation

would produce similar pathology (neurotransmitter disruptions across the lifespan and behavioral changes) as does ambient UFP exposure during gestation. **Methods:** Pregnant C57BL/6J mice were exposed to spark generated ultrafine Fe₂O₃ particles or HEPA-filtered air for 4 hours/day from gestational day 0.5 - 16.5 through whole body inhalation. The average mass concentration of Fe in the exposure group was approximately 4.54 µg/m³ across the exposure dates and average particle diameter was 18.8 nm. Subsets of pups were sacrificed on postnatal day (PND) 14 and PND60. Microdissected striatum and frontal cortex from brains from these mice were analyzed by LC-MS/MS to quantify levels of neurotransmitters, their metabolites, and turnover as well as amino acids. Another subset of mice were assigned to either a behavioral testing group or a non-behavioral group. The behavior group underwent testing on a battery of behaviors including locomotor assessment, novel object recognition, sociability and social novelty preference, social conditioned place preference, and various operant schedules of food reward (fixed interval, fixed ratio - waiting for reward, progressive ratio, and differential reinforcement of low response rates), while the non-behavior group were undisturbed in their home cages. Both groups were sacrificed at PND265. Similar to the other time points, the striatum and frontal cortex from these mice were analyzed by LC-MS/MS for neurotransmitter quantification. **Results:** Male and female mice exposed to Fe (mostly Fe₂O₃) during gestation showed sex-dependent behavior changes on the operant schedules of reward. Male Fe exposed mice had increased response rates on a fixed interval schedule. On a fixed ratio - waiting for reward schedule, male Fe exposed mice displayed increased run rates and decreased long wait times compared to male control mice. Taken together these data indicated a decreased ability to inhibit responding. Conversely, female Fe exposed mice exhibited decreased response rates on both the fixed interval and fixed ratio wait schedules of rewards when compared to the female air exposed control mice. In LC-MS/MS analyses of neurotransmitters, there were significant sex differentiated effects of Fe exposure across the life span, including changes in the dopaminergic system and amino acids in the female striatum, effects recapitulating those previously observed in response to ambient UFP exposures during gestation. **Conclusions:** Gestational exposure to Fe alone reproduces some behavioral and neurotransmitter changes produced by exposures to ambient UFP, indicating that Fe is a contributing factor to UFP-induced toxicity. While exposures during the postnatal phase often shows a stronger male bias, a female specific phenotype of behavioral and neurotransmitter changes emerges after gestational exposure. This indicates that females may be differently sensitive to UFP exposures based on critical window and/or route of exposure. Future research should interrogate these sex-differentiated phenotypes in mice and potential implications to phenotypic differences in humans. Supported by NIH Grants R35 ES031689, R01 ES032260, T32 GM007356, T32 GM152318

PS 3135 Parameterizing a mechanistic model of thyroid hormone kinetics and dynamics for cross species extrapolation using a three dimensional *in vitro* model of the developing brain

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Background and Purpose: Thyroid hormones (TH) are critical for neurodevelopment across vertebrates. Multiple OECD test guideline studies require measurement of TH, however the magnitude of disruption in animal studies required to indicate a neurodevelopmental concern in humans is not established. The use of mathematical modelling may facilitate cross-species extrapolation of TH disruption and allow for the establishment of a protective threshold for human risk assessment. Currently insufficient data are available in the published literature to parameterize such a model. This project addresses these data gaps through applying varying, physiologically relevant, concentrations of TH to sophisticated *in vitro* models of the developing rat and human brains. Assessed endpoints include neuronal migration and differentiation, as well as single-cell transcriptional changes. **Methods:** We applied the human Neurosphere Assay, which forms part of the OECD-endorsed developmental neurotoxicity (DNT) *in vitro* battery (IVB) and models seven key neurodevelopmental processes (KNDPs) *in vitro*. Human neural progenitor cell (hNPC) neurospheres were exposed to increasing concentrations of triiodothyronine (T3) and thyroxine (T4), and effects on hNPC proliferation, radial glia migration, neurite outgrowth, neuronal and oligodendrocyte migration and differentiation, as well as oligodendrocyte maturation, were assessed. Time-matched rat NPCs were similarly exposed within the Neurosphere Assay to delineate species-specific differences in TH-regulated brain development. Mathematical modelling was then applied to derive threshold concentrations defining the lower limits of TH required to maintain normal regulation of KNDPs. To elucidate the cell type-specific mechanisms underlying the observed phenotypic effects, single-cell RNA sequencing was performed on proliferating (day 0) and differentiating (days 2, 4, and 8) human and rat NPCs following T3 exposure. **Results:** We observed marked species differences in the regulation of key neurodevelopmental processes by thyroid hormones. NPC proliferation was regulated by physiological concentrations of T4 in both species (0.81 nM in human vs. 1.63 nM in rat), whereas T3 regulated proliferation exclusively in rat NPCs (0.037 nM). In contrast, neuronal migration and differentiation were controlled by low nanomolar concentrations of both T3 and T4 in the human model (0.14-0.18 nM), while developing rat neurons were largely insensitive to TH modulation. Oligodendrocyte maturation

tion was promoted by T3 in both species at comparable physiological concentrations (27 nM in human vs. 3 nM in rat); however, oligodendrocyte differentiation was stimulated by low picomolar concentrations of T3 only in the rat model (16 pM). Moreover, T4 promoted oligodendrocyte differentiation and maturation in rat NPCs (1.2 nM and 30 nM, respectively), but not in their human counterparts, which appeared insensitive to T4. Initial transcriptomic analyses are ongoing to identify molecular signatures underlying these divergent species-specific responses. **Conclusions:** This study indicates that clear species differences exist in neurological development processes *in vitro* in response to altered TH levels. This has implications for the appropriate use of developmental neurotoxicity studies in rodents for human risk assessment of substances which alter the homeostasis of TH. Overall, this study demonstrates the utility of organotypic *in vitro* systems to parameterize mathematical models for human risk assessment.

PS 3136 Emerging Neurotoxicants: PFAS induce Alpha Synuclein Aggregation and Mitochondrial Dysfunction in SH-SY5Y Neuronal Model

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are a class of synthetic organic compounds valued for their chemical stability and widespread use but increasingly recognized as persistent environmental pollutants with developmental neurotoxicity potential. While extensive data describe their systemic toxicity, the molecular effects of PFAS on neuronal proteins and organelles remain poorly understood. This study compares the effects of several PFAS and classical neurotoxins on protein aggregation, mitochondrial function, and stress signaling in dopaminergic SH-SY5Y wild-type and A53T α -synuclein-GFP cell models during neuronal differentiation. **Methods:** To determine whether the mechanism(s) behind PFAS-induced α -synuclein aggregation and mitochondrial dysfunction vary by concentration and chemical class, differentiated WT and A53-T α -synuclein-GFP SH-SY5Y were treated with PFOA, PFOS, PFDA, PFNA, rotenone, or paraquat (0 μ M-30 μ M). Cytotoxicity, neurite outgrowth, and α -synuclein aggregation were measured via fluorescence microscopy and confocal microscopy. Western blot analysis of α -synuclein, phospho-S129, HSP70, and protein disulfide isomerase (PDI) evaluated proteostasis disruption and oxidative stress responses. **Results:** PFAS exposure altered significant changes in neuronal morphology, including increased α -synuclein aggregation and elevated expression of pS129, PDI and HSP70, indicating proteotoxic stress. Each compound produced distinct yet convergent developmental neurotoxic patterns, suggesting multiple mechanistic routes to similar cellular outcomes. **Conclusions:** These findings indicate that structurally distinct PFAS elicit divergent mechanisms of developmental neurotoxicity, highlighting their potential role as environmental triggers of neurodegenerative disease. Ongoing work aims to elucidate upstream signaling networks and identify PFAS and protein interactions driving these effects.

PS 3137 Neurotoxicity of glyphosate and dicamba exposures on zebrafish (*Danio rerio*) embryo-larval model and adult systems.

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Background and Purpose: Glyphosate is the most widely used agricultural herbicide in the US and over the last 20 years, usage and production tripled. Historic usage has resulted in glyphosate resistant weeds. To combat this issue, alternative formulations such as Roundup Ready Xtend containing a mixture of glyphosate with dicamba is being used with approval in 36 states. Glyphosate can move into drinking water sources and is regulated at 700 parts per billion (μ g/L) by the US EPA. While glyphosate is highly used in the US, it is banned in several countries due to the risk of carcinogenicity (IARC Group 2A, probably carcinogenic to humans) and general toxicity. Dicamba, on the other hand, has not historically been used at a high rate but is changing with the introduction of this new herbicide formulation. Based on historic low use there are no drinking regulatory levels for dicamba currently in the US but with increased use comes additional contamination risks. Currently, there are limited toxicity studies with dicamba but those that exist report genotoxicity and biochemical toxicity. The current study was designed to study the sublethal effects of developmental glyphosate and dicamba exposure alone and in a binary mixture to evaluate developmental neurotoxicity using the zebrafish (*Danio rerio*) to test the hypothesis that binary mixtures of these herbicides are more toxic than singular exposure to either of these herbicides with exposure resulting in developmental and late-life neurotoxicity and genotoxicity and cause neurobehavioral impairments and potential neurodegeneration related outcomes. **Methods:** For the larval phase, zebrafish embryos were collected, and exposed starting 2 hours post fertilization (hpf) to 2 concentrations of glyphosate (700, 7000 ppb), dicamba (100 ppb), 2 binary mixtures (700 or 7000 ppb glyphosate/100 ppb dicamba), or a negative control (0 ppb). Fish were exposed until 72 for qPCR analysis or 120 hpf for neurobehavioral analysis. Behavior was assessed using DanioVision tracking software which altered the light phases into 3 dark and 2 light phases alternatively and tracked the movement of the larvae. For the genotoxicity assay the larvae were rinsed at 72 hpf and qPCR analysis was done for the genes in the BDNF/APP pathway. For the adult phase, zebrafish embryos were exposed to 700 ppb glyphosate, 10, 100 ppb dicamba, or 2

binary mixtures with 700 ppb glyphosate/100 or 10 ppb dicamba, from 2 - 120 hpf after which the larvae were rinsed with filtered water, raised for 8 - 12 months and neurobehavior analysis conducted during the adult phase. Three neurobehavioral tests were completed to assess anxiety (novel tank diving test), sociability (Social Preference Test) and learning and memory (Novel Object Recognition Test). After the neurobehavior was completed, the brains of the adult zebrafish were harvested and qPCR analysis of the genes in the same (BDNF/APP) pathway was assessed to determine adult neurotoxicity. **Results:** Results in the larval behavior assay indicated glyphosate, dicamba and the binary exposures led to a significant reduction in the distance moved, velocity, and time spent moving ($p < 0.05$), indicating hypoactivity amongst the exposed groups with the binary exposures showing a greater significant reduction as compared to the individual exposure treatment groups. From the qPCR analysis, it was found that glyphosate and the binary mixtures downregulated the expressions of *bdnf*, *ntkr2b*, *akt1*, *mapk6*, *appa*, *appb*, *bace1* and *sor11* ($p < 0.05$). In the adult neurobehavior assay, the results of the Novel Tank test and Social Preference Test indicate that the exposed fish are hypoactive and are more anxious, show more social avoidance behavior and impaired learning and memory behavior when compared with the adult control fish. From the qPCR analysis, it was observed that all the genes in the BDNF/APP pathway were upregulated. **Conclusions:** Overall, results show neurobehavioral alterations at environmentally relevant concentrations for single and binary exposures in both larval and early life exposed adult zebrafish. There is also an observed immediate or prolonged dysregulation of the genes in the BDNF/APP pathway indicating that early life exposure to these herbicides do have long life-lasting effects. Also, the binary exposures are more toxic than singular exposures supporting a need for a better understanding of the risks associated with the mixture exposure when considering the composition of the Roundup Ready Xtend.

PS 3138 Enhancing Mechanistic Neurotoxicity Evaluations with *in vitro* LUHMES-based Gene Co-expression Network Analysis

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Background and Purpose: Next-generation risk assessment (NGRA) is reshaping chemical safety by integrating efficient, cost-effective, and human-relevant new approach methodologies (NAMs). Toxicogenomics offers potential to extract mechanistic depth from these assays, yet current implementations rarely venture past isolated genes and non-specific gene sets. Since adverse outcomes are frequently preceded by coordinated and tissue-specific transcriptomic perturbations, it is critical that we advance safety assessment with this context in mind. For hepatotoxicity and nephrotoxicity, the TXG-MAPr webtool provides an accessible and interactive platform to explore early events and evaluate novel compounds in liver- and kidney-based test systems. No equivalent resource currently exists for neuronal assays. **Methods:** Here, as part of the EFSA TXG-MAP project, we present the first neuronal TXG-MAPr, built to assess toxicity in developing human dopaminergic neurons. For this purpose, we used LUHMES cells; an immortalised human mesencephalic neuronal cell line that can be reproducibly differentiated into post-mitotic, dopaminergic-like neurons within six days. At the early differentiation stage (days 2 to 3), when neurons are undergoing neurite outgrowth and are highly sensitive to perturbation, LUHMES cultures were exposed to 42 mechanistically informative compounds across multiple concentrations and timepoints, eliciting a range of distinct adaptive and adverse cellular stress responses. **Results:** The resulting network, built using weighted gene co-expression network analysis (WGCNA), comprises functionally-specific gene modules capturing both pathways preserved across tissues, such as oxidative and endoplasmic reticulum (ER) stress, as well as ones specific to the neurodevelopmental setting. We validate and showcase the utility of this TXG-MAPr tool for early-stage compound assessment and predictive toxicology by projecting independent external datasets onto the LUHMES-derived network. Moreover, we link module activity to phenotypic readouts, exploring how transcriptional modules connect to functional perturbations that precede neurotoxic endpoints. **Conclusions:** Taken together, this work expands the TXG-MAPr framework to a neuronal test system, advancing NGRA and promoting scalable, mechanistically-grounded evaluation of developmental neurotoxicity moving forward.

PS 3139 Adolescent Exposure to Ultrafine Particulate Matter Induces Sex-Specific Alterations in Behavior, Myelin and Transcriptome in the Medial Prefrontal Cortex

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Background and Purpose: Early-life exposure to air pollution is strongly linked to increased risk for cognitive and psychiatric impairments in later life. Adolescence, a developmental period marked by extensive morphological and functional changes of the brain, coincides with the emergence of multiple psychopathologies and may therefore represent a window of heightened vulnerability to environmental insults such as air pollution. Yet, the neurobiological mechanisms mediating these adverse

neurobehavioral effects remain poorly understood. **Methods:** We exposed adolescent mice to ultrafine particles (UFPs) from postnatal day 21 to 49 and examined behavioral consequences and underlying neurobiological alterations in a sex-specific manner. In tandem, a cohort of New York City children aged 8-14 years were recruited to evaluate their executive functioning and overall problems in relationship to their PM_{2.5} exposure level. **Results:** Adolescent UFP exposure induced sex-dependent behavioral alterations in young adult mice. These behavioral differences correspond to sex-specific changes in myelin content and internodal length within the medial prefrontal cortex (mPFC). Transcriptomic profiling of the mPFC revealed a threefold more differentially expressed genes (DEGs) in females than in males, with minimal overlap between sexes. Gene set enrichment analysis identified female-specific enrichment of pathways related to myelination, oligodendrocyte differentiation, and response to oxygen levels, and male-specific enrichment of pathways associated with triglyceride biosynthesis and circadian clock entrainment. *In vitro*, UFP exposure impaired mitochondrial function in oligodendrocyte progenitor cells from both sexes. Finally, in a cohort of New York City children aged 8-14 years, higher PM_{2.5} exposure was associated with greater difficulties in behavioral regulation, executive functioning problems, and overall problem behaviors in boys, but not in girls. **Conclusions:** These findings demonstrate that adolescent exposure to air pollution induces sex-specific neurobehavioral outcomes, accompanied by distinct transcriptional, structural and metabolic alterations, particularly involving myelin, in the mPFC. Together, the results provide a mechanistic framework linking adolescent air pollution exposure to differential susceptibility in males and females.

PS 3140 Disruption of CYP-Mediated Metabolism Modulates Region- and Dose-Dependent Effects of Developmental Exposure to 3,3'-Dichlorobiphenyl (PCB 11) on Neuronal Morphology in Juvenile Mice

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Background and Purpose: Developmental exposure to polychlorinated biphenyls (PCBs) is implicated in the etiology of neurodevelopmental disorders (NDDs), in part through their potential to disrupt normal patterns of neuronal connectivity during critical periods of brain development. While research on PCB developmental neurotoxicity (DNT) has largely focused on higher-chlorinated congeners found in legacy mixtures, lower-chlorinated (LC) PCBs predominate in contemporary environmental samples and human tissues. Of particular concern, PCB 11, an LC congener with documented widespread human exposure, was identified as the second-most abundant PCB congener in the serum of pregnant women at an increased risk of having a child with an NDD. But whether PCB 11 alters neuronal connectivity *in vivo* remains unknown. LC-PCBs are more readily metabolized than HC-PCBs, but the relative contribution of the parent congener vs. their metabolites on neurodevelopment has also not been rigorously addressed. **Methods:** To explore these data gaps, we quantified the dendritic morphology of pyramidal neurons of male and female pups exposed developmentally to PCB 11 in the maternal diet of wildtype (C57BL/6J), cytochrome P450 *Cyp2abfgs* gene cluster knockout (*Cyp2abfgs*-null or CYP-null), and CYP-null mouse that overexpress human CYP2A6 (2A6-Hum) mouse dams. Adult female mice (8-10 weeks old) were exposed to vehicle (peanut oil) or PCB 11 at 0.1, 1.0, or 6.0 mg/kg/d in peanut butter throughout gestation and lactation, and neurodevelopmental outcomes were assessed in their pups. Brains were collected at postnatal day (P) 35 and stained using Golgi-Cox methods. The stained neurons were visualized using a Leica Spinning Disc Confocal Microscope at 40x magnification and traced using Neurolucida software. Pyramidal neurons were analyzed from layer III/IV of the medial prefrontal cortex (mPFC), layers IV/V of the piriform/entorhinal cortex, and the hippocampus, with two to three neurons assessed per animal per region. **Results:** Developmental exposure to PCB 11 produced significant dose-, region-, and genotype-dependent alterations in dendritic arborization. In wild-type mice, exposure to 0.1 and 1.0 mg/kg/d PCB 11 decreased dendritic complexity in mPFC pyramidal neurons, reducing total dendritic length, whereas 6.0 mg/kg/d exposure increased arborization, evident as an increased number of primary dendrites. These effects were absent in CYP-null mice but recapitulated in 2A6-Hum mice, suggesting the dendritic effects of PCB 11 may be mediated by PCB 11 metabolites. Similar genotype-dependent patterns were observed in the piriform cortex, where 6.0 mg/kg/d PCB 11 increased dendritic branching, node number, and total length in wildtype and 2A6-Hum mice, but not in CYP-null mice. These effects on dendritic arborization occurred independent of changes in cell body area. PCB 11 had no effects on dendritic arborization in hippocampal neurons across genotypes. **Conclusions:** These findings extend previous reports that dendritic arborization and neuronal connectivity are vulnerable targets of PCB exposure and identify PCB 11 metabolism as a critical mediator of this developmental neurotoxicity. Given the critical role of dendritic morphogenesis in establishing neural circuitry during brain development, and the evidence from human studies linking disrupted neuronal connectivity to intellectual disabilities and the behavioral and cognitive abnormalities characteristic of NDDs, our findings—together with the strong correspondence between dendritic and previously characterized behavioral effects—suggest that dendritic alterations may underlie the behavioral deficits associated with developmental PCB exposure. This work was supported by NIEHS (R01 ES014901 to XD, HJL

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PS 3141 The zebrafish microbiome modulates neurobehavioral responses to azoxystrobin

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Background and Purpose: The gut microbiome is essential for neurodevelopment via bidirectional gut-brain axis signaling. Environmental chemicals can potentially disrupt this communication by altering community structure and xenobiotic metabolism, yet the functional consequences of such interactions for the host nervous system remain poorly understood. To address this gap, we investigated whether exposure to the broad-spectrum strobilurin fungicide azoxystrobin, a known metabolic disruptor, modulates microbiome composition and function to influence neurobehavioral outcomes. By integrating a controlled microbiota system (simplified human gut microbiota model, SIHUMix) with a vertebrate host model (larval zebrafish), we gain mechanistic insight into microbiota-mediated xenobiotic effects and clarify potential risks posed by azoxystrobin exposure. **Methods:** After stabilization, SIHUMix was exposed to azoxystrobin for 7 days at 10% of the acceptable daily intake (ADI), followed by a 4-day recovery phase. Temporal and functional responses were assessed via integrated metaproteomic and metabolomics profiling. To evaluate host-level consequences of azoxystrobin exposure, microbiome-depleted zebrafish larvae were derived, and a subset was colonized with SIHUMix at 1-day post-fertilization (dpf). Larvae were exposed at 4 dpf and neurobehavioral function was assessed at 5 dpf using the Visual and Acoustic Motor Response (VAMR) new approach method (NAM), complemented by targeted metabolomics. **Results:** Azoxystrobin exposure significantly altered SIHUMix community structure and metabolite production, resulting in a persistently altered microbiota state even after chemical removal. Integrated metaproteomic and metabolomic analyses revealed a functional reprogramming of the microbiota, with upregulation of pathways involved in vitamin and cofactor biosynthesis, nutrient acquisition, and detoxification. Decreased activity in carbohydrate fermentation and amino acid turnover was also observed, consistent with reduced short-chain fatty acid (SCFA) levels. These findings indicate a coordinated microbial stress response and impaired metabolic homeostasis. In zebrafish, azoxystrobin exposure disrupted non-associative habituation learning independent of microbiome status. Notably, azoxystrobin induced dark phase-hyperactivity exclusively in colonized larvae, demonstrating a microbiome-dependent neurobehavioral phenotype. Targeted metabolomics revealed higher baseline levels of serotonin in the colonized control, which decreased following exposure. In microbiome-depleted larvae, serotonin levels were significantly lower than in colonized larvae and remained unchanged after exposure, indicating that microbiota-derived metabolites are required for azoxystrobin-induced serotonergic alterations. This pattern suggests that azoxystrobin and microbiota jointly shape serotonergic signaling, supporting a potential mechanistic pathway by which microbiome metabolic reprogramming contributes to the observed neurobehavioral effects. **Conclusions:** Collectively, these results reveal that chemical-microbiome interactions can manifest at the level of host neurobehavior by linking microbial metabolic reprogramming to disrupted serotonergic signaling. This integrated *ex vivo-in vivo* approach supports the concept that the microbiome is a key determinant of neurotoxic responses and underscores the importance of incorporating microbiome-mediated effects into chemical risk assessment frameworks.

PS 3142 PFAS as myelin-targeting developmental neurotoxicants: cross-model evidence for persistent white-matter and sensory circuit vulnerability

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Background and Purpose: PFAS are bioaccumulative pollutants crossing placental and blood-brain barriers, exposing fetal and neonatal brains. As myelin is lipid-rich and a potential PFAS sink, early exposure may impair white-matter integrity and behaviours relevant to multiple sclerosis (MS). This work analysed whether PFOS and PFOA exposure affect myelin physiology and health consequences. **Methods:** (1) Conditional demyelination was induced in *Xenopus laevis* Tg(*mbp:GFP-NTR*) tadpoles exposed to PFAS during remyelination, assessing visual consequences. (2) Mouse cerebellar slice cultures were demyelinated with lysolecithin and treated with PFAS +/- thyroid hormone (TH). (3) Perinatal mouse PFAS exposure enabled

quantification of neurogenesis in the subventricular zone (SVZ), and consequences on oligodendrocyte maturation, myelin ultrastructure and adult olfactory memory. (4) Giant unilamellar vesicles (myelin-lipid GUVs) assessed PFAS-induced myelin stability. (5) Spatial epidemiology examined links between PFAS exposure and MS prevalence. **Results:** PFOS, but not PFOA, inhibited functional remyelination in *Xenopus*. In mouse cerebellar slices, PFOS reduced remyelination rescued by TH co-treatment, suggesting TH-sensitive pathways. Perinatal PFOS exposure accumulated in myelin and resulted in myelin thinning and axonal damages. SVZ neurogenesis was reduced at P21, but recovered by adulthood, while oligodendrocyte maturation deficits remained. PFOS reduced dopaminergic interneurons in olfactory bulbs, causing lasting olfactory memory deficits. GUV assays demonstrated PFOS-driven alterations in myelin biophysical properties, consistent with myelin destabilization. Lastly, exploratory spatial epidemiology suggested geographic correlation between PFAS contamination and MS prevalence. **Conclusions:** The findings show that PFOS acts as a myelin-targeting developmental neurotoxicant that damages myelin and sensory circuits. This is linked to TH-responsive remyelination and myelin-lipid perturbation. Cross-species and cross-scale data (ultrastructural, biophysical, cellular, behavioural,) highlight the toxicological relevance of this issue and support heightened public-health vigilance on early-life PFAS exposure as a potential modifier of MS risk and sensory health.

PS 3143 Sirtuin pathway under model of persistent methylmercury neurotoxicity in hiPSC-derived cortical neurons

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Background and Purpose: Methylmercury (MeHg) is a well-known environmental neurotoxicant with human exposure primarily through dietary sources such as fish and rice. MeHg exposure is strongly linked to oxidative stress, disruption of redox homeostasis, and mitochondrial dysfunction. Developmental exposure to MeHg is of particular concern, as it can lead to long-lasting neurotoxic effects that persist in adulthood. Sirtuins (SIRT1-7), are deacetylases or ADP-ribosyltransferases that require NAD⁺ for their enzymatic activity (SIRT1-7) and play a critical role in cellular responses to oxidative stress, cell proliferation and cell death. Given their involvement in neurodevelopment and mitochondrial biogenesis, dysregulation of SIRT1 and SIRT3 signaling has been implicated in various neurodegenerative disorders, including Alzheimer's and Huntington's disease. Studies suggest that the sirtuin pathway may also mediate the toxic effects of environmental pollutants, including mercury. Previous scRNA-seq and proteomics analysis from our lab demonstrated that the sirtuin pathway is significantly altered after developmental exposure to MeHg. Therefore, we hypothesized that developmental exposure to MeHg disrupts SIRT-dependent signaling, leading to persistent neurotoxic effects, and that pharmacological modulation of this pathway could either mitigate or exacerbate MeHg-induced phenotypes. **Methods:** Human induced pluripotent stem cells (hiPSCs) were exposed to non-cytotoxic doses of MeHg (1, 10, and 100 nM) during a developmental window of cortical neuronal differentiation (D7-D13). At D40, the expression and localization of SIRT1 and SIRT3 were assessed by immunofluorescence and quantified using Fiji. SIRT1 activity was evaluated by analyzing its acetylated downstream targets, including NF- κ B and Ac-p53. scRNA-seq and proteomics data were analyzed using R and Ingenuity Pathway Analysis (IPA) to identify the genes and proteins associated with the sirtuin signaling pathway and to explore their connectivity to the different isoforms of SIRT1. Additionally, we modulated SIRT1 and SIRT3 activity using selective inhibitors (i.e. EX-527/Selisistat and 3-TYP, respectively) to evaluate their impact on mitochondrial respiration (SeaHorse XF Mito Stress) and energy production efficiency (SeaHorse XF Real-Time ATP Rate). **Results:** 40 days after cessation of exposure, SIRT1 protein expression was not significantly altered, nor was its nuclear localization. Consistently, there was no impairment of SIRT1 activity as indicated by the absence of increased nuclear translocation of NF- κ B p65, a downstream target of SIRT1 deacetylation. However, scRNA-seq analysis revealed a significant enrichment of the sirtuin signaling pathway, with most of the differentially expressed genes being linked to mitochondrial function. This suggests that while SIRT1 expression and localization may remain unchanged, downstream signaling or potential redistribution between the nucleus and cytoplasm could still be affected. Furthermore, it indicates that SIRT3, localized in the mitochondria, could be further affected by MeHg exposure. To address this, we are investigating SIRT3 activity as well as mitochondrial health after pharmacological modulation of SIRT1 and SIRT3. **Conclusions:** Together, these data help clarify which SIRT1s contribute to the enrichment of the sirtuin signaling pathway and the extent to which each contributes to the long-term effects of MeHg-induced persistent neurotoxicity. This work was supported by NIH [R01 ES07331 (ABB/MA) and R01 AG080917].

PS 3144 Developmental Exposure to PFOA and a Novel Resource Deprivation Stress Enhances Sex-specific Deficits in Motivation and Motor Deficits

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Background and Purpose: Neurobehavioral disorders have complex etiologies with multiple environmental factors (e.g. toxicants, stress, nutritional deficits) potentially conferring risk. However, animal models evaluating combinatorial or complex mixtures effects are limited. Per-fluorinated compounds, such as perfluorooctanoic acid (PFOA), are known to disrupt the formation of cholesterol, dysregulate crucial steroidogenesis, and alter corticotropin-releasing hormone necessary for Hypothalamic-Pituitary-Adrenal (HPA) Axis signaling. This shared biological target provides the foundation for our hypothesis that exposure to PFOA combined with perinatal, maternal stress will result in enhanced, sex-specific neuroendocrine and behavioral dysregulation. **Methods:** To test this hypothesis, pregnant C57BL6/J mouse dams were exposed to either perfluorooctanoic acid (PFOA) - 100 μ g/kg/day, or vehicle (Veh), via oral treat, from gestational day (GD) 0.5 to birth. Mice were housed in modified cages designed for resource deprivation (RD) stress. RD stress is a novel stressor based on unpredictable and uncontrollable access to high-value treats. Animals are paired and separated by a wire mesh. One animal receives the treat while the other is deprived of the treat. The deprived mouse can see, hear, and smell the other mouse and treat. Dams were paired by treatment (PFOA or Veh) on GD7 and randomly assigned to either Enriched or RD group until weaning. Enriched dams received mealworms three times daily. Deprived animals receive three treats, then one treat, then no treats across gestation and lactation. Time to pregnancy, pregnancy duration, number of pups, sex ratio, pup loss, and pup weights were recorded. Behavioral assessments, performed from PND60 onwards, included locomotion, cognitive function, and motivation. Mice were euthanized for blood and tissue collection, where brains were micro-dissected into specific regions and flash frozen. Serum was collected from blood samples to measure peripheral hormones. Neurotransmitters, amino acids precursors, and metabolites were measured via liquid chromatography mass-spectrometry (MS) MS from frontal cortex and striatum. Two-way analysis of variance test, and subsequent post-hoc tests (student's t-test) depending on significant outcomes, were conducted stratified by sex. **Results:** In an open field, RD males had increased vertical and jump time, and there was a marginal interaction where Vehicle-RD exposed males had increased average ambulatory speed. Enhanced effects were observed uniquely in PFOA+RD exposed males. PFOA+RD males have increased stereotypic time and decreased resting time, indicating increases in localized, potentially self-directed, movement. In females, exposure to RD marginally decreased ambulatory time, distance traveled, and number of ambulatory episodes. For the elevated plus maze, male PFOA-RD mice spent an increased amount of time in and number of entries into the center of the maze compared to the PFOA-Enriched mice. Additionally, RD males had significant increase, while females had a marginally significant increase in entries into the open arms. RD stress exposed females had an increased number of total lever presses in a high effort progressive ratio operant paradigm, while no effects were seen in males. RD exposed females also had a uniquely high number of lever presses, higher response rate, and total number of rewards during a 60 second fixed interval task. These data indicate that females exposed to stress had increased motivation for food reward. Neuroendocrine and biochemical shifts were observed in PFOA and RD exposed mice in a sex, region and time point dependent fashion. **Conclusions:** Our results show *in-utero* exposure of RD stress and PFOA alter offspring behavior in a sex-dependent manner. Males exposed to RD present a hyperlocomotive phenotype, while females exposed to RD show evidence of increased motivation for food-mediated reward. Enhanced effects of PFOA and stress were seen in male mice with increased stereotypies and behaviors in the center of the EPM maze. These data indicate that additional stress physiology and CNS systems underlying food reward may have been reprogrammed by these development insults, necessitating more research.

PS 3145 The Effect of Juvenile Polychlorinated Biphenyl Exposure on Autism Spectrum Disorder Related Behaviors and Dendritic Morphology in Mice

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Background and Purpose: Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder with complex etiology affecting 1:36 children in the United States in 2020, as compared to 1:68 children in 2010. ASD is characterized by alterations in connectivity of key neuronal networks that give rise to phenotypic symptoms like difficulties in socialization and communication, as well as increased repetitive behaviors. Gene by environment risk interactions are posited to contribute to the rise in ASD cases. Mutations in the Shank3, a scaffolding protein in the post synaptic density of dendritic spines, account for 1-2% of ASD cases. Polychlorinated Biphenyls (PCBS) are a group of persistent environmental chemicals identified as risk factors for ASD. The MARBLES PCB mixture represents the 12 most abundant PCB congeners in the serum of pregnant women who already had one child with ASD. *Developmental* exposure to the MARBLES PCB mixture has been shown to reduce social behav-

ior and increase repetitive grooming in wild type mice. However, individuals are exposed to PCBs throughout life, and the juvenile time period (3-6 weeks) is a key period of dendritic pruning and maturation of dendritic spines to form stable connections. Here we test the hypothesis that *juvenile* exposure to MARBLES PCB mixture increases ASD related behavioral phenotype severity in wild type mice and that these PCB effects will be more severe in Shank3 mutants **Methods:** Wild type (WT) and heterozygous Shank3 (B6.129-Shank3tm2Gfng/J) mice of both sexes (n=10-15/group/sex) were orally dosed from weaning (p21) until six weeks of age (p42) with MARBLES PCB mixture at 0, 0.1, or 1mg/kg/d suspended in peanut oil and mixed into peanut butter. ASD related behavioral phenotypes were assessed using social approach, repetitive grooming and social odor discrimination. Mice were euthanized per approved protocol and brains removed. Brains were bisected and stained according to the manufacturers instructions using FD Rapid GolgiStain kit. Neurons from somatosensory cortex, hippocampal CA1, and hippocampal CA3 were visualized using Keyence-BZ X710 in brightfield. Image analysis was conducted using Keyence Analysis software. Sholl analysis was conducted to assess dendritic complexity and the number or primary and secondary dendrites, total dendritic length, and number of dendritic tips were visually assessed by researchers blinded to treatment conditions. **Results:** 3 chamber social approach: Vehicle control WT males spent more time engaged with a novel mouse as compared to a novel object, as expected. PCBs had no effect on this parameter in WT males. Interestingly, vehicle control WT females displayed more variability in social approach, with the amount of time spent with a novel mouse not reaching significance. Female WT mice treated with 0.1mg/kg PCBs did display a significant amount of time in social engagement, but 1.0mg/kg PCB treated females did not. Repetitive grooming: PCBs did not significantly alter time spent self-grooming in WT male mice. However, WT female mice treated with 0.1mg/kg PCBs displayed less time grooming than vehicle control or 1.0mg/kg treated mice. Analysis of PCB effects in Shank mutants is underway. Dendritic morphology: effect of PCB exposure on dendritic complexity using Sholl analysis is underway. **Conclusions:** Juvenile exposure to MARBLES PCB mixture does not reduce time spent engaged in social behavior as measured by social approach in wild type mice, as expected. In some cases, PCB treatment resulted in more time spent engaged in social behavior. Female mice displayed greater variability in behavior and less time spent in social behavior in WT vehicle controls, indicating female ASD related behavior is less consistent than in male mice. Time spent in repetitive grooming behavior in WT mice was similarly not altered by PCB exposure. PCB treated animals trended to have less time spent grooming in both male and female WT mice. Overall, exposure to 0.1mg/kg and 1.0mg/kg MARBLES PCB mixture in a juvenile time of 3-6 weeks does not worsen ASD related behavioral phenotypes. These results highlight the importance of timing of PCB exposure on behavioral consequences and suggest that juvenile exposures may not be as influential as developmental exposures in producing behavioral phenotypes in mice.

PS 3146 Gestational and lactational exposure to the synthetic cannabinoid nabilone at clinically relevant dose levels does not adversely affect offspring development or neurobehavior in rats

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Background and Purpose: Accumulating evidence indicates that the developing brain is vulnerable to the effects of cannabinoids and that gestation may be a critical window of exposure to these compounds. Nabilone is a synthetic cannabinoid that is approved for the oral treatment of nausea and vomiting associated with cancer chemotherapy in patients who have failed to respond adequately to conventional antiemetic treatments. Nabilone is structurally similar to the psychoactive cannabinoid delta-9-tetrahydrocannabinol (delta-9-THC) naturally occurring in *Cannabis sativa L.* (i.e., marijuana) and is thought to act via the cannabinoid receptor 1 (CB1). Despite increasing studies demonstrating adverse effects of gestational cannabinoid exposure on neurodevelopment, it is not fully understood whether these effects extend to synthetic drug cannabinoids such as nabilone. Therefore, a peri- and post-natal developmental toxicity study was conducted in rats to evaluate the effects of gestational nabilone exposure on offspring neurobehavior at clinically relevant dose levels. **Methods:** Experimental work was completed at Charles River Laboratories (Horsham, PA). Pregnant Sprague-Dawley rats (main study, N = 22 dams/group; toxicokinetic [TK] phase, N = 3-6 dams/group) were administered nabilone via oral gavage as a suspension at dose levels of 0 (vehicle), 0.25, 1, or 2 mg/kg/day once daily beginning on gestational day (GD) 7 through postnatal day (PND) 20. Dose levels were selected based on maternal and offspring body weight reductions in a dose range-finding study which resulted in systemic plasma exposures up to 2-fold clinical exposures achieved at the maximum recommended human dose (MRHD). Dams (F₀) were evaluated for viability, clinical signs, bodyweight, food consumption, parturition, litter outcomes, maternal behavior, number of implantation sites, and gross lesions. Nabilone concentrations in maternal plasma and milk were determined by a validated LC-MS method. Offspring (F₁) were evaluated for viability, clinical signs, bodyweight, food consumption, sexual maturation, behavior assessments (functional observational battery [FOB], passive and active avoidance, social interaction, motor activity, acoustic startle habituation, Morris water maze [MWM]), reproductive capacity, and gross lesions. **Results:** In F₀ dams, nabilone reduced gestational and lactational bodyweight gain in a dose-dependent manner with corresponding reductions in food consumption. There were no additional effects of nabilone on the dams at any dose level, including delivery and litter parameters. Offspring bodyweights were reduced in a dose-dependent manner in both sexes throughout the lactation period

and persisted in males out to PND 57 but not in females. Nabilone did not have additional effects on clinical observations, food consumption, sexual maturation, mating/fertility, or necropsy observations in F₁ offspring at any dose level. In social interaction evaluations, high dose males displayed increased number of crossovers away from a social affiliation animal; however, the total number of crossovers (to and from the social affiliation animal) was not affected. Females exhibited increased arousal/alertness in the FOB in a dose-dependent manner without corresponding alterations in open field behaviors, indicating a lack of biological relevance. Nabilone did not significantly affect any other neurobehavioral endpoints at any dose level. **Conclusions:** Overall, this study demonstrates that gestational and lactational exposure to nabilone does not significantly impact offspring neurodevelopment in rats when administered at clinically relevant dose levels. These findings indicate a potential for differential neurodevelopmental toxicity risk based on the type of cannabinoid and underscore the need for continued research into this area.

PS 3147 Expanding the assessment of learning and memory with the Cincinnati water maze

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Background and Purpose: With increasing concern around developmental neurotoxicity (DNT), regulatory agencies are encouraging the expansion of learning and memory (L&M) assessment. The Morris water maze (MWM) is widely used and well-established for evaluating spatial/allocentric L&M. The Cincinnati water maze (CWM) under infrared lighting has demonstrated high sensitivity for assessing egocentric L&M. Including the CWM under infrared lighting in regulatory guideline test settings would broaden the evaluation of different parts of the brain and multiple aspects of L&M. Regular conduct in several labs considerably expands the knowledge base of this aspect of neurobehavioral testing, which is currently mainly based on research in a single academic lab. **Methods:** This study aimed to establish and validate the 10T CWM under infrared lighting in an industry setting. Following a successful feasibility study using 10 male and 10 female young adult Wistar Han rats (PND 60), a validation study was conducted with the same number of animals per test group. Scopolamine was administered as a positive control to assess sensitivity. Latency and number of errors were used to assess L&M over 20 days with 2 trials per day. **Results:** Young adult Wistar Han rats can complete the CWM. A sex difference was observed. The validation of CWM was successful; administration of scopolamine demonstrated the ability of the CWM to detect adverse effects on L&M using both latency and number of errors. **Conclusions:** This study demonstrates that the CWM under infrared lighting can be implemented in other laboratories outside of the original Cincinnati site. However, the inclusion into rigid and complex testing setups, like the OECD 443 study requires consideration due to the required time necessary to perform the CWM. This research also expands the knowledge on a different strain of rat, the Wistar Han, which is a common rat strain used in toxicological studies, but had not previously been assessed with the CWM. Ongoing studies are examining the feasibility of testing weanlings (PND 25) in the CWM.

PS 3148 A data-driven approach to identify performance compounds for development of in vitro blood-brain barrier new approach methods

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Background and Purpose: The Organisation for Economic Co-operation and Development has recommended an *in vitro* battery (IVB) of new approach methods (NAMs) for evaluation of potential developmental neurotoxicity (DNT). These NAMs capture fundamental cellular processes critical to neurodevelopment; however, none of these approaches incorporate or model the blood-brain barrier (BBB). The BBB is imperative for elimination or minimization of toxic insult to the central nervous system; thus, NAMs which recapitulate chemical-induced effects on barrier formation and/or integrity are essential to the interpretation of potential DNT. As such, NAMs developers will require a set of chemicals expected or not expected to be BBB permeable, with or without anticipated DNT, to demonstrate assay performance. To create a list of performance compounds, the present work utilized a data-driven approach in which publicly available datasets were curated to identify applicable chemicals. **Methods:** To start, a dataset of 1004 chemicals with or without *in vivo* evidence of BBB permeability applied to train two quantitative structure activity relationship (QSAR) models was curated - 944 chemicals were applicable for this analysis. These chemicals were then batch searched by CASRN on the EPA CompTox Chemicals Dashboard - this search identified 629 chemicals. These chemicals were then merged with the 509 chemicals evaluated in at least one DNT IVB assay (ToxCast *invitrodb* v4.2) - this merge identified 47 chemicals. For these 47 chemicals, sensitivity, specificity, and accuracy of the two QSAR models was determined with the *in vivo* evidence applied as the actual value. Moreover, selective activity in the DNT IVB assays for each chemical was calculated. Finally, the chemicals were further curated and ranked - 31 chemicals were identified as potential performance compounds. Those chemicals predicted to cross the BBB that were evaluated in more DNT assays (≥10) and tested across multiple institutions that were selectively active in more assays or not selectively active in any assays, were ranked as high

confidence active or inactive performance compounds, respectively. **Results:** The sensitivity, specificity, and accuracy of QSAR models 1 and 2 were 91%/67%/85% and 97%/46%/83%, respectively. With the assumption that no *in vivo* evidence had been available, QSAR model 1 was applied for the BBB permeability prediction of the 47 chemicals - 35 chemicals were predicted to be BBB permeable, while 12 were not. In addition, 26 chemicals were selectively active in at least one DNT IVB assay, while 21 were not selectively active in any. Furthermore, 16 chemicals were excluded from consideration due to volatility (1), classification as a scheduled drug (8), or evaluation in only one DNT IVB assay (7). Sixteen chemicals were ranked as potential active performance compounds, while 15 were ranked as potential inactives. As an example, bisphenol A was ranked as a high confidence active performance compound because it was predicted to cross the BBB, and it was tested in 14 DNT IVB assays and selectively active in five. Conversely, D-mannitol was ranked as a low confidence inactive performance compound because it was not predicted to cross the BBB, but it was tested in 14 DNT IVB assays and only selectively active in one. **Conclusions:** This data driven approach which relied on *in silico* and *in vitro* datasets, identified 31 chemicals that could be applied as potential performance compounds in the development of *in vitro* BBB NAMs. Moreover, the nuance behind the classification and ranking of these chemicals, highlights the need for BBB NAMs to enhance interpretation of DNT IVB assays.

PS 3149 Disruption of Mesolimbic Dopaminergic Neuron Numbers in the Prairie Vole Brain by Developmental Exposure to a Common Flame-Retardant Mixture

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Background and Purpose: Our laboratory uses the prairie vole (*Microtus ochrogaster*), a socially monogamous, biparental rodent, and model organism for neurodevelopmental disorders (NDDs), to explore how early life chemical exposures impact the development of human-relevant circuits and systems essential to prosocial behaviors such as attachment. Here we focused on two sexually dimorphic subpopulations of mesolimbic dopaminergic (DA) neurons, some of which express ESR1 (estrogen receptor α). Exposure was to vehicle or one of three doses of Firemaster 550 (FM 550), one of the most prevalent commercial flame retardant (FR) mixtures in the US, and containing a near 50:50 ratio of brominated and organophosphate ester components. Here we present the results from the lowest dose from the ongoing study. It has repeatedly been shown in vertebrates that developmental FM 550 exposure sex-specifically alters socioemotional behaviors, including complete loss of pair bonding in male prairie voles. The mechanism remains unknown, hence we examined three highly sexually dimorphic regions underlying attachment behavior: the bed nucleus of the stria terminalis (BNST), the medial preoptic area (MPOA), and the anteroventral periventricular nucleus (AVPV). The mesolimbic dopamine system is essential for attachment behaviors including pair bonding, selective aggression, and paternal behavior. DAergic neurons, identified by tyrosine hydroxylase (TH) immunoreactivity (TH-ir), are more abundant in the male BNST with little to no sex difference in the MPOA or AVPV. Levels of estrogen receptor α (ER α) co-expression in the BNST are strongly related to degree of male prosociality. Additionally, prior work has implicated ER α as a potential hub target of FM 550 exposure. We hypothesized that developmental FM 550 exposure sex and dose-specifically alters TH-ir cell numbers including those co-expressing ER α . **Methods:** Prairie vole dams were injected with 0, 500 (data herein), 1000 or 2000 μ g FM 550/day across gestation and lactation, and the offspring euthanized by transcardial perfusion as adults. Brains were removed, cryoprotected, and frozen flash on postnatal days (PNDs) 77-85, then sectioned at 40 μ m for immunolabeling. TH-ir and ESR1-ir were imaged by confocal microscopy, and analyzed using Image J. Cell numbers of TH-ir with/without ESR1-ir in each section were then confirmed by hand. Data were analyzed by ANOVA with sex and exposure as main effects. **Results:** As anticipated, BNST TH-ir cell numbers were higher in males, especially in the posterior BNST (pBNST). A sex difference was also found in the periventricular (Pe) area of the MPOA, but not in the AVPV. AVPV and MPOA TH-ir numbers were higher in the 500 μ g males while, in females, exposure increased TH-ir numbers in the BNST. In females, the number of TH and ESR-1 co-localized cells were also increased by exposure in all three regions. **Conclusions:** This work will begin to help identify the potential mechanisms by which chemical exposures disrupt the neurodevelopment of circuits and systems underlying socioemotional deficits common to neurodevelopmental disorders. The data also build on our previous work showing that developmental FM 550 exposure disrupts ER α -ir neuron numbers in social brain nuclei and is thus endocrine disrupting.

PS 3150 Leveraging HiBiT cell lines for assessing off-target degradation of developmental and hematopoietic related proteins

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Background and Purpose: Targeted protein degraders (TPDs) are a new modality that leverage the ubiquitin-proteasome system to target a protein of interest. Cereblon-based TPDs hijack the cereblon E3 ubiquitin ligase and tag target proteins for proteasomal degradation. However, cereblon-based TPDs can also cause degra-

dation of multiple neosubstrates (e.g., off-target proteins) leading to unwanted toxicities. Herein, we use HiBiT cells to assess degradation of neosubstrate proteins upon treatment with cereblon-based TPDs, focusing on proteins relevant to developmental toxicity (SALL4, GSPT1 and CSNK1A1) and proteins relevant to hematopoietic toxicity (IKZF1, IKZF3 and CSNK1A1). **Methods:** Targeted protein degraders (TPDs) are a new modality that leverage the ubiquitin-proteasome system to target a protein of interest. Cereblon-based TPDs hijack the cereblon E3 ubiquitin ligase and tag target proteins for proteasomal degradation. However, cereblon-based TPDs can also cause degradation of multiple neosubstrates (e.g., off-target proteins) leading to unwanted toxicities. Herein, we use HiBiT cells to assess degradation of neosubstrate proteins upon treatment with cereblon-based TPDs, focusing on proteins relevant to developmental toxicity (SALL4, GSPT1 and CSNK1A1) and proteins relevant to hematopoietic toxicity (IKZF1, IKZF3 and CSNK1A1). **Results:** Each compound had a unique degradation profile. CC-885 degraded all 5 neosubstrates - SALL4, GSPT1, CSNK1A1, IKZF1 and IKZF3. Thalidomide degraded SALL4, and DEG-77 degraded CSNK1A1. Ibrandomide, lenalidomide and pomalidomide degraded SALL4, IKZF1 and IKZF3. N-methylated pomalidomide did not cause degradation of any neosubstrates - SALL4, GSPT1, CSNK1A1, IKZF1 and IKZF3. **Conclusions:** The results demonstrate that cells engineered with HiBiT tag onto cereblon neosubstrates are a useful model for assessing degradation of specific neosubstrates by cereblon-based TPDs and therefore could be used to identify potential developmental or hemopoietic toxicity liabilities of novel TPDs. With further characterization and development, these HiBiT cell lines could also be used to elucidate structure-activity relationships.

PS 3151 Environmentally Relevant Concentrations of Triphenyl Phosphate (TPHP) Impact Development in Zebrafish

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Background and Purpose: Triphenyl phosphate, or TPHP, is a common plasticizer and flame retardant. Studies of tap water as well as lakes and rivers frequently report TPHP in the range of a few to hundreds of ng/L. Zebrafish have emerged as a model to study TPHP toxicity, but most studies have only explored the impact of mg/L exposures which caused pericardial edema and cardiac looping defects. Our question was rooted in the developmental effect of environmentally relevant exposures to TPHP. **Methods:** Developing zebrafish at epiboly were chronically dosed with nM concentrations of TPHP in their media (0.3x Danieau's) and phenotypic and gene expression data was collected for 5 days. **Results:** At the phenotypic level, we found no cardiac looping but chronically exposure 5dpf animals were shorter in length and displayed pericardial edema. Given its impact on the heart, RNA-Seq was conducted at three different time points in heart development—early (18hpf), mid (48hpf) and late (120hpf). 48hpf, where the heart is looped and consists of the atrium and ventricle separated by the atrio-ventricular canal (AVC), had the most differentially expressed transcripts between control and treated samples. TPHP did not alter the RXR pathway as had been shown at the uM level. However, a novel pathway involving the *tbx5a* transcription factor cascade including natriuretic peptides (*nppa* and *nppb*) and bone morphogenetic protein 4 (*bmp4*) were dysregulated. We also demonstrated that TPHP is a weak pro-oxidant, as it increases the oxidative stress response within hours of exposure. **Conclusions:** Our data indicate that TPHP can affect animal development at environmentally relevant concentrations and its mode of action involves multiple pathways that are unique to sub-acute exposures.

PS 3152 Acute and Developmental Toxic Effects of Dichloroacetic Acid in Zebrafish: Role of Oxidative Stress

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Background and Purpose: Dichloroacetic acid (DCA), a halogenated byproduct of water chlorination and a metabolite of industrial solvents such as trichloroethylene and tetrachloroethylene, has been detected in U.S. groundwater and surface water distribution systems at mean concentrations of 7-20 μ g/L. Although DCA is classified as a likely human carcinogen by the U.S. Environmental Protection Agency (EPA) based on sufficient evidence from animal studies, research on its developmental toxicity at environmentally relevant concentrations and the exact molecular pathways affected by DCA exposure during development remain unclear. In this study, the zebrafish (*Danio rerio*) biomedical model was used to test the hypothesis that DCA induces acute and developmental toxicity in environmentally relevant concentrations through oxidative stress as an underlying mechanism. **Methods:** An LC50 assay was conducted at concentrations up to 100 ppm. To assess toxicity at environmentally relevant levels, wild type zebrafish embryos were exposed to 0, 5, 50, or 500 ppb DCA from 1-120 hours post fertilization (hpf). Embryo survival and hatching were monitored every 24 hours, while larval morphology, heart rate, and visual motor response were assessed at 120 hpf. Embryonic photo motor response was evaluated at 24 hpf. At 120 hpf, activities of key antioxidant enzymes, mitochondrial superoxide dismutase (MnSOD), cytosolic superoxide dismutase (Cu/ZnSOD), catalase (CAT), and glutathione peroxidase (GPX), were measured. Expression of oxidative stress related genes (SOD1, SOD2, CAT, GPX) was also analyzed to identify molecular alterations induced by DCA. **Results:** The 120 hpf LC50 of DCA was greater than 100 ppm. At environmentally relevant concentrations, DCA did not significantly affect survival or hatching rates. At 5 ppb, head and body lengths increased, but the head width to

body length ratio, otolith diameter, and jaw length decreased, with no changes in pericardial area or eye diameter. At 50 and 500 ppb, overall head and body lengths were unaffected, though the head to body length ratio increased. These groups also showed reduced eye diameter, jaw length, otolith diameter, and pericardial area. All exposure groups exhibited elevated heart rates. In behavior assays, the 50 ppb group showed reduced distance moved and velocity in the visual motor response, and both 50 and 500 ppb groups spent less time moving. In the photo motor response, all groups displayed increased burst activity, duration, and count. In antioxidant defenses, cytosolic SOD activity increased at 50 and 500 ppb, while mitochondrial SOD activity decreased at 50 ppb. Glutathione peroxidase activity decreased at 50 ppb but significantly increased at 500 ppb. Catalase activity was unchanged. Gene expression of SOD2, CAT, and GPX was unaffected, but SOD1 expression was significantly reduced in the 500 ppb group. **Conclusions:** The results suggest that DCA disrupts zebrafish development through oxidative stress, resulting in morphological, behavioral, and cardiac impairments.

PS 3153 Developmental Toxicity of Microplastics in Zebrafish (*Danio rerio*): Insights into Larval Behavior

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Background and Purpose: Microplastics (MPs) are microscopic plastic particles that commonly originate from the breakdown of plastics. Their ability to cross biological barriers, bioaccumulate, and transport harmful agents poses a risk to exposed organisms. However, effects of MPs during developmental stages remain poorly understood. **Methods:** In this study, we aim to describe the effects of two different MPs on zebrafish larval behavior. At twenty-four hours post fertilization (hpf), embryos were mechanically dechorionated, and exposed to 0, 0.1, 1, or 10 µg/ml of MP solutions derived from disposable red plastic drinking cups (solo cups) or plastic forks. Larval behavior was monitored in response to light and tapping stimuli at 120 hpf. For the visual motor response (VMR) test, larvae were exposed to a series of 10-minute periods that alternate between dark and light for a total of 50 min. Data for movement endpoints such as distance moved, velocity, time spent moving, turn-angle, angular velocity, mean meander, and turning direction were collected. For the tapping response test, a solenoid inserted at the bottom of the base of the behavior chamber was used for mechanical stimulation. Each trial consisted of 10 min of acclimation, followed by a single tap (level 8 [most intense]), to elicit startle responses in darkness. For startle responses, the same movement endpoints were analyzed over a period of 3 s after the tap. **Results:** We found no statistically significant differences in behavior after the tapping stimulus for both MP solutions. In the VMR assays, MP exposure induced source- and concentration-dependent behavioral effects in zebrafish larvae. Fork MPs promoted hyperactivity at a low concentration (1 µg/ml) but reduced locomotion and angular activity at a higher concentration (10 µg/ml), whereas cup MPs predominantly impaired locomotion and turning behaviors across concentrations. **Conclusions:** These findings indicate that MP origin influences toxicity profiles, with distinct behavioral phenotypes emerging from different particle sources.

PS 3154 Perfluorohexane sulfonate (PFHxS) induced developmental toxicity is susceptible to photoperiod perturbations

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Background and Purpose: Light is a fundamental environmental cue shaping the biology of nearly all organisms. In vertebrates, rhythmicity is maintained by molecular feedback loops involving circadian clock genes, synchronized to external light through retinal photoreceptors and the suprachiasmatic nucleus (SCN). These 24-hour rhythms regulate key physiological processes including metabolism, hormone secretion, redox balance, DNA repair, and xenobiotic detoxification. Seasonal variations in photoperiods, or artificial light exposure from devices and shift work, can therefore alter the toxicity of chemicals acting through these same pathways. Yet, photoperiod remains an overlooked variable in toxicology—particularly for persistent pollutants such as perfluoroalkyl substances (PFAS). Among them, perfluorohexane sulfonate (PFHxS) has gained concern due to its extreme persistence, strong C-F bonds, and bioaccumulation potential. PFHxS exposure has been associated with metabolic and neurological disturbances regulated by circadian systems. Our prior larval photomotor response (LPR) data showed that PFHxS produces photoperiod-dependent behavioral effects, emphasizing the need to study light as a determinant of developmental toxicity. This study addresses the gap in understanding how photoperiod modulates PFHxS toxicity by integrating transcriptomic and molecular analyses. **Methods:** Embryos were exposed to 0, 0.0025, or 25 µM PFHxS from 2-120 hpf under 14L:10D (14 hr light: 10 hr dark) or 24D (24 hr dark) photoperiod conditions. LC-MS was conducted to analyze PFHxS uptake and mRNA sequencing was conducted on an Illumina platform. Transcription factor-gene interactions were inferred using g:Profiler, TFmiR3, and Cytoscape, with hub networks identified via cytoHubba. To assess reactive oxygen species (ROS), embryos were stained with H₂DCFDA and imaged on fluorescence microscope. DNA damage was assessed by phospho-H2AX whole-mount immunohistochemistry. For assessing cell proliferation, embryos were

pulsed with BrdU and stained with anti-BrdU antibody before imaging and categorical scoring. **Results:** LC-MS data showed equivalent uptake under both photoperiods. At 25 µM, 578 and 2912 genes were differentially expressed under 14L:10D and 24D, respectively—indicating stronger responses in 24D. KEGG analysis showed shared disruption of cell cycle, DNA replication, and xenobiotic metabolism pathways, with upregulation of metabolic and neuroactive ligand-receptor signaling. Unique responses were observed: 14L:10D specifically affected pyrimidine metabolism and PPAR signaling, while 24D uniquely altered MAPK, autophagy, insulin signaling, and base excision repair. TF network analysis further highlighted photoperiod specificity. Under 14L:10D, hubs included MCM2, MYB, UHRF1, E2F8, and IGFBP1, emphasizing developmental and metabolic control. Under 24D, hubs shifted toward stress and repair, including E2F1, JUN, MCM2, MCM5, and PTTG1, reflecting damage-responsive regulation. This network configuration reflects a transcriptional emphasis on DNA damage response, redox imbalance, and cell cycle checkpoint activation, indicating that photoperiod disruption reorients PFHxS responses away from developmental and metabolic programs toward pathways that prioritize cellular defense and repair. Since our transcriptomic data suggested stress and DNA repair signaling, we further conducted assays for ROS, DNA damage, and cell proliferation. Our ROS assay revealed a 26.42% increase under 14L:10D and a 49.91% increase under 24D, indicating that changes in photoperiod impact ROS levels. Likewise, for DNA double-strand breaks, we observed a 32.4% increase in H2AX staining under 14L:10D, but a more pronounced 80.6% increase under 24D, suggesting a photoperiod-specific effect. We also observed an inhibition of cell proliferation, as indicated by BrdU staining, but these effects were not affected by photoperiod changes. **Conclusions:** Our findings showed that photoperiod is a critical factor in shaping the developmental toxicity of PFHxS. Differences in photoperiod altered baseline circadian programs and modulated the severity of PFHxS-induced effects on gene expression, oxidative stress, DNA damage, and cell proliferation. Incorporating photoperiod as an experimental variable is therefore essential for understanding chemical toxicity.

PS 3155 Developmental Exposure to Bisphenols and Maladaptive Behavioral Stress Responses: Causal Links and Mechanistic Insights

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Background and Purpose: Bisphenol A (BPA) is a synthetic monomer used worldwide in products containing plastics and resins. Its extensive use has led to widespread human and environmental exposures linked to adverse health outcomes, largely via its estrogen-mimicking activity. Epidemiological studies also link developmental BPA exposure to stress-related disorders such as ADHD, depression, and anxiety. In response to regulatory and consumer pressure, BPA is increasingly being replaced by alternative bisphenols. However, the safety of these alternatives remains poorly characterized, particularly regarding stress-related neurobehavioral outcomes. To assess the risks associated with exposure to these chemicals, we investigated the effects of developmental exposure to BPA and three common alternatives (BPF, BPAF, and BPS) using the zebrafish (*Danio rerio*) embryo model. **Methods:** Dechorionated 6-hour postfertilization (hpf) embryos were exposed to 008, .04, .2, 1, and 5 µM of each chemical with daily media changes. At 120 hpf, we assessed mortality, morphological deformities, resting activity, behavioral responses to acoustic and visual stimuli as a measure of stress response, and locomotor capacity. Subsequently, at 144 hpf, we measured light-dark preference in larvae exposed to selected concentrations of each bisphenol, as choice-based behavior offers deeper insight into anxiety-related behavior. RNAseq was performed on brains of 120 hpf larvae exposed to 1 µM BPA and exhibiting maladaptive stress behaviors. **Results:** Our target exposures were sublethal; nevertheless, BPA, BPF, and BPAF produced significant developmental defects, and BPS caused a dose-dependent reduction in total larval length. All bisphenols except BPS significantly altered stress behavioral responses, with BPA producing depressant effects across all concentrations tested. BPA increased dark avoidance under control conditions but not under stress; BPF and BPS increased dark avoidance under stress, and BPAF exaggerated differences between control and stress conditions. Transcriptomics suggested perturbation of serotonergic signaling, among other pathways altered. **Conclusions:** These findings support a causal link between developmental bisphenol exposures and maladaptive stress behaviors, highlight risks posed by alternatives to BPA, and offer insights into underlying mechanisms. With stress-related disorders on the rise, it is crucial to assess risks of widely used industrial chemicals and to ensure that alternatives genuinely offer a safer option.

PS 3156 Aryl Hydrocarbon receptor pathway drives TBBPA induced Cartilage development and hemoglobin disruption defects.

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Background and Purpose: Tetrabromobisphenol A (TBBPA) is one of the most widely produced brominated flame retardants, incorporated into most consumer products such as textiles, foams, furniture, resins, and electronics, which makes human exposure nearly unavoidable through ingestion, inhalation, and skin contact

Biomonitoring studies have detected TBBPA and its metabolites in urine, placenta, breast milk, and cord blood, with concentrations reaching up to 20 nM in adult plasma and 5.3 nM in cord plasma, underscoring widespread prenatal exposure and potential developmental health risks. Our studies using zebrafish as a model show that, within the tissues most at risk from TBBPA exposure, cartilage is particularly important, as it forms the craniofacial skeleton essential for feeding, respiration, and sensory function. TBBPA also inhibits hemoglobin levels, which play a critical role in establishing hemoglobin production and ensuring adequate oxygen transport. Importantly, these effects were seen in low, environmentally relevant concentrations at nM levels. Disruption of both these tissues can significantly impact embryonic health, physiology and function. Our follow-up transcriptomic analysis revealed that TBBPA exposure significantly dysregulated cartilage-associated genes such as *mmp9* (+3.09) and *sox12* (-0.86), hemoglobin production genes such as *tbx6* (+1.35) and *gata2b* (-0.627). Furthermore, along with robust induction of aryl hydrocarbon receptor (zebrafish *ahr2*) signaling targets, including *cyp1a* (+6.16), *cyp1c1* (+3.74), *ahrra* (+2.32), and *foxq* (+2.59), confirming TBBPA as a potent *Ahr2* agonist. Based on this data, this study aims to leverage zebrafish embryos as a model to study mechanisms of TBBPA impacts on cartilage development and hemoglobin production with the hypothesis that these effects are mediated through the *Ahr2* pathway. **Methods:** Embryos were exposed to 0-0.5 μ M TBBPA from 6 to 120 hpf, with daily solution renewals. To validate transcriptomic results, we first conducted concentration-dependent whole-mount immunohistochemistry (IHC) and examined *Ahr2* and *Cyp1A* protein expression at 24 hpf and 72 hpf (N=10). Subsequently, embryos were co-exposed to 0.5 μ M TBBPA and 1.25 μ M GNF-351- a known *Ahr2* antagonist- to evaluate whether inhibition of *Ahr2* signaling rescues TBBPA-induced phenotypes. Cartilage defects (at 120 hpf) and hemoglobin disruption (at 72 hpf) were assessed using Alcian Blue and o-dianisidine staining, respectively (N=10-15). To further confirm these findings, an *Ahr2* translation-blocking morpholino was injected at the 1-2 cell stage, followed by exposure to 0.5 μ M TBBPA at 6 hpf, and Alcian Blue and o-dianisidine staining as described previously. Statistical analyses were conducted using either one-way or two-way ANOVA, coupled with relevant post hoc tests. **Results:** At 0.005 μ M TBBPA (environmental levels), IHC showed significant upregulation of *Ahr2* and *Cyp1A* proteins, showing 50% and 25% increases at 24 hpf and 150% and 50% increases at 72 hpf, respectively. Co-exposure with the *Ahr2* antagonist GNF-351 partially reversed these effects, reducing *Ahr2* expression by 15% and *Cyp1A* expression by 25% at 24 hpf and 37.5% at 72 hpf. Functional rescue was further evident at the tissue level: o-dianisidine and Alcian Blue staining demonstrated that GNF-351 significantly rescued TBBPA-induced suppression of hemoglobin production and craniofacial cartilage structures respectively. Consistent with these findings, *Ahr2* morphants exhibited a similar pattern of recovery, with partial rescue of TBBPA-induced protein increases by 66.67% for *Ahr2* at 24 hpf and 33.33% for *Cyp1A* at 72 hpf. Correspondingly, o-dianisidine and Alcian Blue staining revealed that *Ahr2* knockdown significantly rescued TBBPA-induced adverse effects on hemoglobin levels and craniofacial cartilage structures. **Conclusions:** TBBPA disrupts craniofacial cartilage formation and hemoglobin production by aberrantly activating *Ahr2* signaling. Acting as an *Ahr2* agonist, TBBPA induces downstream targets such as *Cyp1A* and alters transcriptional programs critical for chondrogenesis and erythropoiesis. Partial rescue by *Ahr2* inhibition or morpholino knockdown confirms the pathway's specificity in mediating these developmental defects, providing mechanistic insight into how early *Ahr2* activation perturbs differentiation processes.

PS 3157 Single-Cell Transcriptomic Profiling of Bisphenol-Induced Disruption in Human Embryonic Stem Cell Osteogenesis

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Background and Purpose: Bisphenols (BPs), particularly bisphenol A (BPA), are well-known endocrine disruptors with widespread environmental occurrence. While BPA's toxicological effects are well documented, its analogs bisphenol F (BPF) and bisphenol S (BPS), commonly used as BPA substitutes, remain less understood, particularly regarding their impacts on bone development. BPA has been shown to inhibit osteoblast differentiation and induce osteoblast apoptosis, compromising bone integrity. However, the developmental bone toxicity of BPF and BPS has not been thoroughly characterized. **Methods:** Here, we investigated the effects of BPA, BPF, and BPS on osteoblast differentiation using human embryonic stem cells (hESCs; H9 line) osteogenically induced with 1,25(OH)₂ vitamin D₃ (VD3), β -glycerophosphate (BGP), and ascorbic acid (AA). Human ESCs were exposed to increasing concentrations (0.0001-100 μ M) of each BP during differentiation. **Results:** BPA and BPF caused dose-dependent reductions in osteoblast viability and differentiation, as measured by MTT, alkaline phosphatase (ALP) assay, and bone-specific marker expression. Interestingly, BPS did not induce cytotoxicity even at 100 μ M but inhibited osteoblast differentiation. To elucidate the molecular mechanisms underlying these effects, single-cell RNA sequencing (scRNA-seq) was performed on cells exposed to BPA, BPF, and BPS at IC10, IC25, and IC50 concentrations at three key osteogenic time-points, day 0 (untreated), day 7, and day 20. This comprehensive single-cell analysis enabled high-resolution mapping of transcriptional and pathway alterations during osteogenesis in response to BP exposure. Integration of scRNA-seq data revealed compound- and dose-dependent disruptions in osteogenic lineage specification and cellular heterogeneity. **Conclusions:** Collectively, these findings demonstrate distinct developmental and molecular toxicity profiles of BPA analogs and underscore the need for continued evaluation of bisphenol substitutes in human bone development.

PS 3158 Investigating impact of gene-environment interactions on developmental toxicology of methylmercury; characterizing CRISPR-induced mutations in glutathione pathway genes in zebrafish

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Background and Purpose: The glutathione (GSH) pathway is an antioxidant pathway found in humans and zebrafish. This GSH pathway plays a critical role in detoxifying cells from substances such as methylmercury (MeHg), which is prevalent in the Great Lakes and a concern for public health. There are known genetic variations in GSH genes that affect methylmercury detoxification efficiency in adult tissue; however, less is known about the environment-gene interactions throughout embryonic development. To investigate this context, past lab members created mutations in zebrafish for 2 GSH genes, *gstp2* and *gclm*. Mutations in these genes, however, have yet to be fully and confidently genotyped (*gclm*) or sequenced to confirm mutation sequence (*gstp2*) in the zebrafish. The purpose of this project is to confirm these mutations and generate homozygous mutant zebrafish lines to study how these genes influence methylmercury detoxification in development. **Methods:** Mutations were created by microinjection of CRISPR reagents into 1-cell zebrafish embryos. Zebrafish larvae containing potential mutations were identified using high-resolution melting analysis (HRMA) genotyping, and these fish were raised to maturity. Offspring of mature zebrafish identified with potential mutation were genotyped to characterize the DNA sequence at the CRISPR target area using PCR + restriction enzyme digestion with gel electrophoresis. Gel extraction of the altered PCR amplicon and subsequent DNA sequencing were performed to identify sequence changes. DNA sample concentration was measured by spectrophotometry before being submitted for commercial DNA sequencing. Computational analyses using public tools were used to determine associated DNA sequence changes in protein structure/function. Identified mutant allele carriers were bred to propagate and generate additional mutant zebrafish. **Results:** DNA sequencing revealed a loss-of-function allele was confirmed in the *gclm* zebrafish fish. Computational analyses determined the *gclm* mutation results in a truncated protein 1/6th of the size of the WT protein. Across breeding and genotyping experiments, a limited number of female homozygous *gclm* mutants were identified. These homozygous *gclm* mutant zebrafish were bred with heterozygous *gclm* mutant zebrafish to produce a stock of mutant allele-carrying fish to be genotyped. The *gstp2* project is in earlier stages with a mutation at the CRISPR target site confirmed via gel electrophoresis. Preliminary results indicate germline inheritance of a *gstp2* mutation for one mutant allele, resulting in heterozygote and homozygote *gstp2* mutant zebrafish. **Conclusions:** The *gclm* mutant zebrafish line was successfully confirmed through DNA sequencing and gel electrophoresis, revealing a loss-of-function mutation predicted to produce a truncated protein. A stock of *gclm* mutant allele-carrying fish was produced from the confirmed *gclm* homozygous mutants. They will be genotyped using a Mendelian genetics approach by crossing the mutant allele-carrying fish with a WT and genotyping their offspring. These fish will be maintained for future developmental toxicology studies. In contrast, the *gstp2* line is still in its early stages, with preliminary confirmation of a mutation at the *gstp2* CRISPR target site through restriction digestion and gel electrophoresis. This *gstp2* mutation is yet to be sequenced. Overall, these projects are advancing the goal of producing loss-of-function mutant zebrafish lines for key GSH pathway genes. These lines of fish will enable studying how human genetic variations in GSH genes affect methylmercury detoxification during development.

PS 3159 Assessment of Developmental and Exocrine Pancreatic Toxicity of Aqueous Film Forming Foams (AFFF) in Larval Zebrafish (*Danio rerio*) and its Potential Modulation by the Nrf2 Pathway

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Background and Purpose: Per- and poly-fluoroalkyl substances (PFAS) are ubiquitous, anthropogenic chemicals that bioaccumulate and cannot be metabolized or degraded. While studies have elucidated the adverse effects of individual PFAS chemicals on developmental, reproductive, and endocrine functions, less is understood about PFAS mixtures. Aqueous Film-Forming Foams (AFFF), mixtures dominated by PFAS chemicals, have been detected as drinking water contaminants. AFFF consists of six regulated PFAS chemicals: perfluorooctanoic acid (PFOA), perfluorooctane sulfonic acid (PFOS), perfluorohexane sulfonic acid (PFHxS), perfluorononanoic acid (PFNA), perfluorobutane sulfonic acid (PFBS), and GenX chemicals (HFPO-DA), that have been extensively studied in a wide range of animal models. The aim of this study is to comparatively assess the developmental toxicity of mixtures of 27 PFAS in AFFF and 6 regulated PFAS, while investigating the role of Nrf2—a transcription factor known to modulate oxidative stress known to be induced by PFAS chemicals. **Methods:** A mixture of 27 PFAS chemicals and a mixture of 6 regulated PFAS chemicals were synthesized to mimic the composition of a previously identified in a legacy AFFF sample comparable to 3% AFFF. Homozygous Nrf2a wildtype and mutant (*nrf2a*^{th318-/-}) zebrafish with a loss-of-function mutation were crossed with Tg(*ptf1a:GFP*) zebrafish to investigate potential modulatory effects of the Nrf2 pathway through visualization of the exocrine pancreas. Zebrafish embryos were exposed to three different sub-lethal concentrations of 0.00005%, 0.001%, and 0.0025% AFFF, corresponding to total PFAS concentrations of 197

ppb, 4.10 ppm, and 10.07 ppm. 0.1% methanol served as a solvent control made in Danieau's solution. Exposures were static and larvae were imaged using brightfield and fluorescence microscopy at 4 days post fertilization (dpf). Images were blinded and analyzed using ImageJ, and morphological assessments included fish length, pancreas length, pancreas area, yolk sac area, and pericardial area. **Results:** Our results show that the 27 PFAS mixture significantly decreased fish length in wild-type and mutant larvae exposed to 0.001% and 0.0025% AFFF. Yolk sac area was significantly increased in 0.0025% AFFF for both wildtype and mutant, compared to their respective controls. The ratio of exocrine pancreatic length to fish length was significantly lower at 0.0025% AFFF in mutant zebrafish, but not in wildtype fish. In comparison, mutant zebrafish exposed to the 6 PFAS mixture had significantly increased yolk sac area at 0.0025% AFFF, with the other morphological endpoints were not altered significantly. **Conclusions:** The results of this study show that exposure to PFAS mixtures adversely impacts morphology and pancreatic development in larval zebrafish in a dose-dependent manner, modulated by Nrf2a pathway, with implications on metabolic processes. This work is supported by R01ES025748.

PS 3160 Microengineered Palate Systems Reveal How Matrix Mechanics Shape Sonic Hedgehog Signaling

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Background and Purpose: Orofacial clefts are among the most common congenital malformations, but the etiology of these conditions is largely unknown due to the complexity of craniofacial development and the current limitations of experimental models. Lip and palate formation is successful when there is proper organized signaling between epithelial and mesenchymal cells that is controlled primarily by the Sonic Hedgehog (SHH) pathway. The SHH protein secreted from epithelial cells diffuses in the extracellular matrix and binds to and activates mesenchymal cell receptors, triggering genetic programs that instruct cells on how to grow, divide, or differentiate. When the face does not develop normally due to a mutation, toxin exposure, or imbalance of signaling, it leads to a cleft lip, cleft palate, or other malformation. To gain further insight into how SHH signaling disruptions result in abnormal morphogenesis, a 3D microphysiological model (MPM) was developed to mimic the epithelial-mesenchymal interface of the developing palate, adapting prior microphysiological approaches for Hedgehog signaling [1, 2]. The system is designed to increase human relevance and to be used as a platform for screening environmental or pharmaceutical agents that alter SHH-dependent developmental processes. This study further evaluates the model's reproducibility and accessibility by testing its performance when implemented by a first-time user with limited prior experience in microphysiological systems, showcasing its potential for broader adoption across research skill levels. **Methods:** MPMs were built with 96-well plates milled using Computer Numerical Control (CNC) and seeded with mesenchymal 3T3 SHH Light II reporter cells suspended in a thiol-modified hyaluronic acid hydrogel (Hystem-C) [2]. Epithelial control and SHH-overexpressing GMSM-K RFP cells were cultivated in side channels and on top of the hydrogel to create an epithelial-mesenchymal signaling axis. Hydrogel crosslinker ratios 1:4 and 1:6 (Extralink:Glycosil) were used to tune matrix stiffness, mimicking differences between mature and embryonic connective tissue. Luciferin-based luminescence imaging quantified SHH pathway activation, and results were assessed using a 0-20 performance rubric evaluating gel integrity, 3T3 distribution, GMSM-K channel alignment, GMSM-K distribution in the well, and luminescence signal. Scores of 0-4 indicated failed wells, 5-10 poor, 11-15 good, and 16-20 excellent performance. This rubric provides an objective, reproducible framework for comparing results across plates and users, allowing for quick assessment of experimental quality. **Results:** During multiple experimental runs (9/10/25 - 10/13/25), SHH signaling was detected in >80% of the wells loaded, with mean luminescence levels of 1.23×10^4 to 2.7×10^4 photons/sec across regions of interest (ROIs). More diffuse, higher-intensity SHH responses were obtained with softer hydrogels (1:6 ratio), whereas stiffer matrices produced sharply peaked but spatially restricted gradients. Highest-scoring wells recorded scores of 15-17 ("Good-Excellent") with aligned epithelium and strong reporter activation. Variability in lower-scoring wells (≤ 10) was often linked to gel polymerization inconsistency, barrier failure, or media leakage. Consistent signal detection across experimental repeats by a first-time operator underscores the MPM's reproducibility and practical usability, even in the hands of a novice user. Low-level SHH activity in overexpressing controls remained after 7 days, suggesting possible endogenous signaling or diffusion artifacts. **Conclusions:** This MPM successfully mimics the epithelial-mesenchymal SHH signaling microenvironment and provides a quantifiable, reproducible system for evaluating developmental processes in orofacial morphogenesis. The standardized scoring rubric improved data consistency and comparability between plates. These results demonstrate that matrix stiffness influences SHH diffusion and activity and highlight the model's value for studying morphogen gradients and chemical perturbation in craniofacial development. Importantly, users with varying experience levels can effectively adopt this system with a high success rate. Future work will aim to use human-derived mesenchymes to improve physiological relevance and will further apply this model to developmental toxicology and risk assessment.

PS 3161 Mitochondrial and neural developmental disruption by ractopamine exposure in zebrafish: Integrated transcriptomic and functional analyses

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Background and Purpose: Ractopamine (RAC), a β -adrenergic agonist widely used as a feed additive, has raised global food safety concerns regarding its residues in edible tissues and potential developmental impacts. The present study aimed to evaluate the developmental and physiological effects of RAC exposure using an integrative zebrafish model. **Methods:** Zebrafish embryos were exposed to food-relevant (10-100 $\mu\text{g/L}$) and supraphysiological (500-2000 $\mu\text{g/L}$) concentrations of RAC. Transcriptomic profiling was performed to identify molecular pathways perturbed by RAC exposure, while phenotypic and functional assays were conducted to assess cardiovascular and behavioral responses. Quantitative analysis of RAC uptake in embryonic tissues was determined by competitive ELISA. **Results:** Low-dose RAC (10-100 $\mu\text{g/L}$) exposure perturbed genes involved in mitochondrial ATP synthesis, purine metabolism, and neurogenesis pathways, indicating compromised energy homeostasis and neuronal differentiation. At higher concentrations (1000 $\mu\text{g/L}$), RAC triggered broad transcriptional alterations associated with embryonic patterning, morphogenesis, neural axonogenesis, visual perception, and oxidative phosphorylation. Functional assessments revealed dose-dependent increases in heart rate, blood flow velocity, and locomotor activity in embryos exposed to 500-2000 $\mu\text{g/L}$ RAC, despite only 14-18% uptake of nominal concentrations and the absence of overt structural malformations. **Conclusions:** RAC exposure, even at food-relevant concentrations, disrupted mitochondrial metabolism and neurodevelopmental gene networks in zebrafish embryos, while higher doses further impaired cardiovascular physiology and behavioral performance. These findings provide mechanistic insights into the developmental risks associated with RAC exposure in vertebrates.

PS 3162 Transgenic zebrafish embryo models for AOP-based developmental toxicity evaluation in craniofacial and cardiac anomalies

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Background and Purpose: Craniofacial anomalies and heart defects are among the most common congenital malformations worldwide and are most often caused by a combination of genetic and environmental factors, including pharmaceuticals and chemical agents. Understanding conserved developmental mechanisms underlying each defect is essential for evaluating teratogenicity and constructing AOP (Adverse Outcome Pathway)-based evaluation frameworks. Zebrafish embryo have recently emerged as a powerful alternative model for developmental toxicity evaluation owing to their high-throughput capabilities, conserved embryogenesis, and ability to visualize complex developmental processes. In this study, we established transgenic zebrafish reporter lines Tg(sox10:EGFP), Tg(myf7:EGFP), Tg(kdr:mRFP), and Tg(gata1:mKate2) to visualize cranial neural crest cells (CNCCs), cardiomyocytes, vascular endothelial cells, and blood cells, respectively. Using these lines, we investigated chemical-induced craniofacial anomalies and cardiac malformations (Liu et al., *Tox.Sci.*, 2023, 2025). These studies aim to establish a scientifically robust, alternative developmental toxicity evaluation system and challenge its potential application toward OECD test guideline development. **Methods:** Zebrafish (*Danio rerio*) strains RIKEN WT (RW), sox10:EGFP, myf7:EGFP, kdr:mRFP, and gata1:mKate2, all on the RW background, were used. Fertilized eggs were exposed to test compounds from 4 to 96 hours post-fertilization (hpf). Morphological observations were performed using immunofluorescence staining, live and time-lapse imaging via fluorescence, confocal, and light sheet microscopy. Median malformation dose (EC50) and median lethal dose (LC50) were calculated to determine the Teratogenic Index (TI), with TI > 2 defined as positive. In addition to the endpoint reported by similar researchers using wild-type embryos, mechanistic endpoints associated with AOP key events were assessed through gene expression analysis and stage-specific imaging of CNCC migration and cardiac morphogenesis. **Results:** Exposure to known mammalian teratogens impaired CNCC migration, disrupted CNCC-related gene expression, and affected the morphogenesis of the first pharyngeal arch (PA1) at 24 hpf, resulting in craniofacial anomalies. These findings indicate that KEs underlying chemical-induced craniofacial defects include altered cell proliferation and apoptosis in CNCCs and dysregulation of CNCC-related genes, leading to impaired PA1 formation, a process conserved among vertebrates. Similarly, defects in cardiomyocytes, endocardial cells, and cardiac looping resulted in morphological and functional abnormalities. Live imaging revealed that teratogen exposure caused marked malformations in cardiac morphogenesis, such as abnormal heart tube formation, incomplete looping, and reduced chamber size. These teratogens also disrupted the expression of conserved cardiac progenitor markers such as *nkx2.5* and *islet*, suggesting impaired cardiac progenitor development. This sequence of alterations is considered a key event leading to cardiac malformations. Using the KEs as an endpoint, evaluation of more than 40 compounds, including pharmaceuticals and industrial chemicals, showed high concordance with mammalian data (87.0% sensitivity, 88.2% specificity, and 87.5% accuracy). Incorporating mechanistic observations enhanced detection of teratogenic mechanisms in craniofacial and cardiovascular systems compared

with wild-type embryos. These results demonstrate that transgenic zebrafish embryo models can elucidate AOP key events across molecular, cellular, and tissue levels, enabling mechanism-based developmental toxicity evaluation. **Conclusions:** The established transgenic zebrafish embryo models visualize conserved KEs underlying craniofacial and cardiac malformations, supporting predictive evaluation of human developmental toxicity. This AOP-based approach provides a promising platform for developmental toxicity assessment. Our ultimate goal is to conduct integrated validation studies within the NGRA framework, combining AOP and IATA concepts with RAX and *in vitro/in silico* assays, to propose a defined approach toward OECD test guideline development.

PS 3163 Whole-Body Teratogenicity Imaging by Tissue Clearing and Light Sheet Microscopy Combined with AOP-Based Assay in Transgenic Zebrafish Embryo

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Background and Purpose: Teratogenicity refers to the induction of structural malformations during embryonic development, often resulting from exposure to pharmaceuticals or environmental chemicals. Accurate detection of affected tissues is essential for assessing developmental toxicity. These structural defects such as craniofacial malformations arise through complex morphogenetic processes involving cranial neural crest cells, and their malformations often present subtle or spatially distributed patterns that require high-resolution, three-dimensional imaging for proper evaluation. Zebrafish (*Danio rerio*) embryos provide a suitable model for teratogenicity assay due to their external development, optical transparency, and genetic homology to humans. Light sheet fluorescence microscopy (LSFM) enables rapid three-dimensional imaging, allowing comprehensive analysis of whole-embryo phenotypes. In this study, a workflow of tissue clearing and LSFM combining transgenic zebrafish embryos was established for whole-body teratogenicity assays. Furthermore, by utilizing a sox10:EGFP line developed for AOP-based assay (Liu et al., Toxicological Sciences 2023), we expanded its applicability to diverse chemical classes including agricultural chemicals, and demonstrated a method for detecting craniofacial and systemic malformations throughout the entire embryo. **Methods:** Zebrafish embryos were used to evaluate the teratogenic effects of model compounds and pesticides. Test concentrations followed Liu et al. (Dev. Dyn., 2020) and Narumi et al. (Front. Cell Dev. Biol., 2020): valproic acid and warfarin were used for imaging studies, and six pesticides were selected for AOP-based assessment. Fertilized embryos were exposed to the test compounds from 4 to 96 hours post-fertilization (hpf) with daily renewal of exposure medium. At 24 and 96 hpf, embryos were fixed and stained with anti-collagen II, lectin PNA, and DAPI for nuclear staining. Samples were cleared using CUBIC or ethyl cinnamate (ECi)-based protocols for three-dimensional visualization. Prior to LSFM, samples were refractive index-matched using a 4:6 mixture of silicone and mineral oils and imaged with the UltraMicroscope Blaze. For AOP-based assays, samples were mounted in low-melting-point agarose and observed using confocal and fluorescence microscopy. Acquired LSFM images were processed and reconstructed with IMARIS software. **Results:** Exposure of zebrafish embryos to valproic acid and warfarin induced craniofacial and systemic malformations, including small jaw and cleft palate, consistent with their known teratogenic profiles. Whole-body imaging following tissue clearing revealed detailed three-dimensional morphological alterations that were challenging to capture using conventional confocal microscopy due to its limited imaging depth and longer acquisition time, despite its higher spatial resolution. Both CUBIC and ECi clearing methods achieved high optical transparency suitable for LSFM observation, enabling visualization of whole-body and skeletal structures at single-cell resolution. In the AOP-based assay, several triazole fungicides induced dose-dependent craniofacial abnormalities and alterations in the first pharyngeal arch (PA1) visualized in sox10:EGFP embryos, suggesting potential mechanistic links to craniofacial malformations. Notably, negative control compounds did not alter PA1 morphology, indicating that, within the AOP framework, this transgenic line can effectively distinguish teratogenic from non-teratogenic chemicals. **Conclusions:** This study demonstrates that combining transgenic zebrafish embryos with tissue clearing and LSFM provides a powerful platform for AOP-based teratogenicity testing within the NAMs framework. The approach enables high-resolution, whole-embryo visualization of developmental abnormalities and facilitates mechanistic interpretation of chemical-induced structural perturbations. The workflow established here expands the applicability of sox10:EGFP zebrafish to diverse chemical categories and supports the reduction and replacement of mammalian teratogenicity assays through advanced imaging-based phenotypic assay.

PS 3164 Application of Chick Embryo-Based Models for Non-Clinical Developmental Toxicity Screening

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Background and Purpose: Non-clinical developmental toxicity studies are essential to evaluate the potential toxicological effects of drugs and chemicals on embryonic and fetal development. Conventional mammalian models, such as rats and rabbits, have long been the standard for regulatory toxicity studies; however, they involve ethical concerns, high costs, and prolonged study durations. To address these limitations, alternative models have been explored. This study aimed to confirm the applicability of chick embryo-based systems as a complementary approach for developmental toxicity assessment. **Methods:** Studies utilizing chick embryo-based assays in toxicology were examined, focusing on their use in evaluating teratogenic and developmental effects. Comparative analyses were made between chick embryo models and conventional mammalian or *in vitro* systems in terms of biological relevance, experimental feasibility, and ethical considerations. **Results:** Chick embryos have been successfully used in research related to cardiovascular development, oncology, and vaccine studies, demonstrating their developmental relevance and accessibility. The results identified key advantages of chick embryo-based models, including cost-effectiveness, real-time visualization of embryogenesis, and reduced ethical burden. However, limitations were noted, such as interspecies differences and the absence of maternal-fetal interactions. Recent advances in imaging, molecular profiling, and automation have enhanced the applicability of this model for developmental toxicology. **Conclusions:** Chick embryo-based systems provide a promising bridge between *in vitro* assays and mammalian *in vivo* studies. Their integration into non-clinical developmental toxicity studies could improve screening efficiency and reduce animal use, aligning with the 3Rs principles. Further standardization and validation are needed to establish chick embryo-based assays as a practical tool for non-clinical developmental toxicity evaluation.

PS 3165 Developmental and Behavioral Effects of Micro-/Nanoplastics (MNPs) in Zebrafish Embryos and Larvae

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Background and Purpose: Plastic use is pervasive across modern society and the environment, and the continuous accumulation of plastic waste leads to the formation of micro-/nanoplastics (MNPs) through environmental weathering. These particles are now widely detected in aquatic ecosystems, raising concern about their potential impacts on aquatic organisms. Although many studies have examined the toxicity of pristine microplastics, information on the developmental and behavioral effects of environmentally relevant, UV-weathered MNPs remains limited. In this study, we investigated the developmental and neurobehavioral toxicity of microplastics (MPs) in zebrafish embryos, focusing on the comparative effects of plain versus UV-weathered MPs for three years having, various sizes distinct particle shapes. **Methods:** Field-emission scanning electron microscopy (FE-SEM) and field-emission transmission electron microscopy (FE-TEM) were employed to characterize the surface morphology, particle size, and internal structure of the prepared UV-weathered MP. FE-SEM images were obtained using a LEO SUPRA 55 (Carl Zeiss, Germany) operated at an accelerating voltage of 10 kV. For TEM analysis, the samples were dispersed in an ethanol-water mixture, dropped onto a carbon-coated copper grid, and subsequently dried in a vacuum oven. TEM images were acquired using a JEM-2100F (JEOL, Japan) operated at an accelerating voltage of 100 kV. Developmental toxicity testing was conducted according to OECD Test Guideline 236 using zebrafish embryos. From 4 to 120 hours post-fertilization (hpf), embryos were exposed to plain or UV-weathered polystyrene microplastics in two size classes (8 μm and 500 nm), each classified as spherical or fragmented. The four categories were defined as pristine spherical polystyrene (PSPS), UV-weathered spherical polystyrene (USPS), pristine fragmented polystyrene (PFPS), and UV-weathered fragmented polystyrene (UFPS). MNPs were dispersed with 0.01% DMSO and MNP concentrations were 0 (solvent control), 1.25, 2.5, 5, and 10 ppm, with half of the exposure medium renewed every 24 h. Developmental endpoints are hatching rate, survival, malformation incidence, body length, and eye diameter, measured by light microscopy (Nikon, Japan). Larval locomotor behavior was assessed using the light/dark transition test on a ZebraBox system (ViewPoint, France). **Results:** The zeta potential and dispersion stability increased in the order of USPS > UFPS > PFPS > PSPS, with the USPS-treated 500 nm PS showing the most pronounced surface modification and highest colloidal stability. FE-SEM images revealed uniform spherical shapes without significant deformation, while TEM analysis confirmed well-dispersed particles with clearly defined boundaries, indicating that the surface treatments did not alter the particle core structure. Exposure to UV-weathered MPs significantly reduced the hatching rate at 96 hpf. In contrast, the survival, malformation incidence, body length, and eye diameter were not significantly affected. Notably, plain and UV-weathered 500-nm MPs produced significant increased light/dark transition behaviors. Within this size class, the spherical MPs increased and fragmented MPs decreased locomotion.

tor activity. **Conclusions:** Overall, these findings demonstrated that MPs, particularly UV-weathered and nanoscale particles (500-nm particle size), caused morphological defects to zebrafish embryos. Behavioral assays provided a more sensitive measure of MP-induced toxicity. The observed size- and shape-dependent effects emphasize that UV-weathered and nanoscale particles can impact aquatic organisms under environmentally relevant conditions. These results underscore the need for additional research on neurodevelopmental effects of micro-/nanoplastics.

PS 3166 Integrated risk assessment using NAMs and PBK modeling indicates low teratogenic risk from empirical cosmetic-use exposure to Glucosamine

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Background and Purpose: Glucosamine, an amino sugar widely used in cosmetics for skin conditioning, is considered safe at current use levels by the 2022 Cosmetic Ingredient Review, which reports inclusion as glucosamine hydrochloride up to 0.9% in leave-on products for face/neck and up to 5% in rinse-off products. However, results of OECD TG 414 prenatal developmental toxicity studies for glucosamine are not publicly available. Concurrently, societal expectations and regulatory drivers to minimize animal testing have accelerated adoption of integrated safety assessments that leverage new approach methodologies (NAMs). Although several promising NAMs for developmental toxicity have been developed, few case studies have demonstrated their integrated use in safety evaluation. In the present study, we conducted a case study combining complementary human-relevant NAMs with physiologically based kinetic (PBK) modeling to evaluate whether empirically tolerated human exposure to glucosamine is consistent with a low teratogenic risk under cosmetic use conditions. **Methods:** Two mechanistically distinct NAMs were used. The devTOX quickPredict™ (devTOXqP) assay employing human iPSC-derived cells assessed cytotoxicity and developmental perturbation using the ratio of the metabolite biomarkers ornithine to cystine (o/c ratio) after 48 hours of exposure to glucosamine at 0.0128-1000 µM. A zebrafish embryo assay was conducted using transgenic embryos expressing fluorescent reporters in neural crest cells, cardiomyocytes, and vascular endothelium to increase detection sensitivity and mechanistic insight. Embryos were exposed continuously from 5 hours post fertilization (hpf) to 4 days post fertilization (dpf), and morphological and functional endpoints, including lethality and malformations, were evaluated at 4 dpf. In the zebrafish embryo assay, glucosamine was tested at 62.5-1000 µM. To compare NAMs-derived no-effect concentrations with human internal exposure, a PBK model was built in PK-Sim™. Model performance was evaluated against published human intravenous bolus (IV) clinical data, first post-dose plasma concentration (10 minutes after the IV bolus) and area under the plasma concentration-time curve (AUC). A cosmetic use scenario assumed repeated application of a 0.9% face cream, with daily dose, application area, and dosing interval informed by the SCCS Notes of Guidance 12th revision, and percutaneous absorption flux derived from the literature. A conservative 100% dermal absorption assumption was included to overestimate exposure. **Results:** In the devTOXqP assay, glucosamine showed no cytotoxicity and did not shift the o/c ratio to the developmental toxicity positive threshold up to 1000 µM, indicating a no-effect level at the highest concentration tested. In the zebrafish embryo assay, the positive control (e.g., valproic acid) induced craniofacial defects consistent with neural crest migration defects and also caused significant malformations in cardiac morphogenesis. In contrast, glucosamine produced no statistically significant increase in embryo lethality or malformation incidence compared with controls, at concentrations up to 1000 µM. The PBK model reproduced key features of the human IV clinical data, with initial plasma concentration and AUC predictions within 0.5- to 2-fold of observed values. Under the conservative dermal scenario (0.9% face cream, repeated use, 100% dermal absorption), the predicted steady-state peak plasma concentration was approximately 1 µM. Thus, the modeled human internal exposure is about three orders of magnitude below the no-effect concentrations determined from both NAMs (≥1000 µM). **Conclusions:** Across two distinct NAMs, glucosamine did not elicit developmental toxicity signals up to 1000 µM, which is approximately 1000-fold higher than human internal exposure from representative cosmetic use, even under conservative dermal absorption assumptions. Together, our integrated approach strongly supported a low teratogenic risk for glucosamine under typical cosmetic use conditions.

PS 3167 The Behavioral Toll: PFAS Compromises Anti-Predator Behaviors of Larval Fish

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are ubiquitous environmental contaminants suspected to be neurotoxic, and exposure to high concentrations are associated with altered neurobehavioral development. Their potential to disrupt behavioral endpoints of ecological importance in larval fish following embryonic exposure to environmentally relevant concentrations of PFAS are not understood. **Methods:** This study examines the impacts of early

developmental exposure to 0.8-800 ng/L perfluorohexane sulfonic acid (PFHxS) or perfluorooctane sulfonic acid (PFOS) in two species of fish, zebrafish (0-5 days) and fathead minnow (0-8 days). Embryos were exposed through water with daily 100% renewal (n=12 with 10 embryos per exposure group). In addition to assessing survival, hatching and growth, we compare innate behavioral responses (embryonic motor activity and startle response) in conjunction with functional behavioral tests (predatory escape assay) across PFAS and species. **Results:** Survival, hatching and growth were minimally impacted; however, the startle reflex was impaired following exposure to either PFAS in both species. PFAS-exposed larvae were less able to escape predation, which was most closely correlated with alterations in maneuverability. Interestingly, impacts are independent of the type of PFAS, and fathead minnows appear more sensitive than zebrafish. **Conclusions:** Our data suggests that concentrations of PFOS and PFHxS commonly detected in aquatic ecosystems are sufficient to disrupt survival by making larvae more susceptible to predation. The inter-species differences observed in this study warrant further study to fully understand the underlying mechanisms that account for impaired neurobehaviors and the ecological risks that PFAS pose to aquatic ecosystems.

PS 3168 Tetrabromobisphenol S (TBBPS)-induced developmental toxicity in zebrafish embryos

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Background and Purpose: Tetrabromobisphenol S (TBBPS) is a new brominated flame retardant (BFR) that serves as an alternative to tetrabromobisphenol A. Previous research has revealed that TBBPS can readily accumulate in environmental matrices and potentially pose risk to environmental and human health. However, being an emerging BFR, there is limited knowledge of its toxic effects. The objective of this study is to use zebrafish as a model and determine the effects of TBBPS exposure on early embryogenesis, encompassing gastrulation- a stage of development when cell fate decisions are made. **Methods:** We initiated TBBPS exposures (0 or 40 µM) at 0.75 hours post fertilization (hpf) and phenotyped their progression hourly through cleavage, blastula and gastrula stages (till ~8 hpf). To examine the genetic basis of TBBPS-induced effects, we conducted mRNA sequencing on exposures from 0.75 to 7 hpf. We then immunostained for a series of cytoskeletal and tight junction proteins (α-tubulin, β-tubulin, ZO-1, F-actin) following exposures to a wide range of TBBPS concentrations (0, 0.004, 0.04, 0.4, 4 and 40 µM), including environmentally levels such as 0.004 µM. To determine whether activation of p38, a key regulator of F-actin polymerization, plays a role in TBBPS-induced F-actin disruption, we co-exposed embryos to TBBPS (0 or 40 µM) along with a known p38 inhibitor (5 µM SB 203580) and assessed developmental phenotypes and F-actin levels. **Results:** Our data revealed that TBBPS-treated embryos show delay in development starting at ~4 hpf (dome stage)- a stage constituting early gastrulation, with significant mortality in late-gastrulation. Gene ontology and transcription factor analyses revealed downregulation of cytoskeletal organization and tight junction assembly as potential pathways for TBBPS-induced developmental delays, processes that are crucial for proper gastrulation progression. Cytoskeletal staining showed that F-actin expression was consistently upregulated across all exposed TBBPS concentrations, including at environmentally relevant concentrations (0.004 µM). F-actin polymerization is known to be regulated by the p38 protein, encoded by *mapk14b*, which showed a ~4.7-fold overexpression in our mRNA-seq data. Therefore, we co-exposed embryos with TBBPS and p38 inhibitor SB 203580 and demonstrated a significant recovery in both developmental delay and F-actin levels, with co-exposure groups exhibiting cell-stages and F-actin expression similar to controls. **Conclusions:** Collectively, our genetic and phenotypic data shows that embryonic physiology is vulnerable to early TBBPS exposure, with developmental delays and genetic disruptions during gastrulation stages. We further demonstrate that these effects may be driven by cytoskeletal proteins with MAPK signaling as a mechanistic driver of effects. Therefore, we demonstrate a mechanism for TBBPS developmental toxicity, where TBBPS exposures increase p38 and *mapk14b* levels, disrupting MAPK signaling and resulting in increased F-actin polymerization. This potentially impacts cell migratory movements and gastrulation progression, resulting in developmental delays. Ongoing studies are aimed at detailed assessments of the mechanism underlying TBBPS-p38 interactions.

PS 3169 Butyrate rescues chlorpyrifos-induced social deficits through inhibition of class I histone deacetylases

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Background and Purpose: Chlorpyrifos (CPF) is an organophosphate pesticide linked to autism spectrum disorder (ASD) and gut dysbiosis, but the molecular basis of its neurodevelopmental toxicity remains unclear. This study examined whether the gut metabolite butyrate can counteract CPF-induced social deficits in zebrafish and identified the histone deacetylase (HDAC) mechanism underlying this behavioral rescue. **Methods:** Zebrafish embryos were exposed to CPF (15 µM, 0-3 dpf), and juvenile social behavior was quantified at 25 dpf using the Fishbook assay. Seven gut- or liver-derived metabolites (10 µM each) were screened for behavioral recovery, followed by a dose-response test of sodium butyrate (1.25-40 µM). Class-specific HDAC involvement was evaluated by pharmacological inhibition and CRISPR-

Cas9 knockouts of class I and class IIa HDACs, including individual disruption of *hdac1*, *hdac3*, and *hdac8*. Whole-brain RNA-seq assessed transcriptional changes, while untargeted gut metabolomics and metagenomics characterized metabolic and microbial alterations. Nitrite levels were determined by Griess assay. **Results:** Embryonic CPF exposure produced persistent, dose-dependent social deficits that were completely rescued by 10 μ M butyrate ($p < 0.0001$). Valproic acid, a class I/IIa HDAC inhibitor, and CRISPR knockout of class I HDACs mimicked the rescue, whereas class IIa knockout suppressed baseline social behavior. Among single-gene knockouts, *hdac1* loss strongly enhanced social behavior in both control and CPF-treated fish, while *hdac3* or *hdac8* loss provided partial rescue. The HDAC1-selective inhibitor BRD-6929 restored social scores in a dose-dependent manner, confirming HDAC1 as a key mediator. Transcriptomic profiling revealed that CPF downregulated neuronal and synaptic genes, including 14 ASD-associated genes (e.g., *GRIA2*, *GRIA3*, *GRIN1*), whereas butyrate significantly restored their expression. CPF exposure also elevated circadian gene expression and altered nitrogen-metabolism pathways in the gut. Metagenomic data showed increased denitrifying bacteria (*Pseudomonas*, *Microbacterium*) and depletion of nitric-oxide-sensitive *Aeromonas*, accompanied by higher nitrite levels (~2-fold). The nitric-oxide donor SNAP reproduced social impairment, indicating an NO-dependent mechanism. **Conclusions:** Butyrate reverses CPF-induced social and transcriptional abnormalities through inhibition of class I HDACs, primarily HDAC1. CPF disrupts the gut microbiota and nitrogen metabolism, increasing NO production that perturbs HDAC activity and suppresses neuronal-gene expression. These findings define an NO-HDAC regulatory axis linking environmental pesticide exposure to microbiome dysbiosis, epigenetic imbalance, and ASD-related behavioral deficits, highlighting HDAC1 and NO signaling as potential intervention targets in developmental neurotoxicology.

PS 3170 Nanoplastic Exposure Differentially Impacts Placenta Function in a Sex Specific Manner

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Background and Purpose: Plastic production has been increasing exponentially. Throughout their lifespan, plastics degrade into smaller particles that accumulate in our bodies and the environment. Recent studies found these plastic particles can cross the placental barrier and reach the fetus. However, the impact of plastic particles on placental function is still unknown. **Methods:** To understand the impact of plastic particles on the placenta, we orally exposed pregnant CD-1 mice to 50 nm or 200 nm polystyrene plastic particles from gestation day 8 to day 15 at a concentration of 5 mg/kg/day. Placentas were collected and sliced in half with one half used for gene expression analysis and the other half for morphological analysis. We determined the sex of each placenta by measuring expression of *Sry* and *Rbm31y*. For morphological analysis, the placentas were embedded in paraffin, sectioned into 8 micron sections and then stained using hematoxylin and eosin. We measured the relative area of the key layers of the placenta (the labyrinth zone, basal zone, and decidua), the maternal and fetal blood spaces, and the intrahemal barrier between them. **Results:** We observed a statistically significant decrease in the area of the decidua in the placentas for the 200 nm treatment group and a borderline significant decrease in decidua area for the 50 nm treatment group compared to control. However, when we separated by sex, only the male deciduals were significantly decreased in the 200 nm group. The labyrinth zone was borderline significantly increased for females in the 50 nm group and the decidua was borderline significantly decreased for the males in the 50 nm group compared to controls. For the blood space measurements, the maternal blood space was significantly decreased in size and the intrahemal barrier was borderline significantly decreased for the 200 nm males compared to controls. **Conclusions:** We observed sex specific changes in placental morphology with different effects at different plastic sizes. The overall effect appears to be driven by males. The decidua is a key site of hormone production, so future studies will evaluate the impact of plastics on hormone production and signaling during development. Additionally, we will look to enhance our understanding of sex specific differences following exposures to different plastic types.

PS 3171 Linking Developmental Toxicity and Oxidative Stress to Alkyl Substituted Naphthalenes in Zebrafish

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Background and Purpose: Polycyclic aromatic hydrocarbons (PAHs) form through incomplete combustion of organic materials and anthropogenic sources such as crude oil processing and landfill waste degradation. Increasing fossil fuel use and record-breaking wildfires in recent years have heightened concerns about PAH impacts on human and environmental health. Naphthalene and its alkyl substituted derivatives are the most abundant detected in air and water monitoring samples, posing significant human exposure risk. However, the comparative toxicity of these structurally similar compounds remains poorly characterized. **Methods:** We evaluated 24 alkyl substituted naphthalenes using a high-throughput developmental

zebrafish toxicity assay (6-120 hours post fertilization (hpf)). Based on preliminary results suggesting volatilization issues, modified exposure designs were made using sealed vials with reduced headspace vials and tumbling to ensure uniform uptake. Embryos (n=8/vial) were exposed to five concentrations (3.125-50 μ M) plus a control, normalized to 0.5% DMSO. At 24 hpf, embryos were transferred to 96-well plates for morphological and behavioral assessment at 24 and 120 hpf. Morphological screening revealed that alkyl substituted derivatives were more developmentally toxic than naphthalene, clustering into five distinct clusters based on shared endpoints. We selected one representative compound from each cluster (1, 7-, 1, 5-, 2, 7-dimethylnaphthalene, 2, 4, 5-trimethylnaphthalene, and naphthalene) for body burden quantification at 24 and 48 hpf following 20 μ M exposures. Biospyder Tempo-seq targeted transcriptomics assessed gene expression patterns across all 24 compounds. Finally, reverse molecular docking against ~30,000 predicted human protein-binding pockets identified potential molecular targets, with the top-scoring proteins subsequently validated through follow-up docking. **Results:** Body burden analysis at 24 and 48 hpf revealed that all alkyl-substituted naphthalenes had greater body burden trends compared to the parent compound, with 1, 7-dimethylnaphthalene showing the greatest concentration. Notably, 1, 7-dimethylnaphthalene also formed a distinct morphological cluster and exhibited the highest number of differentially expressed genes (DEGs), suggesting a unique mode of action. Targeted transcriptomics across all 24 alkyl substituted naphthalenes identified 5 frequently dysregulated genes: *ctss2.1*, *fgbp2b*, *kif9*, and *fkbp5*. Of these, *fkbp5*—a glucocorticoid-linked stress modulator in the FK506 binding protein family—was differentially expressed in 18 of the 24 chemicals, implicating oxidative stress pathways in naphthalene-induced developmental toxicity. Reverse docking against ~16,000 human protein structures identified NT5E as a top predicted binding target, ranking first for binding affinity in 17 of the 24 compounds. NT5E encodes proteins essential for immune cellular response that has been linked to oxidative stress regulation. Follow up docking with both human and zebrafish NT5E structures (open and closed conformations), showed comparable binding scores across species with alkyl-substituted naphthalenes displaying higher predicted affinity than the endogenous ligands, adenosine and adenosine monophosphate (AMP). The zebrafish ortholog CD73 is expressed around 24 hpf, coinciding with the exposure window, suggesting disruption of oxidative stress-related immune signaling may contribute to observed developmental effects. **Conclusions:** Alkyl substituted naphthalenes were significantly more toxic than the parent compound, with higher bioaccumulation and distinct developmental effects. The variability in toxicity across chemicals highlights that structure modification dramatically alters naphthalene toxicity. The increased expression of genes related to glucocorticoid signaling (*fkbp5* and *kif9*) could relate to oxidative stress induced toxicity. Predicted binding to NT5E/CD73 suggests the potential for disruption to purinergic signaling which may relate to the observed gene expression changes. Future work will further characterize the interactions between naphthalenes and NT5E *in vivo* and *in vitro* and assess the role of oxidative stress in the observed toxicity.

PS 3172 Optimizing Dosing Initiation in Rabbit Embryo-Fetal Development Toxicity Studies: Avoiding the Pre-implantation Window

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Background and Purpose: According to ICH S5(R3), embryo-fetal development (EFD) toxicity studies are required in both rodents and non-rodents (typically rabbits). The assessment stage, covers the period from implantation to closure of the hard palate, and the recommended dosing period for rabbits is specifically suggested as GD (Gestation Day) 6/7 to GD19. However, our facility observed two instances of significantly reduced pregnancy rates in rabbit EFD studies that employed the GD6-GD19 dosing regimen. Therefore, this integrated approach aims to provide a critical analysis and discussion on the time of dosing initiation in rabbit EFD studies. **Methods:** A retrospective analysis was performed on 66 GLP-compliant EFD studies in New Zealand White rabbit from our facility (2020-2025). Data from control groups, including the dosing initiation day (GD6 or GD7) and the corresponding pregnancy rates, were compiled and analyzed. For the two rabbit EFD studies initiating dosing on GD6 that demonstrated apparently reduced pregnancy rates, a detailed analysis of the data and potential underlying causes was performed in combination with rabbit EFD studies of 54 FDA-approved new drugs between 2023 and 2024, and the research data of rabbits in our facility. **Results: 1. Historical Data Analysis Reveals a Generally Stable Test System** A summary of 66 GLP-compliant EFD studies in Rabbits revealed that 62 out of 66 studies initiated dosing on GD6, with control group pregnancy rates (24 animals/group/study) ranging from 61.5% to 100% (Mean $\pm$ SD: 88.8% $\pm$ 10.2%). The remaining 4 out of 66 studies initiated dosing on GD7, and the control group animals exhibited pregnancy rates ranging from 79.2% to 95.8% (Mean $\pm$ SD: 89.6% $\pm$ 7.2%). The overall pregnancy rates demonstrated no marked differences compared to those observed in studies initiating dosing on GD6, and the natural conception rate in Rabbits remains relatively stable. **2. Case Studies Illustrating the Risk Associated with GD6 Dosing Initiation** Despite the overall stability of the test system, two projects exhibited severe pregnancy loss linked to GD6 dosing initiation. For a GnRH receptor antagonists, GD6-GD19 dosing resulted in 0% pregnancy in the high-dose group in the pEFD study; however, in the subsequent GLP study, initiating dosing on GD7 achieved viable litters in all groups (n $\geq$ 16). Similarly, for a non-hormonal compound, the pEFD study (dosing GD6-GD19) showed reduced pregnancy rate (50%) at the high dose without maternal toxicity, which was restored to 91.7%-100% in the GLP study at higher doses by shifting the dosing initiation to GD7. These cases underscore that for compounds with potential or uncharacterized effects on

implantation, initiating dosing on GD7 is a critical strategy to avoid pre-implantation loss and ensure reliable teratogenicity assessment. **3. Industry Trends and Biological Evidence Support GD7 Initiation** A review of new drugs approved by the FDA in 2023–2024 identified 54 compounds for which rabbit EFD studies were conducted. Among these, dosing commenced on gestation day (GD) 6 in 19/54 (35.2%) studies, while 35/54 (64.8%) initiated dosing on GD7. This distribution indicates that, while operating within regulatory guidelines, nearly two-thirds of recent development programs prioritize the post-implantation period for teratogenicity assessment. To investigate this, an internal study in rabbits ($n=6$) found that uteri on GD6 were indistinguishable from non-pregnant animals, with no implantation sites or blastocysts observed on the endometrial surface of the uterine lumen; however, by GD7, implantation sites become macroscopically identifiable as distinct swellings on the uterine wall. Thus, initiating administration on GD6 carries a substantial risk of compromising pregnancy outcome. **Conclusions:** While ICH S5(R3) provides flexibility for initiating dosing in rabbit EFD studies on GD6 or GD7, our integrated analysis demonstrates that GD6 administration coincides with a critical and vulnerable pre-implantation window. This poses a substantial and avoidable risk of compromising pregnancy rates, particularly for compounds with potential effects on implantation, thereby jeopardizing the primary objective of the EFD study—the assessment of teratogenicity. Therefore, we strongly recommend initiating dosing in rabbit EFD studies on GD7 as a standard practice.

PS 3173 Ultrafine particulate inhalation during pregnancy results in placental adaptation and fetal hyperglycemia in rats

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Background and Purpose: Inhalation of particulate matter air pollution during pregnancy is associated with fetal growth restriction (FGR). Of particular concern is ultrafine particulate matter (<100 nm in diameter), which can readily cross the alveolar epithelial barrier, enter the circulation and localize in extrapulmonary tissues including the placenta. The placenta plays vital role in the transport of growth-related nutrients such as glucose to the fetus. We hypothesize that transport of nutrients to the fetus will be altered following maternal exposure to ultrafine particulate matter and that this contributes to FGR. **Methods:** Pregnant Sprague Dawley rats were exposed to 9.54 ± 0.28 mg/m³ nano-sized titanium dioxide (nano-TiO₂) (157.30 ± 1.85 nm) as a proxy for ultrafine particulate matter and compared to naïve controls. On GD 20, fasting maternal and fetal blood glucose was measured using a OneTouch Ultra 2 glucometer. Maternal plasma was isolated to evaluate insulin levels using an ELISA kit (Thermo Fisher). Placentas from both male and female fetuses were collected and fixed in formalin for histological evaluation. Paraffin embedded tissue slices were stained with hematoxylin and eosin to evaluate placental zone size and blood space number. Immunohistochemistry and immunofluorescence were used to evaluate the abundance and localization, respectively, of glucose transporter (GLUT) 1, GLUT3 and GLUT4. *Ex vivo* placental perfusion was conducted to assess real-time transport of glucose across the placenta. **Results:** Inhalation of nano-TiO₂ had no effect on maternal glucose or insulin. Conversely, fetal blood glucose was significantly greater in the nano-TiO₂ exposed group when compared to controls (48.2 ± 11.0 vs 32.5 ± 3.0 mg/dL). Histological evaluation revealed a 10% decrease in the labyrinth zone area of exposed dams relative to controls. This morphological change was accompanied by an 11% increase in maternal blood space number and a 35% increase in fetal blood space number. Nano-TiO₂ exposure also resulted in a significant reduction in expression of the primary transporter, GLUT1 (relative DAB intensity of 0.9 vs 1.0) and a significant increase in GLUT4 expression (1.7 vs 1.0); a 15% and 25% increase in membrane localization GLUT1 and GLUT4. In parallel, glucose flux across the placenta was increased in the exposed group as compared to control (AUC: 131.6 ± 14.2 vs 68.8 ± 9.4). **Conclusions:** These data demonstrate that gestational inhalation of ultrafine particulate matter negatively affects the area for nutrient transport in the placenta and reduces expression of the primary glucose transporter. Unexpectedly, fetal blood glucose was greater after maternal exposure, despite the FGR phenotype. These findings suggest dysregulated compensatory changes in the placenta following maternal particulate matter exposure resulting in fetal hyperglycemia, which have implications for adverse metabolic regulation during adulthood. Supported by: NIH-R01ES031285; T32ES007148; P30ES005022

PS 3174 Physiological and Functional Responses in Rat Offspring Exposed to Simultaneous 4G and 5G EMFs from Gestation to Weaning

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Background and Purpose: The widespread use of mobile phones and wireless communication devices has led to continuous human exposure to complex radiofrequency (RF) electromagnetic fields (EMFs). Although typical exposure levels are

generally not high, experimental and epidemiological findings have raised concerns about the potential effects of early-life RF exposure on offspring growth and development. According to systematic reviews of experimental and human studies, notable alterations were reported in development and reproductive outcomes (Cordelli et al., 2021; Kim et al., 2021; Bodewein et al., 2022). Similarly, a NTP report described changes in pup body weight and neurobehavioral parameters associated with the RF exposure from gestation to lactation period (NTP TR 595, 2018). These previous findings, along with public health concerns, support the need for studies evaluating the effects of repeated and long-term RF exposure on offspring. This study aimed to provide additional data to understand such potential effects by evaluating male rat offspring exposed to simultaneous 4G/5G EMFs under real-life exposure conditions in South Korea from the gestation to lactation period. **Methods:** Pregnant Sprague-Dawley rats were exposed to simultaneous 4G (900 and 2120 MHz) and 5G (3650 MHz) EMFs from gestation day (GD) 5 to postnatal day (PND) 21. Exposure was conducted for 9 hours and 10 minutes per day, with a continuous 10-minute on/off cycle for 18 hours and 20 minutes daily, at a total combined exposure level of 4 W/kg. The offspring was exposed under the same conditions with their dams until PND 21. To ensure experimental validity and consistency, the animals were assigned to three groups: a control group, a sham-exposed group, and an RF-exposed group. During the exposure period, food consumption of dams, body weight of offspring (F₁), and developmental and behavioral parameters were monitored. **Results:** The food consumption showed statistically significant decreases in the RF-exposed group compared with the control group on PND 4 and PND 21. Litter-based body weight of offspring tended to be consistently lower in the RF-exposed group compared with the control and sham-exposed groups throughout the study period. In the physical development, a statistically significant difference was observed in eyelid opening between the control and both the sham- and RF-exposed groups. No notable changes showed in reproductive findings. **Conclusions:** The statistically significant changes in food consumption, litter-based body weight, and physical development were observed at certain time points or were of minimal magnitude. Therefore, these findings are not considered adverse effects related to the RF exposure under the present study conditions. Funding Source: This research was supported by Institute of Information & communications Technology Planning & Evaluation (IITP) grant funded by the Korea government (MSIT) (No. RS-2024-00466966).

PS 3175 Human Induced Pluripotent Stem Cell (hiPSCs)-Derived Osteoblasts for *In Vitro* Assessment of Skeletal malformation

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Background and Purpose: Human developmental toxicants can negatively impact embryonic development, causing bone growth retardation and malformations. Assessment of skeletal malformations is a crucial component for regulatory *in vivo* studies addressing prenatal developmental toxicity. Despite the importance of *in vivo* testing, *in vitro* models render themselves highly valuable in the field of developmental and reproductive toxicity (DART) due to the current shift in regulatory requirements and their ability to increase the efficiency of existing DART testing programs. **Methods:** The ReproTracker assay focuses on capturing the teratogenic potential of chemicals by assessing disruptions in the development of human induced pluripotent stem cells (hiPSCs) into neural rosettes, hepatocytes and cardiomyocytes. This study presents the addition of osteoblasts to this platform, expanding the assay's ability to capture the effects of teratogens on bone development. Osteoblast differentiation was determined using the mesodermal biomarker *BRACHYURY*, as well as the bone specific markers *RUNX2*, *SP7*, *COL1A1* and *BGLAP*. In addition, calcium depositions were observed at the later stages of differentiation, further illustrating the functionality of the osteoblasts in culture. **Results:** To validate the capability of these biomarkers for detecting teratogenic potential in the ReproTracker assay, we assessed the effect of well characterized *in vivo* bone developmental toxicants (thalidomide and retinoic acid) and two non-developmental toxicant (saccharin and vildagliptin) at non-cytotoxic concentrations. The tested teratogenic agents decreased the expression of either *BRACHYURY* at day 4, and/or the regulation of the bone biomarkers *RUNX2* and *COL1A1* between days 7 and 14. In contrast, saccharin and vildagliptin did not alter the expression of any of the assessed biomarkers, further illustrating the tight regulation of these biomarkers in osteogenesis and their prospect to identify bone specific teratogens *in vitro*. **Conclusions:** Integration of the osteoblast differentiation in the ReproTracker assay expands the biological coverage of developmental processes recapitulated. Using bone-specific biomarkers as end points to evaluate teratogenic agents, we have developed a tool that not only allows for the detection of skeletal developmental toxicants *in vitro*, but also provides insights into the molecular mode of action of the test article.

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Background and Purpose: Cyanobacterial blooms are expanding worldwide and these blooms release potent neurotoxins that threaten aquatic ecosystems, wildlife, and human health. (+)-Anatoxin-a (ATX-a), a secondary metabolite produced by several cyanobacterial genera, is a fast-acting agonist of nicotinic acetylcholine receptors (nAChRs). Upon binding, ATX-a causes sustained receptor activation and persistent muscle contraction, often resulting in respiratory paralysis in exposed animals. While the acute neurotoxicity of ATX-a in adults has been studied in rodent models, developmental effects in vertebrates remain poorly understood. During early embryogenesis, nAChR signaling is essential for establishing neuromuscular junctions (NMJs), promoting muscle fiber patterning, and guiding motor neuron connectivity. Interference with this signaling could have profound effects on motor system maturation. We hypothesized that transient nAChR activation during discrete developmental windows leads to altered neuromuscular activity and gene expression, revealing critical periods of heightened vulnerability. This study aimed to identify such windows of susceptibility and characterize molecular and cellular responses to ATX-a exposure during zebrafish development. **Methods:** Wild-type (TL strain) zebrafish embryos were microinjected with ATX-a (2 ng per embryo or larva) at five developmental stages (0.25, 1, 2, 3, and 4 days post-fertilization, dpf). Embryos were maintained under controlled conditions, and developmental phenotypes, survival, and behavioral responses were monitored up to 7 dpf. ATX-a was quantified in exposed embryos using LC-MS to determine its persistence and estimate internal dose. Gene expression profiling was performed at 6 and 24 hours post-exposure (hpe) to identify transcriptional changes associated with exposure. To visualize structural effects on motor neurons, we employed Tg(*mnx1*:eGFP) transgenic zebrafish expressing GFP in motor neurons. In addition, muscle birefringence assays were performed to assess alterations in muscle structure and organization following exposure. **Results:** ATX-a exposure at 2 and 3 dpf caused rapid-onset muscle contractions within seconds, followed by a transient loss of touch reflex lasting approximately 3-4 hours, consistent with overstimulation of nAChR signaling. No overt morphological abnormalities were observed at any of the developmental stages. Transcriptomic analysis revealed that embryos exposed during early stages (0.25 and 1 dpf) exhibited substantial differential gene expression at 6 hpe, while responses were minimal at later stages (24 hpe), indicating transient but stage-specific transcriptional perturbations. Enrichment analysis revealed overrepresentation of genes associated with muscle contraction, synaptic organization, neuronal differentiation, and cytoskeletal remodeling, suggesting temporary disruption of NMJ formation and function. Visualization using Tg(*mnx1*:eGFP) embryos confirmed transient neuritic and axonal beading consistent with acute neuronal stress, which resolved within 24 hours. Complementary muscle birefringence assays detected subtle but statistically significant changes in muscle fiber patterning in embryos exposed at 1 dpf, supporting the hypothesis that early nAChR activation interferes with muscle development. **Conclusions:** Our findings reveal a narrow developmental window during which ATX-a exposure disrupts neuromuscular signaling, gene expression, and muscle organization, indicating high sensitivity of the developing motor system to transient nAChR activation. Although many effects appear reversible within 24 hours, transient disruptions during these critical periods may have lasting consequences for circuit formation and motor performance. This work underscores the importance of identifying sensitive life stages when assessing the risks posed by cyanobacterial neurotoxins. Given the increasing global prevalence of harmful cyanobacterial blooms, these findings have broad implications for aquatic ecosystem health and potential human exposure scenarios. This research was supported by the Woods Hole Center for Oceans and Human Health (NIEHS P01 ES028938; NSF OCE-2418297).

PS 3177 Ontogeny of placental FcRn protein during human and animal pregnancy to inform developmental toxicity testing

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Background and Purpose: Species differences exist in Fc domain-containing molecule transfer/biodistribution to the embryo-fetus during pregnancy. Placentation neonatal Fc receptor (FcRn) quantitation in chorioallantoic placenta (CAP) and/or inverted yolk sac placenta (InvYSP) was investigated across species throughout gestation to further understand and inform developmental toxicity testing of Fc domain-containing molecules. **Methods:** Quantitative mass spectrometry was used to quantify FcRn protein in CAP and/or InvYSP throughout gestation in mouse, rat, guinea pig, rabbit, and nonhuman primate (NHP). In human, immunohistochemistry was used to localize and characterize placental FcRn distribution during each trimester. **Results:** FcRn protein was detected and concentration increased throughout

gestation in mouse, rat, guinea pig, and rabbits; with InvYSP concentration markedly higher compared with CAP at all gestational ages. In contrast, NHP FcRn protein concentration in the CAP remained constant throughout gestation. During 1st trimester in human, FcRn occurred in decidua, maternal endothelium, and maternal and fetal macrophages. During 2nd trimester, fetal FcRn increased in syncytiotrophoblasts and fetal chorionic vessels. FcRn expression decreased in maternal endothelium near term. **Conclusions:** FcRn protein was detected throughout gestation (including organogenesis) in placental tissue of all animal species. Regardless of FcRn location, these data support that Fc domain-containing molecules can access the embryo-fetus throughout gestation in all species, with the extent of transfer likely depending on FcRn concentration and size of the placenta. This information on placental FcRn ontogeny will help improve study design and translation of nonclinical developmental toxicity data to safety assessment of human risk of Fc domain-containing molecules.

PS 3178 A Translational *In Vitro* Model of the Human Placental-Embryo Interface for Investigating PFAS Developmental Toxicity

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Background and Purpose: In recent decades, human exposure to PFAS (per- and polyfluoroalkyl substances), particularly perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS), has raised growing concern due to their persistence, bioaccumulation, and adverse effects on reproductive health and embryonic development. Their chemical stability and wide industrial use have contributed to their classification as “forever chemicals.” Studies have shown that PFAS can cross the placental barrier, exposing the embryo during critical stages of growth and potentially leading to long-term consequences. Despite this evidence, the mechanisms underlying their embryotoxicity remain incompletely understood. Therefore, this study aims to contribute to a deeper understanding of PFAS-induced embryotoxicity by developing a reliable experimental model that accurately reproduces the maternal-fetal interface. **Methods:** An *in vitro* placental barrier/simulated embryo model was developed to replicate the maternal-fetal interface in a controlled and reproducible manner. To this end, human placental vascular endothelial (HPVCEs) and human trophoblast stem (hTS) cells were co-cultured on opposite sides of a semipermeable membrane. The hTS cells were induced to differentiate into syncytiotrophoblasts to establish a physiologically relevant model of the placental barrier. Model integrity was validated through transepithelial electrical resistance (TEER) measurements, quantitative Real Time PCR and immunofluorescence analyses. This model was used to study the effects of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) on placental barrier function. Additionally, to mimic the developing embryo, induced pluripotent stem cells (iPSCs) were differentiated into cardiomyocytes and the potential interference of PFOA and PFOS was assessed. **Results:** Exposure of the placental barrier model to PFOA resulted in a marked, dose- and time-dependent reduction in TEER values, indicating compromised barrier integrity. This effect was accompanied by increased lactate dehydrogenase (LDH) release and a significant increase in cell death (up to 40-42% after 48 h at 50-100 µM) demonstrating the cytotoxicity potential of PFOA. Furthermore, immunofluorescence analysis revealed a concentration-dependent impairment in syncytial structure formation, suggesting that PFOA disrupts both trophoblast differentiation and cell viability. In contrast, exposure to PFOS resulted in only a modest decrease in TEER values, which reached statistical significance at the highest concentration (100 µM). LDH assays confirmed limited cytotoxicity (about 6-9% of cell death after 48 h at 50-100 µM). Immunofluorescence analysis showed reduced syncytiotrophoblast differentiation, suggesting that PFOS primarily affects trophoblast differentiation rather than cell survival. Both PFOA and PFOS impaired iPSC differentiation into contracting cardiomyocytes and triggered pro-inflammatory responses. **Conclusions:** By integrating complementary *in vitro* approaches, we established a functional maternal-fetal model in which the placental barrier protects iPSC-derived cardiomyocytes. This model enables the simultaneous assessment of xenobiotic transfer across the placenta and its downstream effects on embryo-like cells, providing a physiologically relevant simulation of embryonic exposure. Our findings demonstrate the translational value of this model for investigating PFAS-induced developmental toxicity. Beyond validating its applicability to PFAS, the results highlight its potential as a versatile platform for exploring the mechanisms of action and developmental impact of other xenobiotics. These insights reinforce the need for deeper mechanistic studies and stricter regulatory measures to limit human exposure to persistent and bioaccumulative chemicals.

PS 3179 Windows of developmental susceptibility to cannabidiol reveal stage-dependent morphological and behavioral effects

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Background and Purpose: Cannabidiol (CBD) is becoming increasingly available and is at times suggested to pregnant women to mitigate pregnancy-related symptoms commonly present during the first trimester, including nausea, insomnia, and pain. The goal of this study is to consider different windows of developmental suscep-

tibility using the zebrafish (*Danio rerio*) model. **Methods:** Wildtype (5D) zebrafish embryos were exposed to control (0.05% DMSO) from 6-102 hours post fertilization (hpf) or 2 μ M CBD from 6-102 hpf, 6-30 hpf, 30-54 hpf, 54-78 hpf, or 78-102 hpf, with mortality and hatching monitored daily. After each exposure window, embryos were collected for gas chromatography-mass spectrometry (GC-MS) to assess CBD bioaccumulation or transferred to clean egg water until 120 hpf. At 120 hpf, behavior (larval photomotor response assay) and morphology, including eye diameter and body length, were evaluated. **Results:** GC-MS quantitation showed that the two later exposure windows (54-78 and 78-102 hpf) exhibited higher larval CBD concentrations than early-stage exposures, but were not significantly different from the full-length exposure (6-102 hpf). Larvae exposed to CBD from 6-102 hpf showed reduced body length, smaller eyes, and decreased locomotor activity, indicating developmental impairment from continuous exposure. Shorter exposure windows caused less pronounced effects, yet incidence of yolk sac edema was increased regardless of exposure window. The embryos in the 30-54 and 54-78 hpf groups had significant growth deficits, while the 78-102 hpf group exhibited dark phase hypoactivity. **Conclusions:** Identifying critical periods of susceptibility will provide insight into how the timing of exposure influences adverse developmental outcomes and can contribute to a better understanding of potential risks associated with CBD use during pregnancy. *This research was supported by grant R01DA057317 from the National Institute of Drug Abuse and National Institute of General Medical Sciences.*

PS 3180 Berberine and Metformin Inhibit Caterpillar Growth and Chrysalis Development

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Background and Purpose: Compounds triggering adenosine monophosphate-activated protein kinase (AMPK) that yield weight loss and anti-diabetic effects are also reported to have geroprotective properties. For example, both the pharmaceutical metformin and herbal supplement berberine aid in managing diabetes and obesity and were found to have lifespan extending effects clinically and in laboratory rodents. Invertebrates such as nematodes, fruit flies, and crickets are emerging as an alternatives to vertebrate animal use to study anti-aging properties of drugs because of their short lifespans. Painted lady butterflies (*Vanessa cardui*) also have short lifespans and are commercially available for controlled studies but are less frequently used in toxicology or aging research. *V. cardui* caterpillars take about two weeks to reach the chrysalis stage, metamorphosis takes about a week, and wild butterflies survive for roughly six weeks to migrate, find mates and start the next generation. The hypothesis for this study was that dietary administration of metformin or berberine to caterpillars would dose-dependently extend the lifespan of *V. cardui* butterflies. **Methods:** To test the research hypothesis *V. cardui* caterpillars were obtained in their second instar, weighed and placed either one or five per cup in clear plastic cups holding caterpillar diets spiked with 0, 0.1, 1 or 10 mg/g metformin or berberine. Caterpillars were monitored for weight on day 5, the date of chrysalis formation, date of butterfly eclosure, butterfly wing size and individual survival (lifespan). After emerging from chrysalises, butterflies were fed watermelon twice per week, so adults were not treated with berberine or metformin. Most data collected were analyzed using one way analysis of variance with Tukey's post-hoc tests to compare dose effects. Survival plots and analyses were used to compare dose effects on the probability of survival. An enzyme linked immunoassay kit was used to measure 20-hydroxyecdysone (20E) in naive chrysalises to assess feasibility. **Results:** Over the course of the study some of the 10 mg/g metformin and all 10 mg/g berberine-exposed caterpillars either failed to form chrysalises or never eclosed from their chrysalises. Most of the 1 mg/g berberine-fed caterpillars failed to molt and died, while 0.1 mg/g berberine had no effect on chrysalis formation or butterfly eclosure. Caterpillars treated with 1 mg/kg berberine or 10 mg/g metformin emerged as butterflies with much smaller wings than control treated butterflies. Treatments of 0.1 mg/g metformin or berberine had no impact on caterpillar development or butterfly size. The lifespan of butterflies did not increase with either metformin or berberine treatments, so the hypothesis that early life AMPK activator exposure would extend the butterfly life span was not supported. Molting hormone (20E) levels in naive chrysalises were 3 ng/ml on average. **Conclusions:** These findings reveal that metformin and berberine fed to *V. cardui* caterpillars dose-dependently inhibited their growth, development, or disrupted their metamorphosis. Metformin in this study had less adverse effects on *V. cardui* development than berberine at matched doses. Both AMPK activators are derived from plant secondary compounds, metformin from French lilac and berberine from goldenseal (among other plants), which may have served to defend these plants from herbivorous insects, especially in immature stages at concentrations in the range of 10 mg/g. Based on these outcomes the hypothesis that metformin or berberine treatments can extend *V. cardui* lifespans should instead be tested by dissolving these compounds at the same dose range in sucrose solutions to administer them to butterflies. Overall *V. cardui* could be a promising alternative to vertebrate animal models for tests of potentially toxic developmental effects of compounds in pre-clinical studies.

PS 3181 From High Dose to High Concern: Are Current Developmental Toxicity Findings for Essential oil- and Fragrance Ingredients Human-Relevant?

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Background and Purpose: European hazard classifications increasingly label fragrance ingredients as reproductive toxicants based on high-dose studies mandated by the European REACH legislation. Many of the molecules which have hence become under regulatory scrutiny in Europe are natural ingredients of plants, spices and essential oils and are part of our natural, daily food. These substances and their close analogues are very efficiently metabolized by liver enzymes, and hence only become toxic at high doses, where the capacity of the liver to detoxify them is saturated, a condition far from realistic human exposure. Developmental effects observed may thus often be under conditions of non-linear kinetics and metabolic saturation, and the effect thus could be specific to the test condition, and not inform about a human-relevant hazard at exposure conditions of workers or consumers, and not even for conditions of accidental spills. **Methods:** A case study is presented for Heliotropine, a natural ingredient of pepper and vanilla. Based on post-implantation loss and skeletal malformations, observed at a dose of 1000 mg/kg bw/d only, a CMR1B label and hence a ban in consumer products was decided in Europe. Metabolism at doses mimicking the doses tested *in vivo* was investigated in plated rat hepatocytes. Internal dose of the key metabolite under the situation of the toxicological tests is estimated based on these *in vitro* data and limited available *in vivo* information. This internal dose is compared to estimates of consumer exposure and the resulting internal dose. **Results:** Heliotropine is detoxified by oxidation and glycine conjugation, with glucuronidation as secondary pathway. This glycine-conjugation pathway is known to be saturable, and this is confirmed by the *in vitro* data for piperonal: Complete elimination of piperonylic acid by this pathway occurs when testing at 50 μ M, while glycine conjugation is saturated above 200 μ M. Comparing these data to the situation in the toxicological test indicates that the effects observed occur at a condition where a large amount of free piperonylic acid is in the circulation and hence the developmental effects observed in the study may reflect metabolic saturation rather than intrinsic developmental toxicity. Based on estimates of consumer exposure from use surveys, this internal dose is several orders of magnitude higher as compared to realistic human exposure. **Conclusions:** Developmental studies are designed in a way that maternal effects must be triggered to validate dose-level selection. However, when developmental effects occur only under maternal stress or metabolic overload, their relevance to human hazard becomes questionable. Authorities often deny a link between nonspecific mild maternal toxic effects and the developmental effects, as this link is difficult to prove at the level of the individual chemical. When high dose studies on natural compounds consistently trigger developmental effects under maternal stress, a sound toxicological discussion is needed whether such effects at conditions affecting the maternal homeostasis are informative for human-relevant hazard assessment. A critical gap are *in vivo* data on toxicokinetics and internal human exposure for workers and consumers to judge human relevance, and this should be considered a critical research need. A regulatory system labeling many ingredients of our natural diet as substances of high concern will not increase public trust in toxicological evaluations.

PS 3182 Photoperiod Dependent Impacts on Toxicity of Nuclear Receptor Ligands

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Background and Purpose: Artificial light has extended human activity beyond natural daylight but has also introduced unnatural photoperiods, distinct periods of light and no light, that can disrupt circadian rhythms. The circadian rhythms synchronize behavioral and physiological processes to environmental cycles of light and dark, which regulate essential functions like metabolism, cell proliferation, and immune response. Nuclear receptors are transcription factors that regulate a battery of metabolic processes and are suspected to be regulated by the circadian clock. Leveraging zebrafish as a model, we sought to investigate the impacts of photoperiod changes on nuclear receptor signaling. **Methods:** To determine the effect of different periods of light and dark (photoperiods) on behavior, we set up 5 plates with zebrafish embryos (N=32 per treatment) at 0.75 hours post fertilization (hpf), one subject to a photoperiod of 24 hours of light/0 hours of no light (24L), a photoperiod of 18 hours of light/6 hours of no light (18L), a photoperiod of 14 hours of light/10 hours of no light (14L), a photoperiod of 6 hours of light/18 hours of no light (6L), and a photoperiod of 0 hours of light/24 hours of no light (0L). Embryos remained in those conditions until 5 days post fertilization (dpf), when a larval photomotor response (LPR) was conducted to assess the effects of photoperiod exposure. Following this, we selected 2 photoperiods, 14L and 0L, and conducted mRNA sequencing. At 5dpf, samples were pooled, RNA was extracted, and sequencing was conducted on an Illumina platform, with bioinformatics analyses being conducted using DeSeq2. Finally, to understand the interaction between photoperiods and nuclear receptors, we screened a nuclear receptor ligand comprised of 74 diHerent nuclear receptor (NR) ligands (Enzo Screenwell Library), including agonists and antagonists of 14 diHerent nuclear receptors. Embryos were plated in embryo media at 0.75 hpf (N=16 per treatment) and subjected to photoperiods of 14L and 0L. At 4 dpf, embryo media were removed, and 100 μ L of chemical ligand solution was added. Following chemi-

cal exposure, embryos were returned to their respective photoperiods for a 10-hour exposure. At 5dpf, larval photomotor response, an assay commonly used as a behavioral readout of physiological health, was conducted to assess LPR following chemical and photoperiod exposure. **Results:** When zebrafish embryos were grown up under the 5 diHerent photoperiods, we saw a significant diHerence in LPR responses, depending on the photoperiod, with embryos showing hypoactivity under 0L photoperiods compared to other periods. Our transcriptomic data revealed disruption of several core clock and clock-controlled genes, including reduced expression of several nuclear receptor target genes. Across the screening of nuclear receptors via the LPR assays, we observed three response patterns: no eHect, a photoperiod independent chemical driven eHect (where we saw eHects irrespective of photoperiods), or a photoperiod dependent eHect, where there was a significant interaction between the chemical and photoperiod based on a 2-way ANOVA. Several nuclear receptor ligands, including PXR, PPAR α , RXR, and RAR showed photoperiod-dependent eHects, signifying photoperiod-driver nuclear receptor activity. **Conclusions:** Our findings demonstrate that nuclear receptor activity is sensitive to photoperiod variation. These interactions have potential implication in drug toxicity, becoming relevant for individuals who are exposed to rapid photoperiod changes and disrupted photoperiods.

PS 3183 Fit-for-purpose refinement and application of a risk of bias assessment on literature reporting prenatal acetaminophen exposure and neurodevelopmental effects

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Background and Purpose: Observational studies are subject to systemic bias and confounding due to the nature of the study design. Risk of Bias (RoB) tools provide a structured and objective way to assess for the presence and seriousness of bias inherent to the study design. These tools are a critical component of the systematic review process and are most informative and transparently applied when refined to the research question, such that bias and confounding are assessed in a manner specific to the exposure and outcome of interest. Herein, the objective is to refine a RoB tool to be topic-specific and subsequently apply the tool to studies investigating prenatal acetaminophen use and potential associations with neurodevelopmental effects, including Attention-Deficit/Hyperactivity Disorder (ADHD) and autism spectrum disorders (ASD). **Methods:** The U.S. National Toxicology Program's (NTP) RoB Rating Tool was selected and refined a priori to the research question. To guide refinements for evaluations of confounding, factors commonly associated with ADHD and ASD were identified from previous literature reviews of risk factors. From such, a directed acyclic graph was developed to facilitate identification a minimal adjustment set of confounders or effect modifiers. Exposure ascertainment methods were refined to support evaluation of the reliability of the exposure measurement (e.g., direct measurement vs. self-reported recall) and the temporal relevance of the exposure, including consideration of the timing of biomarker collection to account for rapid metabolism. The refined tool was applied to studies reporting clinician-diagnosed ADHD and/or ASD. **Results:** Topic-specific refinements included definition of key confounders of interest (including indication of use, familial or genetic factors, maternal comorbidities, and maternal mental health) and explicit definition of exposure characterization related to timing and quantification of APAP exposures. Additionally, clarifying modifications were made to the guidance to assist reviewers with selection of bias determinations. Based on the RoB assessments of eligible studies, bias due to uncontrolled and residual confounding was likely to be present in all studies. Few studies adequately accounted for familial or genetic predisposition ADHD or ASD or maternal comorbidities; no studies comprehensively addressed all critical modifying factors. Exposure misclassification was identified as a major source of systemic bias; all studies relied on retrospective maternal recall, often temporally distant from use without information on timing, duration, or frequency, or on unvalidated biomarkers collected at or near the time of birth. Because criteria for review inclusion involved clinician-diagnosed ADHD or ASD, lower RoB was generally observed for the outcome ascertainment domain. Across the evidence base reviewed, a consistent overall RoB pattern was observed with all studies exhibiting at least one key domain rated as probably or definitely high. **Conclusions:** The assessment demonstrated that available epidemiological data has limitations attributable to systemic bias; these limitations preclude confidence in utilizing available epidemiological evidence to assess causality. This research exemplifies the importance of formal critical appraisal via RoB evaluation when conducting systematic review, particularly when assessing the strength of a body of evidence for causal relationships.

PS 3184 Uncovering lineage-specific molecular mechanisms and gene signatures of developmentally osteotoxic chemicals using human embryonic stem cells

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Background and Purpose: Congenital skeletal malformations represent a significant number of birth defects affecting the population and thus pose a huge public health concern. Among potential environmental contributors to their incidence are commonly used medications, supplements, pesticides, preservatives, and plasticizers. While effective in their respective uses, previous studies suggest they may interfere with developmental processes and increase risk and incidence of skeletal birth defects. Such defects can be found in various skeletal sites in the craniofacial, axial, and appendicular skeleton, which embryologically derive from different progenitor cells. However, it is unclear whether all skeletal teratogens act through common molecular mechanisms independently of the osteogenic progenitors they harm. If identified, such mechanisms could be targeted to intervene in the etiology of adverse effects associated with exposure. **Methods:** We have recently been able to model skeletal embryotoxicity of forty such chemicals *in vitro* using human embryonic stem cells and investigating their cytotoxic and developmental effects. The embryonic stem cell test (EST), through three endpoint analyses, determines concentrations at which a 50% decline is seen in cell viability and differentiation capacity. Based on prior screening data we selected chemicals with previously documented defects specific to any of the three skeletal lineages and three additional chemicals with multi-site effects, culminating in a final list of twelve chemicals that were subjected to RNA sequencing at the half-maximal inhibitory concentration of differentiation inhibition. **Results:** Bioinformatic analysis was performed to shed light on common differentially expressed transcripts and signaling pathways affected in consequence of exposure amongst chemicals in each category. Specifically, we present here data identifying overlapping differentially regulated transcripts amongst neural crest-specific chemicals and associated gene ontology analysis. Indeed, overlapping transcripts categorized as neural crest specific demonstrating the general possibility of identifying common exposure gene signatures specific to a developmental lineage. **Conclusions:** Future studies validating the identified gene and pathway candidates in additional chemicals will confirm these 'toxicity' gene signatures and signaling molecules, paving the way for their manipulation to prevent adverse outcomes. In its totality, this project demonstrates the utility of stem cell-based models in developmental toxicology as a humane and mechanistically relevant alternative to animal testing.

PS 3185 Developmental light conditions modulate basal mRNA profiles in zebrafish

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Background and Purpose: Embryonic zebrafish are widely used to assess chemical toxicity; however, laboratories employ inconsistent lighting protocols during exposure studies. While adult zebrafish are maintained under a standard 14:10 hour white light:dark photoperiod, embryo exposure protocols vary considerably ranging from continuous darkness (to prevent chemical photodegradation) to various light conditions. Beyond standardization concerns, lighting conditions may influence developmental susceptibility to chemical toxicants by modulating biological process during critical developmental windows. Despite this potential, the influence on light exposure on chemically-induced toxicity in developing zebrafish remains poorly characterized. We hypothesize that differential light exposure would alter zebrafish susceptibility to certain chemicals toxicants. **Methods:** Dechorionated zebrafish embryos were exposed to three lighting conditions from 6 to 120 hours post fertilization (hpf): blue light, darkness, or a 14:10 hour white light:dark photoperiod (control). Endpoints included morphological screening, the larval photomotor response (LPR) behavioral assay, and transcriptomics analysis at 24, 48, 72, 96, and 120 hpf (n=96 for morphology and behavior; n=4 biological replicates for transcriptomics). Based on transcriptomic findings and reported light-sensitivity, we then co-exposed embryos to four chemicals individually (diclofenac, bisphenol A, bisphenol F, and diphenhydramine) under different lighting conditions (n=36 per concentration, 7 concentrations plus an on-plate chemical control and light-only control plate). Dose-response curves were analyzed for shifts in EC50, LC50, or lowest effect level (LEL) using F-test (p<0.05). **Results:** Lighting conditions alone did not produce morphological abnormalities at 120 hpf. Transcriptomic analysis revealed light-dependent changes in gene expression, with differentially expressed genes (DEGs) increasing from 247 DEGs at 24 hpf to 983 DEGs at 120 hpf. Pathway enrichment analyses were completed using DEGs shared by the blue light and darkness treatment groups and DEGs unique to each group at each timepoint. The analysis using the shared DEGs by the dark and blue light treatments identified circadian rhythm-related terms across all timepoints, suggesting ongoing light-responsive biological processes were altered throughout development. Additionally, terms unique to blue light and darkness treatments were identified. For example, "lipid metabolic process" was significant at 96 and 120 hpf for the dark treatment group but not the blue light group, indicating unique responses to differential light treatment. Chemical co-exposure demonstrated that lighting conditions altered toxicological susceptibility. Diclofenac exposure under blue light or photoperiod conditions produced behavioral effects (LEL= 0.00195 μ M in the excitatory phase of LPR), while dark-exposed embryos showed no effects at concentrations up to 80 μ M. **Conclusions:** Different lighting conditions during embryonic

development significantly alter the basal transcriptome over developmental time. As embryos mature, the number of transcriptomic changes increases, suggesting that a broader range of biological processes is affected by this non-chemical stressor at later stages. Moreover, lighting conditions influence sensitivity to developmental toxicity from certain chemical exposures, indicating that light exposure is an often-overlooked factor modifying toxicological responses. The observed variation in diclofenac sensitivity across lighting conditions further suggests that inconsistencies among zebrafish toxicity studies may, in part, result from differences in experimental lighting conditions.

PS 3187 Toxicological Evaluation of Age-Dependent Responses to a SARS-CoV-2 mRNA Vaccine Candidate in Juvenile and Adult Sprague-Dawley Rats

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Background and Purpose: mRNA COVID-19 vaccines have received emergency authorization for adolescents (12-17 years) in several countries, and some regulatory authorities have extended approval to children aged 5-11 years. However, age-dependent safety data for mRNA vaccination remain limited. Therefore, this study aimed to evaluate acute systemic responses to mRNA vaccination in juvenile and adult rats. **Methods:** The mRNA vaccine candidate (CUK3/LNP128) was injected intramuscularly into both thigh muscles of male and female SD rats at 100 µg/100 µL per head, twice at 2-week intervals. Adult rats received the first dose at 10 weeks of age and juveniles at 3 weeks, and the necropsy was performed 2 days post-secondary injection (2dpi) for the evaluation of acute systemic responses. **Results:** No notable differences in clinical signs, body weights and blood chemistry were showed similar responses in juvenile and adult rats. In hematological and coagulation changes showed similar responses including increases in WBC, neutrophils, and eosinophils, decreased platelets, and prolonged activated partial thromboplastin time (aPTT) in both groups. However, juvenile group showed more pronounced reduction in reticulocytes and increase in nucleated red blood cells. Both groups showed increases in spleen, liver, and kidney weights, while the decrease in thymus was more marked in juvenile group compared with adult group. **Conclusions:** These findings indicate that the hematological and immune systems during juvenile development may be more sensitive to mRNA vaccination. We consider that these findings will contribute to the development of regulatory guidelines for the safety assessment of mRNA vaccines considering age-related differences.

PS 3188 BTEX constituent effects resultant of low-level exposure during critical windows of early development in zebrafish

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Background and Purpose: Volatile organic compounds (VOCs), such as mixtures of benzene, toluene, ethylbenzene, and xylene (BTEX) are ambient anthropogenic pollutants. Especially prevalent in urban environments, BTEX's are a public health concern because high levels may be found in residential drinking water wells near landfills, waste sites, or leaking underground fuel storage tanks which can enter indoor air through vapor intrusion. The constituents of the BTEX mixture are understudied for many potential adverse health outcomes. Benzene, the most volatile compound of the BTEX mixture, is one of few VOCs with enough human and animal data to constitute carcinogenicity whereas ethylbenzene is labeled as a probable carcinogen. BTEX exposure has also been linked to many non-cancer adverse health outcomes, yet few studies have focused on early developmental windows in which hematological, immune, metabolic, reproductive, and endocrine systems are immature. **Methods:** In this study, the zebrafish model was utilized to carry out controlled VOC exposures during a critical window of development. Sealed glass vials with septum tops, a previously validated method for volatile exposure, were employed with gastight syringes for optimized chemical delivery. Phenotypic data collection was conducted following low-level exposure (0.001-10 ppm) exposure to benzene, toluene, ethylbenzene, or xylene at 5 days post fertilization (dpf) and tissue samples were collected immediately (5 dpf) for transcriptomic endpoints. Behavioral assays were carried out at 24 hours post fertilization (hpf) and 5 dpf to characterize early and very early signs of neurotoxicity. Statistical significance was established at $p < 0.05$, $\log_2\text{fc} > 0.5$. RNA samples were only included for sequencing with RNA integrity numbers (RIN) above 7. **Results:** At 5 dpf, exposure to ethylbenzene and toluene were significantly associated with altered locomotive behavior during the light and

dark periods at levels as low as 0.001 ppm. For toluene, the 0.1 ppm group displayed widespread hyperactivity compared to the control group. Toluene exposure was also shown to affect hyperactivity for the 10 ppm dose in the light and the ultra low (0.001 ppm) dose in the dark. At 24 hpf, spontaneous tail coiling (STC) was significantly increased (1 ppm) by toluene exposure. Skeletal deformities and swim bladder abnormalities were increased in the benzene exposed and ethylbenzene exposed groups respectively. Significant decrease in survival ($p < 0.05$) was observed for the 0.01 ppm, 1 ppm, and 10 ppm doses after a 5-day exposure to ethylbenzene. Many genes and pathways were perturbed in exposure groups and comparative pathways of interest are associated with carcinogenesis, endocrine systems disorders, neurological system disorders, and reproductive system disorders. Key pathways, including insulin secretion signaling and inflammation pathways, were altered ($p < 0.05$) as a result of BTEX constituent exposure. **Conclusions:** The strongest evidence of toxicity in our study was among the F0 group apical endpoints. Behavioral outcomes were significantly altered in the light by multiple doses of ethylbenzene but the 10 ppm exposure group was hypoactive in both the light and dark periods. Similarly, impaired behavior was an important finding for toluene and xylene exposure which can be indicative of early developmental delays. We also establish mortality data that will inform future toxicological study of VOCs and complex VOC mixtures. While there wasn't any one gene or gene family impacted in all BTEX chemicals, we observed a number of genes linked to neurological development and endocrine functioning in the BTEX exposed zebrafish. Due to the high conservation of molecular pathways between zebrafish and mammals, our data are compared to results in other exposure models and cohorts providing further insight into potential mechanisms that could be implicated in the developmental effects of VOCs on public and human health.

PS 3189 ASD-relevant neurodevelopmental toxicities of the monoterpenes Thymol and p-Cymene

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Background and Purpose: The increasing prevalence of autism spectrum disorder (ASD) has drawn significant attention to its environmental origin. Environmental factors are estimated to contribute to 40% of ASD risk. However, the identities of these factors and their mechanisms of action remain poorly understood due to limitations of current research approaches. **Methods:** To bridge this gap, we screened the ToxCast library using a high-throughput social behavioral assay platform, Fishbook, to discover emerging ASD-relevant toxicants. **Results:** From the 140 toxicants screened, we identified Thymol and p-Cymene, both belonging to the chemical class monoterpenes, as potent inhibitors of social behavioral development through embryonic exposure. Monoterpenes are natural compounds commonly found in food flavorings, essential oils, and personal care products. Through a proteome-wide reverse molecular docking screen, we discovered lysine demethylase 5 (KDM5) family proteins, in particular KDM5C and KDM5D, as predicted binding targets of Thymol and p-Cymene. The KDM5 family proteins are histone demethylases that selectively remove methyl groups from histone H3 lysine 4 (H3K4me-2/3), which are epigenetic marks typically associated with active gene transcription. Embryonic exposures to thymol and p-cymene increased the global level of H3K4me3, indicating that these toxicants likely inhibited KDM5 activity. RNA-sequencing detected profound up-regulation of genes associated with genetic risk factors of autism following Thymol and p-Cymene exposure. **Conclusions:** In summary, this work discovered Thymol and p-Cymene as risk factors for social behavioral development and revealed KDM5 as a potential mediator of their neurodevelopmental toxicity.

PS 3190 Early Developmental Neurotoxicity of Quaternary Ammonium Compounds

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Background and Purpose: Quaternary ammonium compounds (QAC) are widely used as disinfectants, antiseptics, and preservatives across medical, household, and industrial applications, with global use surging since the COVID-19 pandemic. As a result, humans are routinely exposed through dermal, oral, and inhalation routes. Biomonitoring and experimental studies confirm widespread exposures (10-150nM in >50% of US subjects) and transfer to the embryo/fetus. Prenatal exposure during neurulation and early organogenesis are of particular concern, as this developmental window is highly vulnerable to structural defects and functional disorders of the central nervous system (CNS). While the mechanisms driving QAC developmental toxicity remain poorly defined, animal models demonstrate that two major QAC subclasses - benzalkonium chloride (BAC) and dialkyldimethyl ammonium chloride (DDAC) - are associated with increased embryo mortality, CNS malformations, and neurobehavioral abnormalities. Additional research is needed to resolve differences in toxicity and potency across QAC subclasses. Here, we profiled BAC and DDAC exposures in rat whole embryo culture (WEC) and human embryonic stem cell (hESC) and their neural progenitor derivatives (NPCs) to elucidate morphological, cellular and molecular mechanisms underlying QAC developmental neurotoxicity (DNT). **Methods:** Rat embryos were cultured ex vivo with BAC-C12 or DDAC-10 (1.5 or 3.0 µM) or vehicle controls (media or 0.1% DMSO) for 48 hours, followed by evalua-

tion of morphological and functional parameters. hESCs and NPCs were exposed to BAC-C12, BAC-C16, DDAC-10, or ODDAC (30µM and 100µM) for 24h, followed by cell viability/proliferation and cell death assessment using neutral red cellular uptake and lactate dehydrogenase (LDH) activity assays, respectively. Benchmark concentration modeling was performed with BMDExpress 2.3. The effects of BAC-C12 and DDAC-10 (0.01-3µM) on neural migration was assessed following 72h of chronic exposure. To identify early molecular targets underlying QAC-induced DNT, we profiled transcriptomic responses to BAC-C12 or DDAC-10 (0.01-3µM) in NPCs at 3h and 6h. Data processing and analyses were performed using R. **Results:** Rat embryos exposed *ex vivo* to BAC-C12 or DDAC-10 exhibited concentration-dependent teratogenicity, with ~30% of embryos malformed at 3.0µM. Both compounds induced axial and midline defects, anterior and caudal neural tube anomalies, and impaired development of forebrain, midbrain, hindbrain, caudal neural tube and heart. Somite number and total morphological score (TMS) were significantly reduced ($p < 0.05$). In hESCs and NPCs, BAC-C12, BAC-C16, DDAC-10, and ODDAC decreased viability (<10%) and increased LDH activity (10%) at concentrations lower than <7µM (24h). At subcytotoxic-concentrations, BAC-C12 and DDAC-10 significantly reduced neural migration (72h). We identified 357 (BAC-C12) and 1,501 (DDAC-10) genes to be differentially expressed (DE) ($p < 0.001$; absolute log2FC > 1.2) in exposed NPCs. In general, changes in expression followed a monotonic pattern with increasing concentrations, and responses were concordant between the 3h and 6h timepoints as well as between the two compounds. Common DE genes ($n=205$) were enriched for cholesterol biosynthesis, one-carbon metabolism, DNA damage response, and cell cycle arrest - pathways previously associated with QAC activity in neurocellular models and/or implicated in NTDs and other neurodevelopmental disorders. These findings suggest that both BAC and DDAC exposures are of concern for the developing CNS and highlight common pathways that could contribute to teratogenicity and neurodevelopmental disorders in humans. **Conclusions:** Overall, our findings from rat embryo culture and human embryonic stem cell models demonstrate that two major QAC subclasses - BAC and DDAC - induces disruption of early neurodevelopment at low concentrations. Ongoing experiments are assessing additional subclasses of QACs and characterizing the molecular alterations following chronic exposure of NPCs derivatives, and the rat whole embryo culture (WEC) model.

PS 3191 Machine-Learning Assisted Predictive Developmental and Reproductive Toxicity Assessment Using *C. elegans* as a New Approach Methodology

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Background and Purpose: Developmental and Reproductive Toxicity (DART) assessment is an essential component of product safety evaluation and is traditionally performed using vertebrate models. The nematode *Caenorhabditis elegans* (*C. elegans*) has emerged as a promising *in vivo* alternative due to its short lifecycle, conserved toxicological pathways, genetic homology with humans, and diverse genetic backgrounds. However, broader adoption of *C. elegans* in DART testing has been constrained by low-throughput methods and a limited range of measurable endpoints. **Methods:** To overcome these limitations, we developed a microfluidic-based imaging platform combined with machine learning (ML) analysis pipelines that enable high-throughput, multiparametric assessment of sublethal phenotypes relevant to DART endpoints. Using a 2.5D U-Net architecture, we achieved precise segmentation of *C. elegans* bodies (average Dice score: 97.80) and accurate quantification of body length, area, and volume. To further assess reproductive toxicity, we developed an *in-utero* embryo detection and classification model based on Masked Autoencoder (MAE) backbone followed by an anchor-free, center-based object detection head. In the first stage, a self-supervised MAE learns robust feature representations from large volumes of unlabeled *C. elegans* data. In the second stage, these pretrained representations are fine-tuned through supervised learning for embryo localization and developmental-stage classification (early stage and late stage). The multiparametric endpoints were integrated to enhance assay sensitivity and reproducibility. **Results:** The *in-utero* embryo imaging approach demonstrated high sensitivity for detecting reproductive toxicity by quantifying embryo numbers under different stage of development. At an intersection of union threshold (IOU) of 0.70 and non-maximum suppression (NMS) of 0.85, the model achieved an F1 score of 95.3% for early-stage embryos and 90.7% for late-stage embryos. Compared to manual scoring, the automated pipeline is 360x faster while maintaining consistent embryo detection and classification accuracy and minimizing observer bias. Our automated DART platform shows highly reproducible data (4-16% coefficient of variance) and validated using concentration-dependent response with two well characterized reference chemicals (methylmercury and propiconazole) and strong concordance with rat data. Additionally, assays measuring systemic toxicity (motility and food consumption) were developed to complement DART testing and serve as internal control benchmarks. **Conclusions:** Our microfluidic and ML-enabled *C. elegans* DART platform provides a rapid, reproducible, and predictive alternative to vertebrate-based toxicity testing. The approach significantly expands the measurable endpoints for *C. elegans* and establishes it as a New Approach Methodology (NAM) for DART. This system enables ethical, high-throughput *in vivo* toxicology testing at a fraction of the cost and time required for mammalian studies.

PS 3192 Multi-Omics Profiling of Zebrafish Embryos Exposed to 3,4-Dichloroaniline: Insights from a Pilot Workflow Study.

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Background and Purpose: Zebrafish embryos are increasingly used in toxicological screening as a New Approach Methodology (NAM) due to their genetic homology to humans, rapid organogenesis, and ethically refined alternative to mammalian systems for developmental toxicity screening. Besides, this NAM aligns with OECD Test Guideline 236 and also offers a promising platform for early-stage chemical safety assessment in drug development. This pilot study aimed to implement a multi-omics workflow for assessing molecular responses to chemical exposure in zebrafish embryos, using 3,4-dichloroaniline (DCA) as a reference compound. **Methods:** Embryos were exposed in 96-well plates to DCA at two concentrations (0.6 and 2 mg/L) from 2-4 hpf and again at 2 dpf. Samples were collected at 4 dpf, pooled in groups of either 4 or 8 embryos, and flash-frozen for omics analysis. Metabolites, lipids, and RNA were extracted at the University of Birmingham, and metabolomics and lipidomics were conducted using four LC-MS assays C18 and HILIC chromatography in both ionization modes. RNA quality and quantity were assessed to confirm suitability for transcriptomics. **Results:** Pooling 8 embryos yielded ~50% more LC-MS features than 4 embryos, though both pool sizes provided sufficient biomass for information-rich metabolic and lipid datasets. Metabolic variability was low across QC samples and acceptable across biological replicates. DCA revealed clustering by dose, though some unexpected sample splitting was observed, likely due to differences in timing during embryo collection, as indicated by internal lab records. RNA yields were consistently high across all samples, confirming the suitability of the workflow for transcriptomic analysis. In addition, DCA was detected in HILIC positive ionization mode, but with low and variable intensity. **Conclusions:** This study successfully implemented a zebrafish embryo multi-omics exposure and extraction workflow, demonstrating its feasibility for future toxicological investigations and regulatory applications. The ability to measure metabolomics and transcriptomics data from the same embryo pool should maximize our ability to integrate these mechanistic responses to chemical exposure. Furthermore, deriving 24 pooled samples for multi-omics per 96-well exposure plate ensures this workflow can be used for high throughput screening. While DCA was useful for workflow testing, its limitations as a metabolomics-positive control highlight the need for more suitable reference compounds. Given these limitations, alternative omics-compatible positive controls are currently under evaluation for future studies. Consequently, next we will focus on refining SOPs to reduce sample variability—particularly by optimizing embryo collection timing and improving washing procedures to prevent cross-contamination between treatment groups—and on selecting omics-compatible positive controls to strengthen the regulatory relevance of this zebrafish-based multi-omics workflow.

PS 3193 Uncovering the Role of Glucocorticoid Signaling in Arsenic-mediated Stress Behavioral Alterations

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Background and Purpose: Arsenic (As) is a widespread environmental contaminant linked to numerous adverse health outcomes. Growing epidemiological and experimental evidence suggest its role as a risk factor for stress-related disorders such as anxiety and depression. However, the neuroendocrine mechanisms driving these stress behavioral alterations remain poorly understood. Our previous work demonstrated As-induced hyperactive stress behavioral phenotypes in larval zebrafish. Glucocorticoid (GC) signaling plays a key role in regulating behavioral stress responses and As has been shown to dysregulate it. **Methods:** In this study, we investigated the role of GC signaling in mediating physiological and As-induced stress behavioral phenotypes in larval zebrafish. To study this link, we used wild-type and transgenic zebrafish lines along with a combination of approaches, including pharmacologic GC modulations, co-exposures with As, high-throughput stress behavioral assessments, and *in vivo* imaging of corticotropin-releasing hormone (CRH) neuronal structure and activity—key regulator of GC signaling. **Results:** Pharmacological modulation of GC signaling (dexamethasone, mifepristone, and metyrapone) produced behavioral phenotypes that mirrored As exposure; while co-exposure studies supported a mechanistic link. Structural imaging of CRH neurons revealed As-dependent changes in the neuronal count. *In vivo* calcium imaging of CRH neurons demonstrated changes in neuronal activity in response to GC pharmacological modulations and As-induced alterations that can be closely linked to previously observed stress behavioral phenotypes. **Conclusions:** Our findings provide deeper insights into the neuroendocrine mechanisms underlying As-mediated stress behavioral disorders and identify GC signaling as a possible driver of effects, highlighting GC-targeting strategies as potential avenues for mitigation and therapeutic interventions.

PS 3193a To Evaluate the Effect of Patch Application in the Reproduction/ Developmental Toxicity Screening Test using Wistar Rats

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Background and Purpose: The study was conducted to evaluate the potential systemic, reproductive, and developmental effects of a dermal patch (applied with 0.2 mL of reverse osmosis (RO) water) in Wistar rats. **Methods:** Adult males and females (n=10/sex/group) were assigned to either a control group (without any treatment) or a dermal patch application group. Males were treated for a duration of 29 days, while female rats were treated from 15 days prior to mating, through gestation phase and lactation phase until postnatal day (PND) 14. Parameters assessed included clinical signs, body weight, food consumption, oestrous cyclicity, mating and fertility indices, gestation outcomes, litter size, pup viability, and developmental milestones. **Results:** No treatment-related effects were observed in mortality, clinical signs, body weight, food intake, or macroscopic findings in adults or pups. Reproductive and developmental endpoints—including mating success, fertility, gestation length, litter size, pup survival, and growth—remained unaffected. While some statistically significant changes were noted in maternal and pup body weights and food consumption, these were deemed incidental and not toxicologically relevant. **Conclusions:** The findings indicate that dermal application of the patch was well tolerated and did not elicit adverse systemic or reproductive effects.

PS 3194 Exploring the Effects of Chronic Exposure to DEHP and DiNP on Uterine Endometrial Glycogen in Mice

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Background and Purpose: Glycogen, a branched glucose reservoir, maintains optimal uterine glucose levels crucial for uterine functions and embryo development. Insufficient glycogen has been linked to infertility and early pregnancy loss, acting as a buffer to store or release glucose when levels are too low or high. Due to the hormonal regulation of glycogen in the endometrium, endocrine-disrupting chemicals, including Di(2-ethylhexyl) phthalate (DEHP) and Diisononyl phthalate (DiNP), may pose risks to endometrial glycogen metabolism. Previous studies have shown that phthalates dysregulate hepatic glycogen by disrupting enzymes involved in both glycogen synthesis and breakdown. As phthalates are known to threaten reproductive health, this study aimed to analyze glycogen in murine uterine tissue chronically exposed to 0.15, 1.5, or 1500 ppm of DiNP. **Methods:** Diastase-labile periodic acid-Schiff staining (PAS/PASD) was used to localize glycogen and reveal its presence in all tissue sections. Immunohistochemistry (IHC) was used to visualize glycogen synthase I, hexokinase I, glycogen phosphorylase, and glucose-6-phosphatase catalytic subunit 3. **Results:** PAS/PASD staining for phthalate-exposed tissues was modestly higher than that of controls. IHC for hexokinase I revealed higher levels in the stroma at 0.15, 1.5, and 1500 ppm compared to the control, suggesting increased glucose uptake in the stroma. Moreover, glycogen phosphorylase immunostaining was higher at 1500 ppm, suggesting increased glycogenolysis. Glycogen synthase I and glucose-6-phosphatase had no differences in staining compared to the control. **Conclusions:** Results indicate that phthalate exposure may impact glucose-relevant enzymes, but future studies are needed to assess additional pathways, including glycolysis and the pentose phosphate pathway. These findings reveal an understudied mechanism of phthalate toxicity, providing a basis for future toxicology studies that assess the impact of endocrine-disrupting chemicals on metabolic pathways crucial for reproductive health.

PS 3195 AhR-Mediated Blood-Testis Barrier Regulation Links PCB Exposure to Sperm DNA Methylation Variability

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Background and Purpose: According to the evolutionary stochastic epigenetic variation hypothesis, stress may increase variation in naturally methylation regions (VMRs) associated with development and morphogenesis to ensure increased phenotypic variability in offspring and improve chances of survival of the corresponding genetic lineage in conditions of a changing environment. The blood-testis barrier (BTB) plays a central role in regulating the microenvironment for spermatogenesis, and recent experimental evidence has demonstrated that tightening of the BTB results in less methylation in VMRs, while the BTB disruption by various stressors results in increased methylation variation in VMRs. Additionally, it was shown that activation of the aryl hydrocarbon receptor (AhR) pathway directly promotes BTB tightening. Persistent environmental toxicants, such as polychlorinated biphenyls (PCBs), can differentially activate AhR signaling depending on their congener structure, potentially influencing BTB integrity and downstream sperm DNA methylation dynamics. This study investigated whether early-life exposure to coplanar and non-coplanar PCBs alters sperm methylation variance in opposite directions.

Methods: Sperm DNA methylation was profiled in 36 young men (age 8-18) participating in a longitudinal birth cohort study. Concentrations of mono-ortho (coplanar) and tri/tetra-ortho (non-coplanar) PCBs were measured in blood collected at ages 8-9. Reduced Representation Bisulfite Sequencing (RRBS) was performed on sperm collected at age 18, covering over 400,000 CpG sites. Variable Methylation CpGs (VMCs) were identified in sperm DNA. Differentially methylated CpGs (DMCs) associated with blood PCBs were identified using linear regression models. Gene Set Enrichment Analysis (GSEA) and functional enrichment were conducted to compare CpGs affected by PCBs and highly variable CpGs. **Results:** GSEA indicated that childhood exposure to mono-ortho PCBs was associated with differential methylation in CpG sites located predominantly within low-variance regions, whereas tri/tetra-ortho PCBs exposure corresponded to changes concentrated in VMCs (FDR<0.05). Functional pathway enrichment showed distinct biological responses between PCB groups. Mono-ortho PCB-associated genes were enriched in nervous system development and Wnt signaling, whereas tri/tetra-ortho PCB-associated genes were enriched in PI3K-Akt-mTOR signaling and extracellular matrix organization. **Conclusions:** Differential activation of AhR signaling by distinct PCB congeners appears to regulate BTB permeability, creating a selective microenvironment against external stressors and thereby shaping sperm DNA methylation variability. Coplanar mono-ortho PCBs activate AhR, tighten the BTB, and constrain epigenetic variance. In contrast, non-coplanar tri/tetra-ortho PCBs fail to engage this pathway, leading to a more permeable BTB and increased methylation variability. BTB-associated epigenetic signatures represent a mechanistic link between toxicant exposure and sperm epigenetic variability, offering a new insight into reproductive toxicology.

PS 3196 Chronic Exposure to an Environmentally Relevant Phthalate Mixture Induces an Immune Response in Young Adulthood that Might Contribute to Oxidative Stress and Aberrant Extracellular Matrix Remodeling in the Ovary Later in Life

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Background and Purpose: Phthalates, endocrine disrupting chemicals that target the ovary, are used as plasticizers and solvents in products such as food packaging, medical tubing, and personal care products. Humans are ubiquitously exposed to a mixture of phthalates daily, primarily through ingestion. Recent research suggests that phthalates might accelerate ovarian aging. Specifically, chronic di(2-ethylhexyl) phthalate (DEHP) exposure can alter markers of ovarian aging, including altering the ovarian and systemic immune response, increasing ovarian fibrosis, and increasing ovarian expression of antioxidant enzymes in mice. Exposure to an environmentally relevant phthalate mixture containing DEHP (Mix) can impair the main functions of the ovary; however, little is known about the effects of Mix exposure on immune response, oxidative stress, or extracellular matrix (ECM) remodeling in the ovary. Thus, we tested the hypothesis that Mix exposure alters the ovarian and systemic immune state contributing to oxidative stress and ECM remodeling. **Methods:** Female CD-1 mice were exposed to Mix (0.15, 1.5, or 1500 ppm) through the chow for 6 or 12 months. After 6 months, ovaries and sera were collected for use in cytokine arrays to examine the immune state. After 12 months, ovaries were collected for qPCR analyses of antioxidant enzymes (*Cat*, *Gpx*, *Gpx7*, *Gsr*, *Prdx1*, *Prdx2*, *Prdx4* and *Sod1*), ECM components (*Emilin1*, *Emilin2*, *Emilin3*, *Hyal1*, and *Hyal3*), and ECM modulators (*Mmp2*, *Mmp9*, *Timp1*, *Timp2*, *Timp3*, and *Timp4*). **Results:** Mix exposure for 6 months significantly altered the ovarian and systemic immune states. In the ovary, Mix exposure increased 14 (0.15ppm), 10 (1.5ppm), and 8 (1500ppm) and decreased 6 (0.15ppm), 4 (1.5ppm), and 6 (1500ppm) cytokines compared to control. In the sera, Mix exposure increased 6 (0.15ppm), 13 (1.5ppm), and 8 (1500ppm) and decreased 1 (0.15ppm), 3 (1.5ppm), and 4 (1500ppm) cytokines compared to control. In both the ovaries and sera, Mix exposure increased IL17A and altered MMP9 (0.15ppm), increased RBP4, CRP, CCL5, and PDGF-BB and altered adiponectin (1.5ppm), and increased PDGF-BB, decreased CCL20, and altered adiponectin and CCL5 (1500ppm) compared to control. Interestingly, at all doses tested, Mix increased CCL5 in the ovaries and increased PDGF-BB in the sera compared to controls. Mix exposure for 12 months significantly increased *Cat* (0.15 and 1.5ppm), *Sod1* (0.15, 1.5, and 1500ppm), and *Mmp9* (1.5ppm), borderline increased *Cat* (1500ppm), *Prdx1* (1.5ppm), *Prdx2* (0.15 and 1.5ppm), and *Prdx4* (1.5ppm), significantly decreased *Hyal1* (0.15ppm) and *Hyal3* (0.15 and 1.5ppm), and borderline decreased *Hyal3* (1500ppm), *Emilin1* (0.15 and 1.5ppm), and *Timp3* (1500ppm) expression compared to control. Mix exposure did not significantly alter expression of any other selected genes. **Conclusions:** Mix exposure for 6 months induced unique systemic and ovarian immune responses in mice, and Mix exposure for 12 months increased expression of antioxidant enzymes and dysregulated expression of ECM components and modulators in mice. Together, these findings suggest that chronic Mix exposure can induce an immune response in young adulthood that might contribute to oxidative stress and aberrant ECM remodeling later in life, potentially accelerating ovarian aging.

PS 3197 **Orphan Nuclear Receptor 4A1 (NR4A1) and NR4A2 Dual Ligands Inhibit Endometriosis Progression via Gas6-TAM and Epithelial-Mesenchymal Transition Pathways**

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Background and Purpose: Endometriosis is a chronic inflammatory disease affecting over 5.5 million women in the United States and approximately 176 million worldwide. It is characterized by the ectopic implantation of endometrial tissue, resulting in pelvic pain, infertility, and progressive fibrosis. Current treatment options are primarily hormonal and often unsuitable for long-term use, particularly in women desiring fertility. Our previous work identified the orphan nuclear receptor NR4A1 as a pro-endometriotic factor and a viable target for inverse agonists. Recently, we developed a series of 1,1-bis(3'-indolyl)-1-(3,5-disubstitutedphenyl)methane (DIM-3,5) analogs that exhibit high-affinity binding to both NR4A1 and NR4A2. This research aims to investigate the therapeutic efficacy of DIM-3,5 analogs in modulating NR4A1/NR4A2 activity and their roles in suppressing endometriotic cell proliferation, epithelial-mesenchymal transition (EMT), and fibrosis. Additionally, we examine the involvement of the upstream Gas6-TAM receptor axis as a regulatory component in endometriotic signaling. **Methods:** Several DIM-3,5 analogs were synthesized and evaluated in immortalized human endometriotic epithelial (IHEEC) and stromal (IHESC) cell lines. Cell viability was assessed using the XTT assay. Both cell types were transfected with siRNAs targeting NR4A1 or NR4A2 to assess functional roles. Apoptosis and necrosis were quantified post-knockdown via Annexin V/PI flow cytometry. Protein expression changes associated with the Gas6-TAM receptor pathway, cell growth, EMT and fibrosis were analyzed by Western blotting. Additionally, quantitative RT-PCR was performed to assess mRNA expression of genes involved in the Gas6-TAM axis following compound treatment. Chromatin immunoprecipitation (ChIP) and luciferase reporter assays will be performed to confirm the effect of DIM-3,5 ligands on NR4A1/2 and promoter regions of human TAM kinase genes. **Results:** DIM-3,5 analogs significantly inhibited cell viability at low micromolar concentrations in both IHEEC and IHESC cell lines. Independent knockdown of NR4A1 and NR4A2 similarly reduced cell viability and induced both apoptosis and necrosis, as evidenced by Annexin V assays. Treatment with DIM-3,5 compounds led to marked downregulation of genes involved in cell proliferation, survival, fibrosis, and EMT. Comparable effects were observed following NR4A1/NR4A2 knockdown, reinforcing the functional roles of these receptors in maintaining endometriotic cell survival and fibrotic signaling. In addition, DIM-3,5 analogs and NR4A1/2 silencing significantly suppressed Gas6 expression at both mRNA and protein levels. Activity of the associated TAM receptors (Tyro3, AXL, MerTK) was also decreased. Since the Gas6-AXL axis is implicated in downstream pathways promoting cell proliferation and migration, these results support a mechanistic link between NR4A1/2 modulation and Gas6-TAM signaling in endometriotic cells. Together, these findings highlight DIM-3,5 analogs as promising non-hormonal therapeutic agents for endometriosis. **Conclusions:** Dual targeting of NR4A1 and NR4A2 by DIM-3,5 analogs effectively suppresses pro-endometriotic pathways, including those regulating cell survival, EMT, and fibrosis. These findings support the development of a non-hormonal, mechanism-based strategy for endometriosis treatment.

PS 3198 **Gestational Inhalation of Micro-nanoparticles During Pregnancy Alters the Profile of Metabolites in the Mammary Gland of Sprague Dawley Rats**

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Background and Purpose: Fine particulate matter (particles in the air <2.5µm in diameter) is heterogeneous in particle size and chemical composition. Higher concentrations of fine particulate matter have been associated with adverse health outcomes, including pregnancy complications and fetal growth restriction. Both micro-nanoplastics and nano-titanium dioxide (nano-TiO₂) have been found in air samples, indicating the likelihood of inhalation exposure. Presence of micro-nanoplastics (ie: micro-nanoplastics and nano-TiO₂) in human and animal breast milk indicates micro-nanoplastics also cross the blood-milk-barrier. This suggests infants can be exposed to environmental toxins while breastfeeding. Recognizing micro-nanoplastics exhibit distinct behaviors, we investigated their individual impacts on the rat mammary glands during pregnancy. We hypothesized maternal inhalation exposure to inhaled pollutants (micro-nanoplastics or nano-TiO₂) would differentially alter the presence of metabolites within the mammary glands, the initial site of breast milk formation. **Methods:** Pregnant Sprague Dawley rats were randomly separated into naïve or exposed (nano-TiO₂ or polyamide-12 [PPA]) as representative environmental particulates; (n=5-6/treatment group). Whole-body inhalation exposures occurred from gestational day (GD) 5 - GD 19 at aerosol concentrations of ~10 mg/m³ for 4hr per day for both particles. On GD 20, mammary gland tissues were collected for processing. Polar and non-polar metabolomics were acquired using Liquid Chromatography-Mass Spectrometry (LC-MS). Mean differences in mammary metabolites from naïve animals were compared to TiO₂ and PPA groups, respectively via t-test. **Results:** Broad capture targeted LC-MS resulted in acquisition of 76 identified metabolites within the rat mammary glands. Within the nano-TiO₂ exposed mammary glands, 17 (22.4%) metabolites were significantly lower among nano-TiO₂ exposed dams compared to naïve dams (p<0.05). The majority of compounds (58.8%) were amino acids (AA) and AA-metabolites (2 essential AAs, 4 non-essen-

tial AAs, and 4 AA derivatives/intermediates). Remaining compounds were from classes of carbohydrates (17.6%), nucleic acids (11.8%), lipids (5.9%), and vitamins (5.9%). Within PPA exposed mammary glands, 6 (7.89%) were significantly altered by exposure compared to naïve controls (p<0.05). Most (83.3%) metabolites were significantly reduced (xanthine, hypoxanthine, inosine, 4-pyridoxic-acid, ribose) with urocanate being the only metabolite significantly increased by PPA exposure. Both nano-TiO₂ and PPA exposure resulted in a significant reduction in Vitamin B6 (VB6) metabolites. **Conclusions:** Overall, these data suggest micro-nanoplastics have unique impacts on metabolites within the rat mammary glands during pregnancy. Exposure to nano-TiO₂ changed metabolites predominantly related to amino acid metabolism, while exposure to PPA altered metabolites related to purine and histidine metabolism. These outcomes indicate differential physiological impacts within the mammary glands between the particle exposures. Future studies should examine changes in the milk metabolome, lipidome, and proteome near birth to determine whether milk composition differs by exposure to specific micro-nanoparticles.

PS 3199 **A new approached method for long-term evaluation of reproductive toxicity and its recovery using a mouse testis organ culture system**

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Background and Purpose: A rapid *in vitro* evaluation method that shows a high correlation with animal-based toxicity studies would be extremely valuable for the early screening of candidate pharmaceuticals and for designing nonclinical development strategies. Currently, there are no existing alternative *in vitro* methods to animal testing that can evaluate male reproductive toxicity and recovery, comparable to conventional rodent studies. We then developed a new organ culture method using a chip made of polydimethylsiloxane (PDMS) to cover the tissue and press it thin and flat on the agarose gel. This PDMS-ceiling (PC) method, in which the oxygen concentration is lowered from ambient 20 to 15% in an incubator, has been proven to increase spermatogenic efficiency. Cisplatin is a chemotherapeutic agent frequently used to treat numerous solid tumors in pediatric oncology (World Health Organization, 2021). In the present study, we investigated the ability of the PC testis organ culture system to evaluate the effect of clinically relevant cisplatin treatment on male reproductive toxicity and the time necessary for recovery. **Methods:** Acrosin (Acr)-green fluorescent protein (GFP) transgenic mice were used in this study. Seven-day-old mice were used as the source of the testis tissue. The agarose gel was used as platforms for testis tissue placement. A single testis tissue was placed on a gel and a PC chip with a dent depth of 160 µm was placed over the tissue. The culture plates were placed in an incubator at 34 °C with 15% oxygen and 5% carbon dioxide. dMEM was changed once a week and was poured up to half the height of the agarose gel. For cisplatin treatment, a series of culture media were prepared with specified concentrations of cisplatin: 0, 0.4, 1, 4, 12, and 40 µg/mL, designated as cis-0, cis-0.4, cis-1, cis-4, cis-12, and cis-40, respectively. The original medium was replaced with the new media with cisplatin, and testis tissues were exposed to the cisplatin for 24 h. The media containing cisplatin were then replaced with dMEM containing AlbuMAX medium, followed by a series of three washes at two-hour intervals, using fresh culture medium each time to remove any residual cisplatin. The germ cells of Acr-GFP mice expressed GFP in the cytoplasm as they progressed to the pachytene stage (stage IV), allowing the evaluation of the progression of spermatogenesis over time without interrupting cultivation. For semi-quantitative evaluation of Acr-GFP expression, cultured testis tissues were classified into six grades according to the percentage of GFP-positive areas: 0, 0%; 1, 1-20%; 2, 21-40%; 3, 41-60%; 4, 61-80%; and 5, 81-100%. **Results:** At the time of cisplatin treatment (ED0), GFP expression was not observed in any of the tissues as they had only been cultivated for a period equivalent to the maturation stage of eight-day-old tissues. At ED7, GFP expression was almost equal among the control, cis-0.4, cis-1, and cis-4 groups, however, the cis-12 and cis-40 groups showed very little GFP expression. At ED14, the GFP grade in the cis-0.4 and cis-1 groups remained almost equivalent to that of the control. Interestingly, cis-4 showed a significant decrease in GFP expression levels at ED14, followed by a rapid recovery to a level comparable to that of the control at ED35. In cis-12, a partial recovery of GFP expression was observed around ED35, after which GFP expression gradually improved until ED70. However, no GFP expression was detected in cis-40 until ED70. These results were supported by histopathological examination. **Conclusions:** Observed in animal experiments, cisplatin treatment caused tissue volume shrinkage and reduced GFP fluorescence in a concentration-dependent manner. Recovery from male reproductive toxicity was also dependent on the concentration of cisplatin received. These results demonstrated that this novel *in vitro* system can be a faithful replacement for animal experiments to assess the male reproductive toxicity of candidate pharmaceuticals and their reversibility, providing a useful method for drug development.

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Background and Purpose: Spinosad, a naturally derived bioinsecticide widely used in agriculture, is considered a reduced-risk pesticide. Although it can induce oxidative stress in various biological models, its effects on the oxidative balance of mammalian ovarian cells remain unclear. This study examined the time- and dose-dependent effects of spinosad on the total antioxidant capacity (TAC) of bovine granulosa cells *in vitro*. **Methods:** Bovine granulosa cells were isolated from abattoir-derived ovaries, pre-incubated in medium containing 10% newborn calf serum for 24 hours, and then exposed to spinosad (0.1 μ M, 1 μ M, or 10 μ M) in serum-free medium for an additional 96 hours. Subsequently, the culture media were collected at 48 and 96 hours to determine TAC (in mmol/L) using a colorimetric assay. This study was financially supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK, TOVAG-1240439). **Results:** At 48 hours, TAC levels in the 1 μ M dose group were significantly higher than those in the control group ($p < 0.05$). However, neither the lower-dose (0.1 μ M) nor the higher-dose (10 μ M) groups differed significantly from the control. By 96 hours, TAC levels in the 1 μ M group had significantly decreased compared with its 48-hour value ($p < 0.05$). **Conclusions:** Spinosad exposure induced a nonlinear, transient antioxidant response in cultured bovine granulosa cells, characterized by a short-term enhancement of total antioxidant capacity at 48 hours that was not sustained at 96 hours. The 1 μ M dose significantly increased antioxidant capacity at 48 hours, indicating early activation of the antioxidant defense system, whereas both lower (0.1 μ M) and higher (10 μ M) doses produced minimal changes. These findings highlight a possible dose-sensitive adaptive mechanism in granulosa cells under spinosad exposure, warranting further investigation into its reproductive toxicological relevance.

PS 3201 Human extravillous trophoblasts exhibit circadian rhythms responsive to molecular clock regulators and select environmental chemicals

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Background and Purpose: The circadian system coordinates daily timing of cellular and physiological functions; in pregnancy, its dysregulation has been associated with preeclampsia and preterm birth. In placenta trophoblasts, core clock components are rhythmically expressed, and perturbation of clock genes alters extravillous trophoblasts (EVTs) invasiveness. However, whether environmental chemicals, known to impair EVT invasion, disrupt circadian regulation in placental cells remain unknown. Thus, we examined two chemical classes: one linked to circadian disruption in insects (neonicotinoids) and one targeting a central placental pathway (benzotriazole UV stabilizers (BUVs)). Neonicotinoids, widely used insecticides, act at nAChRs receptors, involved in regulating circadian pacemaker activity in invertebrates, whereas BUVs, widely used in sunscreens, affect the epidermal growth factor receptor (EGFR), a major regulator of EVT proliferation and invasion. Because both receptor systems are expressed in EVTs, we hypothesize that exposure to either of these chemicals classes will decrease the circadian clock rhythmicity in EVTs. **Methods:** A stable Period 2::Luciferase (PER2::LUC) reporter was generated in HTR-8/SVneo EVTs to enable real-time tracking of molecular clock dynamics. Cells were first synchronized by serum shock (metabolic cue) or by thymidine block (cell cycle cue), rhythmic expression of PER2::LUC was recorded from circadian time (CT) CT0 to CT96, and evaluated for two known circadian clock modulators: dexamethasone (DEX; 100 nM) and CK1 ϵ / δ inhibitor PF-670462 (10 μ M) was assessed at CT0, CT18, and CT24. Next, thymidine block synchronized cells were exposed to environmentally relevant concentrations of the neonicotinoid 3-OH-imidacloprid (2 μ M and 20 μ M), acetylcholine (a nAChR agonist; 2 μ M and 20 μ M), or BUVs (UV-326 and UV-327; 1 μ M and 10 μ M). All bioluminescence traces were assessed for goodness of fit prior to rhythm parameter (amplitude, period, and phase) analysis. **Results:** Both synchronization methods yielded robust rhythms in HTR8/SVneo EVTs for up to 72 h. In serum shock synchronized EVTs, DEX had no effect on rhythm output when applied at CT0 but caused a slight phase delay at CT18 and increased amplitude with tighter phase clustering at CT24. In thymidine block synchronized cells, DEX disrupted the rhythmicity when applied at CT24 in 75% of the recordings. PF-670462 increased period length at CT0 in both synchronization methods, and at CT18 in serum shock synchronized EVTs, and led to tighter phase clustering at CT24. In thymidine block synchronized cells, PF-670462 induced a 12 h phase delay at CT0. Exposure to 3-OH-imidacloprid, acetylcholine, and UV-326 in thymidine block synchronized EVTs at CT0 yielded rhythms that passed goodness of fit criteria and showed no changes in amplitude, period, or phase. However, exposure to 10 μ M dose of UV-327 showed a trend towards phase advancement at CT0. **Conclusions:** This study demonstrates that EVTs possess robust circadian rhythms, modulated by clock regulators in a time- and synchronization-dependent manner. Most environmental chemicals tested did not alter rhythm parameters, though UV-327 showed a potential phase-shifting effect. Chemical exposures were assessed under thymidine block synchronization, which may yield more tightly phased rhythms, based on differential responses to DEX

and PF-670462, warranting future evaluation under serum shock conditions. Overall, these findings suggest that select environmental chemicals may influence placental clock function and merit further investigation. Funded by R01ES035691 to A.V.-L.

PS 3202 Hormone Secretion and Membrane Transporter Regulation in Human Placental Explants Under Standard and Accelerated Culture Conditions

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Background and Purpose: *Ex vivo* culturing of human placental explants is a physiologically relevant model as cell-cell communication remains intact. During explant culture, syncytiotrophoblasts partially detach during the first 2 days and regenerate through cytotrophoblast fusion to form a new syncytial layer over a period of 5-7 days. Previous studies have suggested that treatment of explants with trypsin accelerates this process by inducing early shedding. However, there has been little investigation into how this accelerated protocol affects hormone secretion, morphological changes, and the expression of nutrient and xenobiotic membrane transporters. Therefore, this study sought to profile the dynamic changes that human placental explants undergo over a 7-day period, with or without trypsin treatment, with a focus on the regulation of hormone production, structure, and transporter enrichment. **Methods:** Six healthy, term placentas (N=3 male; N=3 female) were collected during scheduled Cesarean sections. Placental villous tissue samples were dissected and washed with PBS to remove maternal blood. Villous explants (~8 mm³) were prepared and cultured in 24-well dishes. For the trypsin-treated group, explants were incubated in 0.25 mg/mL trypsin for 20 minutes at 37 °C before replacement with standard culture media on Day 0. Cultures were maintained at 37 °C with 5% CO₂ and 20% O₂ for up to 7 days. Media was collected daily for measurement of human chorionic gonadotropin (hCG) and lactate dehydrogenase (LDH). Fresh media was added each day. Explants were imaged daily using a light microscope. Quantitative Targeted Absolute Proteomic (QTAP) analysis of transporters by liquid chromatography-mass spectrometry and histopathological analyses of explant morphology on Days 0, 1, 2, 3, 5, and 7 are ongoing. **Results:** In the control group, explants appeared similar under a light microscope until around Day 3 to 4 when gradual aggregation occurred, and tissue density increased. In the trypsin-treated group, on Day 0, the extracellular matrix (ECM) was degraded and became gelatinous. By Day 1, the degenerated ECM had disappeared, and the explants aggregated and became smaller in size. In both control and trypsin-treated groups, the hCG concentration peaked on Day 1 and gradually declined over Days 2 and 3. Notably, trypsin-treated explants had 70% higher hCG levels on Day 1 compared to the non-treated group ($p < 0.001$), but no differences were observed on Day 2 or later. LDH levels were also the highest on Day 1, gradually decreased toward Day 3, and stabilized at a low level until Day 7. No significant differences in LDH levels were observed between control and trypsin-treated explants. **Conclusions:** Delineating the morphological and biochemical profiles of human placental explants with and without trypsinization provides a foundation for establishing appropriate and time-efficient models for placental toxicology studies. Supported by Daiichi Sankyo Co. Ltd. and the Integrated Transporter Elucidation Center funded by NIH UC2HD113039.

PS 3203 The Effects of Iodoacetic Acid on Oxidative Stress Markers in the Mouse Ovary

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Background and Purpose: The incorporation of water disinfectants into the main water supply has significantly decreased the incidence of waterborne diseases such as cholera. However, the interaction between disinfectants and organic material generates water disinfection byproducts (DBPs). Iodoacetic acid (IAA) is an unregulated DBP that has been reported as a reproductive toxicant. Our previous studies show that IAA inhibits follicular growth and alters the expression of steroidogenic factors *in vitro*, but its underlying mechanisms of action were unknown. Thus, we tested the hypothesis that IAA exposure causes ovarian toxicity through oxidative stress pathways. **Methods:** Adult CD-1 mice were dosed with vehicle control (reverse osmosis water) or IAA (2.7- 2,695.7 μ M) for 35 days. Following this, ovaries were collected when the mice were in diestrus, and either antral follicles were isolated from the ovary, or the ovary was kept whole. Both antral follicles and whole ovaries were subjected to RNA extraction, reverse transcription, and quantitative polymerase

chain reaction (qPCR) to analyze the expression of several enzymes that regulate oxidative stress (*Gpx1*, *Gpx2*, *Gsr*, *Gss*, *Gsta1*, *Gstm1*, *Gsto1*, *Gstp1*, *Gstt1*, *Sod1*, *Sod2*, and *Cat*). In other experiments, ovarian antral follicles were collected from CD-1 mice between 40 and 42 days of age. The follicles were incubated in individual wells within a 96-well plate in media containing vehicle control $\pm$ the antioxidant Trolox or IAA (2-15 μ M) $\pm$ the antioxidant Trolox for 96 hours. Follicle growth was measured every 24 hours and media were collected for estradiol measurements at 96 hours. **Results:** IAA decreased *Cat*, *Sod1*, *Gss*, *Gsta1*, *Gstp1*, and *Gstt1* and increased *Gstm1* expression in whole ovaries compared to control *in vivo*. IAA decreased *Sod2* and *Gpx2* and increased *Gstp1* expression in antral follicles compared to control *in vivo*. IAA increased *Gpx1* and *Gsto1* expression in antral follicles compared to control *in vitro*. **Conclusions:** IAA inhibited follicle growth and reduced estradiol levels, whereas Trolox rescued follicles from IAA-induced inhibition of follicle growth and estradiol levels *in vitro*. Collectively, these data indicate that IAA exposure causes ovarian toxicity by altering oxidative stress pathways in the mouse ovary. Supported by NIH R21 ES028963.

PS 3204 SILDENAFIL CREAM, 3.6%: VAGINAL DOSE STUDY ON EMBRYOFETAL DEVELOPMENT TOXICITY AND TOXICOKINETICS IN RATS

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Background and Purpose: The purpose of this study was to investigate the effects of sildenafil cream, 3.6% on embryofetal development in rats when administered via vaginal route from Gestation Day (GD) 6 to up to and including GD 17 and to characterize the dose response relationship to any observed toxicity. In addition, the toxicokinetics (TK) in mated females were determined. Sildenafil cream, 3.6% is being developed for treatment of female sexual arousal disorder. **Methods:** 157 mated female rats were randomly assigned to 5 groups with 29 animals in the sham dose control and placebo control groups (25 animals/group in the main study and 4 animals/group in TK study) and 33 animals in the three test article treatment groups (25 animals/group in the main study and 8 animals/group in TK study). Test article groups were administered sildenafil cream, 3.6% at the dose volume of 0.1 mL/animal (once daily), 0.2 mL/animal (once daily) or 0.2 mL/animal (twice daily), equivalent to 3.77, 7.54 or 15.08 mg sildenafil/animal/day. The placebo control group was administered Placebo Cream at the dose volume of 0.2 mL/animal (twice daily). The sham dose control was not administered any test article, but all other procedures were performed as with test article groups or placebo control group. The dose duration was from GD 6 to GD 17 for main study animals and from GD 6 to GD 20 for TK animals. The ovaries and uteri were examined for determination of litter data. The uteri without visible implantations were placed in ammonium sulfide solution for detection of early resorptions. The placentae of live fetuses were examined macroscopically. All the live fetuses were weighed and examined for external abnormalities; their crown-rump lengths were measured, and sexes were determined. Approximately 1/2 of the live fetuses in each litter were fixed with modified Davidson's fixative for soft tissue examination and the remaining fetuses were stained with Alcian blue and Alizarin red solution for subsequent skeletal examination. The visceral and skeletal examination of fetuses was conducted for the high dose and two control groups. In addition, frozen vagina samples were collected from all TK animals at scheduled necropsy. **Results:** In the main study, there were 24/25, 24/25, 23/25, 23/25, and 23/25 pregnant animals in sham dose control, placebo control, low, mid, and high-dose groups, respectively. There were no unscheduled deaths noted in this study. All animals survived to the scheduled necropsy. Test article-related changes were restricted to the transient and slight decreases in food consumption at high dose (7.0%, relative to sham dose control group) mainly during GD 6-12 when compared to the control groups. The changes were reversible during the following period and were considered non-adverse. TK analyses were conducted for dams on GD 6 and 17. For analyte of sildenafil, T_{max} values were observed between 0.5 and 8.0 hours post first dose. The systemic exposure increased dose-proportionally in females on GD 6 and GD 17 as the dosage increased from 3.77 to 7.54 mg sildenafil/animal/day. Generally, no marked drug accumulation in systemic exposure was observed at any dose level. For analyte of *N*-desmethyl sildenafil, T_{max} values were observed at 8.0 hours post first dose. The systemic exposure increased dose-proportionally in females on GD 6 and GD 17 as the dosage increased from 3.77 to 7.54 mg sildenafil/animal/day. There were no test article-related changes on mortality, clinical signs, body weight, macroscopic observation, litter data (number of corpora lutea, implantation sites, live fetuses, fetal death, and death fetuses, resorptions, pre- and post-implantation loss), sex ratio, placental examination, fetal weight and crown-rump length, and fetal external, visceral and skeletal examinations. **Conclusions:** Based on the above results, the No-Observed-Adverse-Effect-Level (NOAEL) of sildenafil cream, 3.6% for maternal and embryo-fetal developmental toxicity was considered to be 15.08 mg sildenafil/animal/day (corresponding maternal mean C_{max} and AUC_{0-24h} of sildenafil in plasma on GD 17 were 3970 ng/mL and 47200 h*ng/mL, respectively; maternal mean C_{max} and AUC_{0-24h} of *N*-desmethyl Sildenafil in plasma on GD 17 were 575 ng/mL and 5040 h*ng/mL, respectively; maternal mean vaginal tissue concentration of sildenafil on GD20 were 30700 ng/g; maternal mean vaginal tissue concentration of *N*-desmethyl sildenafil on GD 20 were 954 ng/g).

PS 3205 Lifelong Exposure to an Environmentally Relevant Endocrine Disruptor Mixture Promotes Preneoplastic Lesions and Reveals Molecular Markers Linked to Poor Survival in Prostate Cancer

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Background and Purpose: Endocrine disruptors (EDs) are environmental contaminants capable of interfering with hormone-regulated tissues, including the prostate. While most toxicology studies evaluate single compounds and short exposure periods, humans are chronically exposed to mixtures. This study investigated molecular and histopathological alterations in the ventral prostate of male rats exposed to a mixture of 12 environmentally relevant EDs from intrauterine life to adulthood. **Methods:** Pregnant Sprague-Dawley rats received either vehicle (corn oil) or an EDs mixture (phthalates, pesticides, UV filters, bisphenol A, and butylparaben; 32.11 mg/kg/day) from gestational day 7 to postnatal day (PND) 21. Male offspring continued exposure until PND 220. Ventral prostates were processed for histopathology (hematoxylin and eosin) and RNA-seq (HiSeq-2500). All procedures were approved by the Animal Ethics Committee at UNOESTE (CEUA 6034) and complied with ARRIVE guidelines. Differential expression was assessed using DESeq2 ($\log_2FC \geq |1.5|$; adj. $p \leq 0.05$), followed by Gene Ontology enrichment and protein-protein interaction (PPI) network analysis (STRING). Prognostic value of selected genes was evaluated using TCGA prostate cancer datasets. **Results:** The EDs mixture induced prostatic intraepithelial neoplasia (PIN) in 80% of exposed animals, characterized by loss of epithelial polarity, prominent nucleoli, and stromal and luminal inflammatory foci, indicating early neoplastic transformation. Transcriptomic profiling identified 48 differentially expressed genes, predominantly downregulated. GO enrichment highlighted disruptions in cellular response to stimulus and intracellular transport, suggesting endoplasmic reticulum stress and impaired vesicular trafficking. PPI network analysis revealed *VCP*, *HSPA5*, *NPM1*, and *ARCN1* as central regulatory nodes, consistent with roles in protein quality control, proliferation, and stress adaptation. Survival analysis demonstrated that high expression of *ARCN1*, *CD55*, *CRIP2*, and *MFSD4A* was significantly associated with reduced overall survival in prostate cancer patients, indicating translational relevance and biomarker potential. **Conclusions:** Lifelong exposure to an environmentally relevant mixture of EDs compromises prostate homeostasis, inducing pre-neoplastic lesions and transcriptional reprogramming involving stress response and proliferative signaling networks. These findings provide mechanistic evidence linking environmental ED exposure to increased prostate cancer susceptibility and identify candidate molecular biomarkers for disease monitoring and risk assessment. (Funding: FAPESP - 2018/24044-0 and CNPq - 305205/2022-6).

PS 3206 Diisononyl phthalate disrupted microRNA regulation of the placental transcriptome

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Background and Purpose: The placenta is a fetal organ that regulates oxygen, nutrient, and waste exchange; immune protection; hormone production; and maternal-fetal communication. We have demonstrated relationships between phthalates and placental mRNA and microRNA (miRNA) expression via epidemiological and toxicological methods. Diisononyl phthalate (DINP) is a high molecular weight phthalate that is metabolized into primary metabolite mono-isononyl phthalate (MINP), then secondary metabolite mono-carboxy-isoctyl phthalate (MCIOP). Our previous human cohort studies have highlighted MCIOP as the phthalate metabolite most associated with both mRNA and miRNA expression in bulk placental tissue. However, there are limited studies of MCIOP or its precursors using *in vitro* models and toxicological methods. The goal of this project is to evaluate miRNA expression in the placental HTR-8/SVneo cell line and investigate the effects of DINP and its metabolites on mRNA and miRNA expression. **Methods:** HTR-8/SVneo cells were treated with DMSO (0.05%) or 90 μ M of DINP, MINP, or MCIOP for 24 hours with 6 samples per treatment. Total RNA was isolated and sequenced to quantify global miRNA and mRNA expression. Differentially expressed genes (DEGs) and miRNAs (DEMs) were evaluated by fitting linear models in EdgeR and using a significance threshold of $FDR < 0.05$. DEGs were assessed for pathway and transcription factor (TF) enrichment through overrepresentation analysis with KEGG pathways and a placental transcriptional regulatory network. The resulting DEGs and DEMs were then mapped onto an existing placenta-specific miRNA-mRNA network based on human data. Global HTR-8/SVneo miRNA expression was compared to bulk placental tissue to determine the utility of this cell line for analyses of placental miRNA expression. **Results:** MCIOP altered both mRNA (4,508 DEGs) and miRNA (68 DEMs) expression, including 9 miRNAs that were part of the placenta-specific C14 miRNA cluster (C14MC). There was no evidence that DINP affects mRNA or miRNA expression, while MINP modestly altered the mRNA transcriptome (23 DEGs). Three DEMs (miR-27a-5p, miR-199b-5p, and miR-30b-5p) and 3 DEGs (*ZNF616*, *KRT10*, and *CPZ*) were also associated with prenatal urinary concentrations of MCIOP in a human cohort study. MCIOP DEGs were enriched for pathways related to DNA replication and repair, protein processing, and oxidative phosphorylation. MCIOP DEGs were also overrepresented as target genes for 57 transcription factors (TFs), of which 6 were nuclear hormone receptors.

Using our placental miRNA-mRNA network, 49 of the MCIOP DEMs are predicted to target 5,607 genes expressed in HTR-8/SVneo cells, of which 2,025 were MCIOP DEGs. Twelve of these miRNAs targeted genes that were enriched within 12 KEGG Pathways related to catabolism and protein processing. HTR-8/SVneo cells and bulk placental tissue both expressed 439 miRNAs; however, 188 miRNAs were exclusively expressed in placental tissue, and 303 miRNAs were exclusively expressed in HTR-8/SVneo cells. Placental-specific miRNAs from the C19 miRNA cluster (C19MC) were not expressed in HTR-8/SVneo cells, but all C14MC miRNA present in the bulk tissue were also expressed in HTR-8/SVneo cells with 19 additional C14MC miRNAs, which could be specific to the extravillous trophoblast. Overall, the expression of shared miRNAs was comparable between bulk tissue and HTR-8/SVneo with an R^2 of 0.64 for all 439 shared miRNAs and 0.83 for overlapping C14MC miRNAs. **Conclusions:** We demonstrated that MCIOP, but not its precursors (DINP and MINP), causes substantial changes to the placental transcriptome at the level of miRNA and mRNA, emphasizing that metabolites, but not parent compounds are responsible for transcriptomic effects, which has important implications for toxicology studies. Overall, HTR-8/SVneo cells demonstrated good coverage of miRNAs expressed in bulk placental tissue with the exception of C19MC, highlighting their potential use for validation of epidemiological studies. Future directions include incorporating proteomics data, expanding the phthalate concentration range, and using placental cell lines representative of other trophoblast phenotypes.

PS 3207 Profiling the novel impacts of the organochlorine compounds tris(4-chlorophenyl)methane (TCPM) and tris(4-chlorophenyl)methanol (TCPMOH) on human placental cells

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Background and Purpose: Tris(4-chlorophenyl)methane (TCPM) and tris(4-chlorophenyl)methanol (TCPMOH) are anthropogenic environmental pollutants that are believed to be byproducts of the organochlorine pesticide dichlorodiphenyltrichloroethane (DDT). TCPMOH is thought to be a metabolite of TCPM. These chemicals persist in the environment, and have been found to accumulate in aquatic mammal tissue and in human breast milk. They are also potential endocrine disruptors, yet no studies evaluating potential toxic effects have been conducted in the placenta, a transient organ with many endocrine functions critical for fetal development. In this study, TCPM and TCPMOH were screened for their cytotoxic effects, differential gene expression, and invasion capacity. **Methods:** For cell viability and cytotoxicity, HTR-8/SVneo cells were treated with medium plus DMSO (control) or TCPM or TCPMOH (1 μ M - 100 μ M) for 24-hours and cytotoxicity was measured using Multiplex Multitox Cytotoxicity Assay. RNA sequencing of HTR-8/SVneo cells was used for differential gene expression after being treated with medium plus DMSO (control), TCPMOH (1 μ M or 10 μ M), or TCPM (1 μ M or 10 μ M). Differential gene expression was analyzed using DESeq2 in R. For invasion capacity, HTR-8/SVneo cells treated with medium plus DMSO (control) or TCPMOH (1 μ M or 10 μ M). For cell cycle determination, HTR-8/SVneo cells treated with medium plus DMSO (control) or TCPMOH (1 μ M or 10 μ M). **Results:** Placental cells significantly decreased in cell viability when exposed to TCPMOH concentrations of 50 μ M or greater, while TCPM did not show a significant decrease in cell viability. Differential gene expression when exposed to TCPMOH for 1 μ M and 10 μ M concentrations yielded 3 shared significantly upregulated pathways: Inflammatory response, Interferon gamma, and KRAS signaling. Invasion capacity for 1 μ M concentrations reduced cell count by 38%, and for 10 μ M concentrations cell count was reduced by 62%. **Conclusions:** Placental cell viability was significantly reduced by concentrations of TCPMOH 50 μ M and greater, while TCPM did not display significant cytotoxicity. Differential gene expression showed an upregulation of pathways related to inflammation as well as cell proliferation at sublethal concentrations. Low concentrations of TCPMOH also affected the invasion capacity of the placental cells.

PS 3208 Effects of S - (1,2-dichlorovinyl) - L - cysteine (DCVC) exposure on mitochondrial respiration in BeWo Cells

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Background and Purpose: Trichloroethylene (TCE) is a widely used industrial solvent and recognized environmental pollutant, present in spray adhesives, paint removers, tool cleaners, and other common household products. It has remained persistent in the environment globally, causing a risk to human health. TCE can be metabolized into the reactive and toxic metabolite S-(1,2-dichlorovinyl)-L-cysteine (DCVC), which induces mitochondrial-mediated apoptosis in trophoblast cells, known to play a crucial role in the early stages of pregnancy and placental development. Mitochondrial metabolism plays a crucial role in trophoblast differentiation, and little is known about how DCVC exposure affects this process. **Methods:** The current study aims to investigate the effects of different environmentally relevant DCVC concentrations on inducing mitochondrial stress and disruptions to the fusion

process, known as syncytialization, in BeWo syncytiotrophoblast cells. To assess the effects of DCVC on the BeWo cell line, we measured live-cell metabolic function using the Agilent Seahorse XF HS Mini Analyzer, conducting a Mito Stress Test assay. This test measures key parameters such as the oxygen consumption rate (OCR) of cells, allowing for real-time monitoring of plate-based live assays. Prior to the assay, BeWo cells were exposed to 10 and 20 μ M DCVC and incubated for 24 hours. **Results:** Following the exposure period, oxygen consumption rate (OCR), proton efflux rate (PER), extracellular acidification rate (ECAR), and seven other parameters were measured via the Seahorse XF Analyzer. A one-way ANOVA was conducted for the basal respiration results for the three trials to assess changes in mitochondrial activity. So far, preliminary data have shown no significant results identified in the DCVC-treated BeWo cells. **Conclusions:** DCVC remains a significant concern in both public health and environmental toxicology. Ongoing studies aim to further characterize its effects, including upcoming experiments that will use polymerase chain reaction (PCR) to quantify mitochondrial copy number and gene expression. Additional work with the fused version of these cells is also underway to provide deeper insight into the mitochondrial dysfunction induced by DCVC in this placental cell line.

PS 3209 Toxicity assessments of perfluorobutane sulfonic acid (PFBS) in two in vitro human placental models

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Background and Purpose: Perfluorobutane sulfonic acid, PFBS, is a compound belonging to the class of human made chemicals, per- and polyfluoroalkyl substances, PFAS, with its greatest source of exposure through drinking water. In humans, maternal PFBS exposure can be associated with adverse birth outcomes and complications for mothers-to-be. Prior studies associate PFBS exposure with preeclampsia, a pregnancy-specific disease characterized by shallow trophoblast invasion and subsequent placental ischemia. **Despite epidemiological evidence, the toxicological mechanisms underlying this association and its effects on the placenta is not clearly known.** The aim of the research is to understand a dose-response threshold and gene expression results related to reproductive toxicity in two *in vitro* human placenta models exposed to PFBS. This research generates a characterization of a toxicity profile for preliminary studies. **Methods:** We measured cell viability in the HTR-8/SVneo cell line using the Promega MultiTox-Glo Cytotoxicity Assay at 0, 0.1 μ M, 1 μ M, 10 μ M, 20 μ M, 50 μ M, 100 μ M PFBS. Transcriptomic via RNAseq analysis was performed in two models: the HTR-8/SVneo cell line and in third trimester tissue explant. RNA sequencing was processed using the R package DESeq2. Pathway analysis was executed through gene set enrichment analysis, GSEA, programming using ranked Log2FC values. **Results:** The cell viability assay showed no dose-response relationship in the live-dead cytotoxicity assay in the cell model. The RNA sequencing results demonstrated concentration dependent effects in the third trimester tissue model, as evidenced by the magnitude of altered gene expression with 20 μ M versus 5 μ M versus 0 μ M PFBS ($FDR > 0.05 + |\log_{10}FC| > 1$ [$FC > 2$]). Gene set enrichment pathway analyses identified upregulation in cell cycle proliferation in cells with 5 μ M treatment and both 5 μ M and 20 μ M treatments in third trimester tissue. Gene set enrichment pathway analyses identified downregulation in inflammation and immune response across all models and doses. **Conclusions:** This study advances understanding of potential risks of PFBS exposure during pregnancy by identifying differentially expressed genes and subsequent pathways in placental cells and tissues.

PS 3210 Chromium(VI) Exposure During Pregnancy Disrupts Placental Development and Fetal Growth by Altering the Epigenome and Transcriptome of the Placental Labyrinth zone

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Background and Purpose: Hexavalent chromium [(Cr(VI))] has been detected in groundwater across the United States due to industrial and military operations, including plating, painting, cooling-tower water, and chromate production. Cr(VI) is a mutagen, teratogen, carcinogen, and a reproductive toxicant and it causes lung cancer, along with evidence of Cr(VI)-induced genetic alterations. Environmental and occupational exposure to Cr(VI) in women adversely affects their reproductive health, causing premature birth (PTB), preeclampsia and fetal growth restriction (FGR). The actual molecular mechanism underlying Cr(VI)-induced FGR is unknown. The placental labyrinth zone (LZ) in rats (which closely resembles the chorionic villi in human placenta) is the primary site for nutrition and gas exchange between the mother and the developing fetus. Disruption of LZ leads to compromised nutritional exchange potentially causing FGR. According to the Developmental Origin of Adult Health and Diseases (DOHAD) theory, exposure to endocrine disruptors during early

life can have serious impact on the offspring's health in later life. We hypothesized that exposure to Cr(VI) induces FGR by altering the gene expression profiles of the LZ, disrupting placental function and fetal development. **Methods:** Timed-pregnant rats were exposed to 0 or 5 ppm Cr(VI) (as potassium dichromate) in drinking water from gestational day (GD) 9.5 to 12.5, representing environmentally relevant exposure levels (eg. drinking water Cr(VI) in Midland, TX is 5.28 ppm). Animals were euthanized on GD 18.5, and pregnancy outcomes, fetal growth indices, and placental morphology were evaluated. Histological, immunohistochemical (IHC), and quantitative RT-PCR analyses were conducted to assess apoptosis, cytotrophoblast (CTB) cells fusion into syncytiotrophoblast (STB), inflammation, oxidative stress, and anti-oxidants expression were determined. In addition, bulk RNA-sequencing of micro-dissected LZ tissues was performed to identify global transcriptomic changes and regulatory pathways using bioinformatics tools. Pathway enrichment and network analyses were visualized through volcano plots, heatmaps, and KEGG/GO functional clustering. **Results:** Cr(VI) exposure significantly increased fetal resorption rates and decreased live birth index and fetal weight compared to controls. Histological evaluation revealed disrupted trophoblast architecture and the affected fusion of CTB leading to abnormal formation of syncytium in the LZ, increased expression of pro-inflammatory and oxidative stress proteins, and downregulation of anti-inflammatory proteins and antioxidants, compared to control. RNA-seq analysis revealed extensive dysregulation of genes involved in oxidative phosphorylation, extracellular matrix organization, angiogenesis, and epigenetic regulation (including DNA methylation and histone modification pathways). Integration of transcriptomic and molecular data demonstrated that Cr(VI) perturbs the epigenomic landscape of trophoblasts, leading to aberrant gene expression profiles associated with trophoblast differentiation, placental pathologies, restricting fetal growth and increasing fetal death. **Conclusions:** Our findings demonstrate that prenatal exposure to Cr(VI), recapitulating the human exposure levels, disrupts placental development by reprogramming the epigenomic and transcriptomic networks of the trophoblasts in the labyrinth zone. These changes compromise placental development and function, resulting in restricted fetal growth and increased fetal loss. This study provides novel mechanistic insights into how developmental exposure to Cr(VI) causes placental perturbations through epigenomic modulation, advancing our understanding of metal-induced reproductive and developmental disorders.

PS 3211 Effects of Prenatal BaP Exposure on Spindle Quality in MII Mouse Oocytes

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Background and Purpose: Benzo[a]pyrene (BaP) is a by-product of the incomplete combustion of organic material and a known endocrine disrupting chemical. Previous studies of the Luderer Laboratory found that gestational BaP exposure induced DNA damage and mitochondria-mediated apoptosis in F1 metaphase II (MII) mouse oocytes. Prenatal BaP exposure is heavily prevalent amongst the general population but the extent of damage to female fertility is not fully understood, specifically on the quality of F1 MII mouse oocytes exposed to human relevant doses. At ovulation, oocytes are arrested at MII during which the meiotic spindle aligns chromosomes in the center of the cell. Errors in chromosome segregation lead to aneuploidy in resulting daughter cells which is linked to miscarriage and increased cancer risk. **Methods:** C57BL/6J F0 pregnant mice were orally dosed with 0 (sesame oil control), 0.033, or 2 mg/kg/day BaP during F1 embryonic ovarian development, E6.5-15.5. The 0.033 mg/kg/day dose over 10 days is roughly 1/3 of the estimated exposure to BaP during pregnancy of a highly exposed woman and 2 mg/kg/day is used for the comparative higher dose. F0 dams were humanely euthanized and F1 MII oocytes were extracted from the fetal ovaries; immunofluorescence was carried out using antibodies against alpha-tubulin and CREST to visualize the meiotic spindle and kinetochores and DAPI to visualize chromosomes. Z-stack images of eggs were collected and IMARIS software was used to analyze spindle width, the distance from the top-most and bottom-most spindle, and spindle disorganization, classified as spindles deviating from the metaphase plate. Spindle intensity was also measured based on the signal of segments from the surface created in IMARIS. **Results:** There was a significant increase in spindle width (GEE, $p < 0.001$) and the prevalence of spindle disorganization (Kruskal-Wallis: $p = 0.033$) in mice exposed to BaP compared to control. Spindle intensity, an indirect measurement of spindle quantity was not altered by BaP exposure (GEE, $p = 0.073$). **Conclusions:** Greater spindle width and disorganization at MII pose a risk for aneuploidy as the likelihood of non-disjunction, or errors in chromosome segregation into daughter cells, drastically increases in the presence of stray spindles. If an egg that underwent non-disjunction were to be fertilized, the resulting zygote may have the improper number of chromosomes leading to developmental defects like Trisomy 21. Given the non-replenishable nature of the ovarian reserve, any environmental factors that reduce oocyte quality are detrimental to overall reproductive health and fertility. In light of recent drops in fertility rates, this research will be important for understanding the extent of BaP-mediated meiotic damage and contribute to standardized precautions against environmental toxins.

PS 3212 Sexually Dimorphic Immune Response in Placentas of Nano-Titanium Dioxide Exposed Rat Dams

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Background and Purpose: The placenta plays a vital role for the developing fetus by providing nutrient and waste exchange, hormone production, and a protective barrier. Two major zones of the placenta include the junctional zone (JZ), which generates hormones and growth factors, and the labyrinth zone (LZ), which is the site of nutrient exchange. Poor placental protection or nutrient delivery can have more immediate effects such as preeclampsia, fetal growth restriction, and fetal mortality, and/or lifelong health effects on the offspring, as described by the Developmental Origins of Health and Disease hypothesis. Therefore, disturbances to the placenta through maternal toxicant exposure can have lasting implications to the baby. In Sprague Dawley (SD) rat dams, gestational inhalation of titanium dioxide nanoparticles (nTiO₂) leads to placental insufficiency, intrauterine growth restriction, and sexually dimorphic metabolic and endocrine dysfunction in adult offspring. These sex differences begin *in utero*, with fewer males being born to nTiO₂ exposed dams, and female fetuses experiencing more growth restriction. Exposed female placentas also had greater changes to relative placental zone mass and Hofbauer cell (placental macrophage) presence. Because immune responses are known to differ significantly by sex, and placental cells have sex chromosomes, we sought to determine if placentas exposed to nTiO₂ have sexually dimorphic and zone-specific immune responses. **Methods:** Female SD rats ($n = 4-6$ per group), approximately 8-week-old, were acclimated for 48 hours before handling and provided standard chow and water *ad libitum*. Females were randomly assigned to be exposed to either nTiO₂ (12 mg/m³) or HEPA-filtered air (25 mL/min) for 6 hours per day for 6 non-consecutive days between GD12 and 19. Dams were euthanized on GD20 for tissue collection and evaluation of litter size, implantation count, pup weight, and placental weight. Placentas were divided by fetal sex, and placental zones (JZ and LZ) were isolated. One male and female placenta per dam was fixed in 4% paraformaldehyde for immunofluorescence of Hofbauer cell polarization. At least one male and female placenta per dam were snap frozen for subsequent protein and RNA analysis. A cytokine proteome array was used to screen whole placentas for changes in 79 cytokines by exposure and sex. Phosphorylation of JNK and NFκB in placental zones was measured by western blot. Relative mRNA levels of cytokines, chemokines, and pro-inflammatory ER stress pathways were determined RT-qPCR. **Results:** In general, nTiO₂ exposure resulted in more cytokine upregulation in the female placenta and more downregulation in the male placenta, as measured by the proteome array. Proteins increased in the exposed female placenta include Galectin-1, Neprilysin, IL-2, IL-3, IL-6, IFNγ, and CCL2, and in the male placenta include Neprilysin and ICAM-1. Proteins decreased in the female placenta include RAGE and IGFBP-3, and in the male placenta include FGF, IGF-1, IL-1β, and CCL2. The mRNA expression of TNFα, IL-1β, IL-10, and IFNγ were all higher in the JZ compared to the LZ in both males and females. However, trends in these cytokines differed by nTiO₂ exposure in the LZ, but not in the JZ. Exposed male JZs had more IL-10 (355%, $p = 0.06$, $n = 3$) and IFNγ (305%, $p = 0.06$, $n = 3$), while female JZs had a trending increase in IL-10 (487%, $p = 0.1$, $n = 3$). Further RT-qPCR analysis will be utilized to further evaluate changes in cytokines, chemokines, and apoptotic pathways by nTiO₂ in placental zones. Western blot of female JZs showed a trending increase ($p = 0.1$, $n = 3$) in JNK phosphorylation by nTiO₂, indicating an increase in apoptosis. **Conclusions:** Altogether, these data demonstrate sexually dimorphic and zone-specific immune responses in placentas of gestational nTiO₂ exposure. In general, the female placenta had higher protein concentrations of cytokines when exposed to nTiO₂, which may contribute to the growth restriction and placental zone adaptations seen in female pups. Furthermore, while the JZ had higher mRNA expression of cytokines, changes due to nTiO₂ occurred more in the LZ. Better understanding the sex differences in placental responses can help us to identify mechanisms, pharmacological targets, and markers of poor pregnancy outcomes specific to the fetal sex.

PS 3213 Thyroid dysregulation secondary to hepatic enzyme induction underlies altered ovarian cyclicity in rats exposed to PF-06835919, a KHK inhibitor

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Background and Purpose: PF-06835919, a ketohexokinase (KHK) inhibitor being developed for the treatment of non-alcoholic fatty liver disease (NAFLD), caused decreased or absent corpora lutea in the ovary and cornified squamous epithelium in the vagina in a 6-month repeat dose toxicology study in rats. These findings occurred in the presence of liver and thyroid effects that were consistent with PF-06835919-related hepatic enzyme induction. Given that there are numerous reports in the literature indicating that thyroid function is associated with the female reproductive cycle, it was hypothesized that the increased incidence of decreased/absent corpora lutea in the ovary was a result of hypothyroidism secondary to PF-06835919-related UGT (UDP-glucuronosyltransferase) enzyme induction in the rat. **Methods:** An investigative study was conducted to further characterize the ovarian findings and to investigate this potential mechanism. In this study the duration of dosing required prior to onset of estrous cycle disruption and the duration of time needed for the animals

to return to normal cycling following suspension of dosing were evaluated following daily oral administration of 100 mg/kg/day PF-06835919 to female Wistar Han rats. TSH (thyroid stimulating hormone), T3, and T4 levels were also measured. All procedures performed on animals were in accordance with regulations and established guidelines and were reviewed and approved by an Institutional Animal Care and Use Committee or through an ethical review process. **Results:** Estrous cycle disruption (persistent estrus) was noted in 10 of 15 females with onset of disruption occurring between days 33 and 81. Higher TSH (3.8x control) and altered T3/T4 were noted on day 37, indicating that thyroid hormone dysregulation preceded the estrous cycle disruption. This aligns with the thyroid hormone effects with longer dosing in the 6-month rat toxicity study, in which elevations in TSH (7.09x), and a reduction in T3 (0.59x) were observed at 100 mg/kg/day. Consistent with these observations, UGT enzyme induction, including UGT1A1, were confirmed to be induced in a separate 2-week study. Recovery of the estrous cycle disruption occurred in 30% of the animals before the study was terminated due to the onset signs of natural reproductive senescence in several animals, including control animals. **Conclusions:** While a direct link has not been established, all data support the hypothesis that PF-06835919 induction of one or more UGT enzymes and consequent thyroid dysregulation is the cause of altered ovarian cyclicity observed in rat. Although dysregulation of thyroid hormones can result in female reproductive effects in both humans and rats, the mechanism underlying PF-06835919-induced thyroid dysregulation (induction of UGTs leading to altered thyroid hormone levels) is rat specific and therefore not relevant to humans.

PS 3214 The effects of a combination of western diet and phthalate exposure on gene expression levels in the ovary

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Background and Purpose: Phthalates are a diverse group of chemicals commonly used in a wide range of products, including food packaging, children's toys, and plastic containers. These compounds have been shown to negatively impact female reproductive health, but their effects on the ovary in conjunction with a western diet—characterized by high fat and processed foods prevalent in the United States—are not well understood. Thus, this study tested the hypothesis that combined exposures to a western diet and phthalates affect the ovarian expression of regulators of immune, steroidogenic, and aryl hydrocarbon receptor signaling pathways (*Il-1α*, *Il-1β*, *Il-1r1*, *Il-1r2*, *Il-4*, *Il-6*, *Il-10*, *Tnf-α*, *17β-Hsd*, *3β-Hsd*, *Cyp17a1*, *Cyp11a1*, *Esr1*, *Esr2*, and *Ahr*) and impair production of estradiol levels. **Methods:** To test this hypothesis, female C57BL/6 mice were divided into six experimental groups: control diet, control diet + di(2-ethylhexyl) phthalate (DEHP), control diet + diisononyl phthalate (DiNP), western diet, western diet + DEHP, and western diet + DiNP. RNA was extracted from the ovaries of these mice and subjected to qPCR for measurement of regulators of immune, steroidogenic, and aryl hydrocarbon receptor signaling pathways. Sera was subjected to estradiol ELISA to measure estradiol levels. **Results:** The results indicate that DiNP exposure significantly increased *Il-1β* and *Cyp17a1* expression in the ovary compared to control, irrespective of whether the mice were on a control or western diet. Furthermore, DiNP exposure + control diet and DiNP exposure + western diet significantly decreased *Il-1r2* and *3β-Hsd* expression in the ovary compared to control. DEHP exposure + western diet significantly decreased expression of *Cyp11a1* and *3β-Hsd* compared to control. In contrast, control diet + DEHP, control diet + DiNP, western diet, western diet + DEHP, and western diet + DiNP did not significantly affect the expression of *Il-1α*, *Il-1r1*, *Il-4*, *Il-6*, *Il-10*, *Tnf-α*, *17β-Hsd*, *Esr1*, *Esr2*, or *Ahr* compared to controls. None of the phthalate or diet treatments significantly affected estradiol levels compared to control, but statistical analysis indicated an interaction between phthalates and diet on estradiol levels. **Conclusions:** Overall, these findings indicate that DiNP and western diet exposure alter the expression of some inflammatory and steroidogenic markers in the mouse ovary. Supported by NIH R01 ES034112.

PS 3215 Single Cell Transcriptomics Reveals that Hexavalent Chromium [Cr(VI)] Exposure During Pregnancy Disrupts Uterine Artery Remodeling by Altering the Extracellular Matrix Network.

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Background and Purpose: Hexavalent chromium [Cr(VI)] is a heavy metal endocrine disruptor (EDC). Occupational exposure to Cr(VI) affects about half a million industrial workers in the U.S. and several million globally. Significant Cr(VI) contam-

ination has been detected in roughly 30% of drinking water sources in California. According to the U.S. Environmental Protection Agency, the safe level of Cr(VI) in drinking water is 0.01 PPM. However, in some severe pollution incidents in the U.S., including Hinkley, CA, and in developing countries, levels of Cr(VI) in drinking water or groundwater have exceeded the EPA limit by 50 to 500 times. For instance, water in Midland, TX, contains 5.28 PPM Cr(VI). Women exposed to Cr(VI) through occupational or environmental sources experience adverse reproductive outcomes such as preterm labor, miscarriage, and fetal growth restriction (FGR). The molecular mechanisms underlying Cr(VI)-induced FGR remain unclear. Trophoblast remodeling of the uterine artery (UA) is a crucial process during pregnancy, where fetal extravillous trophoblasts (EVTs) invade the maternal uterine spiral arteries. This invasion replaces the endothelial cells of the arteries with trophoblasts, transforming them from high-resistance/low-flow vessels into low-resistance/high-flow ones to ensure adequate blood flow to the developing fetus. Failure of proper remodeling can lead to complications such as preeclampsia and FGR. We hypothesize that Cr(VI) exposure disrupts uterine artery remodeling (UAR) by altering the gene network underlying UAR, leading to arterial thickening or stiffness. **Methods:** Pregnant Sprague-Dawley rats were exposed to 5 PPM Cr(VI) (as potassium dichromate) via drinking water from gestational day (GD) 9.5 to 12.5. On GD 18.5, uterine arteries were collected for histology (H&E, Verhoeff-Van Gieson) and immunohistochemistry (Collagen IV, α-smooth muscle actin [α-SMA]). Single-cell RNA-seq was used to define transcriptional changes in fibroblasts and vascular smooth muscle cells (VSMCs). Differentially expressed genes (DEGs) were visualized using volcano plots, and Gene Ontology enrichment identified dysregulated biological pathways. **Results:** Cr(VI)-exposed arteries displayed thickened vessel walls, reduced luminal diameter, and increased elastin and Collagen IV deposition, consistent with structural stiffening. IHC showed elevated α-SMA expression, indicating heightened vascular contractility. Single-cell transcriptomics revealed fibroblast downregulation of vasodilatory and signaling genes (*Ramp3*, *C1qa*, *Cd74*) and VSMC upregulation of ECM and contractile genes (*Col1a1*, *Col3a1*, *Mgp*, *Gsn*). Enrichment analyses demonstrated activation of ECM organization, collagen fibril formation, angiogenesis, and vasculature development, alongside suppression of cell migration, intracellular signaling, and apoptotic regulation. These data indicate that Cr(VI) exposure reprograms both fibroblast and VSMC gene networks governing matrix homeostasis and vessel elasticity. **Conclusions:** These cellular and molecular alterations indicate that Cr(VI) exposure disrupts vascular remodeling by dysregulating ECM, inducing oxidative stress, and increasing arterial stiffness, leading to restricted blood flow and FGR.

PS 3216 Characterization of tight junctions in a JEG3 cell-based human placental model, widely used in reproductive and developmental toxicity studies

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Background and Purpose: In development of medical products, animal tests have begun to be replaced by *in vitro* and *in silico* tests. Indeed, the U.S. Food and Drug Administration just announced "Roadmap to Reducing Animal Testing in Preclinical Safety Studies" in April 2025. Toxicity results from animal testing poorly predict human toxicity, particularly in reproductive and developmental toxicity. Notably, in perinatal medicine, reliable means for extrapolating to human toxicity are limited. Therefore, cell-based models that accurately mimic the human placental barrier are required for toxicity assessment. However, the structural and functional components of the human placental barrier, particularly tight junctions (TJs), have not been fully characterized. In this study, we investigated the contribution of TJs to barrier properties in an *in vitro* human placental model by examining changes in barrier function following treatment with TJ modulators. **Methods:** Human choriocarcinoma JEG3 cells were used as an *in vitro* human placental model. Cells were cultured on polycarbonate Transwell inserts to form a confluent monolayer for eight days. To evaluate how TJ modulators influence barrier integrity, the monolayer was treated with claudin (CLDN) modulators—including CLDN3/4 binder C-CPE 194, broad CLDN binder C-CPE m19, CLDN5 binder C-CPE mt, and a mutant lacking CLDN affinity C-CPE YL—as well as angulin modulators, including angulin-1/3 binder angubindin-1 and a mutant lacking angulin affinity angubindin-1 4ADE. Trans-epithelial electrical resistance (TEER) was monitored at defined intervals during the 24-hour treatment period, and recovery was further assessed for 24 hours after modulator removal. To assess paracellular permeability, the apparent permeability coefficients (P_{app}) for fluorescein and fluorescein isothiocyanate (FITC)-dextran of different molecular weights (4, 10, 40 and 150 kDa) were determined 24 hours after modulator treatment. Baseline permeability of a small molecule drug, metformin, was also evaluated under non-treated conditions. **Results:** C-CPE 194 and m19 significantly reduced TEER to approximately 20 % of baseline, while C-CPE mt lowered it to 34 %. Angubindin-1 moderately decreased TEER to 75 %. In contrast, C-CPE YL and angubindin-1 4ADE showed no effect, consistent with their lack of binding affinity for CLDNs or angulins, respectively. Recovery of TEER was observed 24 hours after removal of the modulators. Permeability assay demonstrated that the apparent permeability coefficients (P_{app}) of all fluorescent tracers increased in association with TEER reduction. Compared with control, P_{app} values increased by 2.0-3.5-fold in C-CPE 194 and C-CPE m19 groups, 1.5-2.4-fold in C-CPE mt group, and 1.1-1.3-fold in angubindin-1 group. No significant changes were observed in C-CPE YL and angubindin-1 4ADE groups. The P_{app} of metformin after 30 minutes was approximately 2.4-fold higher than that

of fluorescein. **Conclusions:** These findings indicate that CLDN3 and CLDN4 play key roles in maintaining the JEG3 monolayers integrity, as shown by the marked barrier modulation following C-CPE 194 and m19 treatment. Angulin-1 and angulin-3 may also contribute to tricellular TJ integrity, as suggested by the moderate TEER reduction induced by anguibindin-1. Considering the gene expression profile of JEG3 cells, CLDN4 and angulin-1 likely constitute the major structural components of the intercellular TJ seals. Permeability data indicate that TJs restrict paracellular influx of molecules; however, the higher permeability of metformin compared with fluorescein—despite their similar molecular size—implies that active transport mechanisms may also contribute. Overall, these results highlight the importance of characterizing both TJ and transporter functions to enhance the physiological relevance and predictive value of *in vitro* human placental models for reproductive and developmental toxicity testing.

PS 3217 Prenatal and Postnatal PFAS Exposure Induces Sex-Specific Hepatic and Reproductive Organ Hypertrophy in CD-1 Mice

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are ubiquitous in the environment. In line with the developmental origins of health and disease (DoHaD) hypothesis, PFAS exposure early in life (e.g., *in utero*) has been associated with adverse health outcomes such as delayed puberty later in life in both human populations and animal models. During pregnancy, placental and fetal endocrine communication governs hypothalamic-pituitary-gonadal (HPG) axis development. Although the mechanisms underlying these adverse health outcomes are not precisely established, they may include disruption of placental hormones such as leptin, progesterone, estradiol, and human chorionic gonadotropin. This study evaluated the effects of prenatal and postnatal PFAS exposure on organ weight in a CD-1 mouse model. **Methods:** Time-mated CD-1 dams were exposed to perfluorooctanoic acid (PFOA; 0.1, 0.3, 0.5 mg/kg/day), perfluorohexane sulfonate (PFHxS; 0.15, 0.4, 0.65 mg/kg/day), or control water throughout gestation and lactation. Offspring were either sacrificed at postnatal day (PND) 23 or maintained on the same drinking water until pubertal timepoints (males: PND 27, 43; females: PND 40, 56). Body and organ weights were collected, including liver, kidneys, brain, testes (males), uterus & ovaries (females), adrenal glands, and mammary glands (females), and subsequently normalized to body weight. Tissues were collected for subsequent gene-expression and hormone assays. **Results:** PFAS exposure produced sex-specific, dose-dependent effects on organ weights normalized to body weight (expressed as g/g). At PND 56, the combined uterus + ovary weight (g) relative to body weight (g) was significantly elevated in 0.65mg/kg PFHxS (mean $\pm$ SEM = 1.93 ± 0.06 ; $n = 5$) compared all other exposure groups ($p < 0.0001$). This represents an approximate twofold increase in weight relative to the control and lower-dose groups, consistent with uterine and ovarian hypertrophy following chronic PFAS exposure. The hypertrophy effect was specific to 0.65mg/kg PFHxS. Male offspring exhibited no significant differences in relative testis weights across exposure levels or time points, highlighting a female-specific sensitivity to PFAS-mediated endocrine disruption. In the liver, PFAS exposure induced consistent, dose-dependent hypertrophy across developmental stages. At PND 23, females in exposed to 0.3 mg/kg PFOA displayed higher relative liver weights (mean = 9.0 ± 2.59) than those in the control (mean = 5.7 ± 0.296 ; $p < 0.05$). By PND 56, females with all PFOA exposures had livers with weights that remained significantly elevated (means = 6.9 - 9.7) compared to the control (mean = 5.1 ± 0.73 ; $p < 0.05$). Males showed similar trends at PND 43, with relative liver weights in 0.5mg/kg and 0.3 mg/kg PFOA (11.5 ± 0.7 and 10.4 ± 1.07 , respectively) exceeding those in the control (6.4 ± 0.56 ; $p < 0.05$). These findings align with PFAS-induced hepatic hypertrophy, a phenotype associated with PPAR α activation and altered lipid metabolism. **Conclusions:** In summary, pre- and postnatal exposure to PFAS in drinking water was associated with liver hypertrophy in both male and female offspring and reproductive organ hypertrophy in female offspring specifically. Future research will investigate changes in the expression of key puberty-related genes in critical organs such as the liver and gonads.

PS 3218 Cross-Laboratory Reproducibility and Age-Adapted Strategies for Learning and Memory Assessment in Rodents Using Morris and Biel Water Maze Paradigms

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Background and Purpose: Learning and memory (L&M) assessments are often considered among the least sensitive endpoints in regulatory safety evaluations. However, cognitive integrity is essential for survival, adaptation, and overall neurological health. As regulatory agencies increasingly emphasize the importance of higher-order cognitive function in developmental neurotoxicity (DNT) and general neurobehavioral assessments, the need for reliable and reproducible testing strategies has grown. Standardization of behavioral assays, including room setup, apparatus, visual cues, and harmonized protocols is critical to achieving consistency across laboratories. This study aimed to (1) evaluate inter-laboratory reproducibility

of Morris Water Maze (MWM) performance across geographically distinct sites, and (2) explore age-adapted strategies for dual L&M testing using MWM and Biel Water Maze (BWM) paradigms within the same animals. **Methods:** Sprague Dawley rats were tested under control and scopolamine-treated conditions across four laboratories: GRN (Greenfield, IN, USA), and Eye, Huntingdon, and HAR (Harrogate, UK). All sites followed harmonized protocols for MWM testing, including standardized visual cues and apparatus. Behavioral endpoints included path length, latency to platform, and probe trial performance. To assess cross-laboratory reproducibility, control data from GRN ($n=498/\text{sex}$) and HAR ($n=139/\text{sex}$) were compared. In a separate study arm, age-adapted testing was conducted using MWM in weanling rats (Postnatal Day [PND] 25) and BWM in adolescent rats (PND 60-70), or vice versa. Scopolamine was used as a positive control to evaluate sensitivity of both paradigms. Animals were tested sequentially in both mazes to assess feasibility, performance trends, and potential carryover effects. **Results:** MWM data revealed consistent acquisition and probe trial outcomes across all four sites, demonstrating strong inter-laboratory reproducibility, with statistically significant L&M impairments specifically associated with scopolamine treatment, which served as the positive control to validate assay sensitivity. Analysis of Historical Control Data from GRN and HAR showed highly comparable performance across all key parameters, including path length and latency to platform, despite differences in sample size and geographic location. Probe trial metrics such as platform zone crossings and time spent in the target quadrant were also aligned, supporting the robustness of standardized MWM protocols. In the age-adapted testing arm, weanling rats successfully completed MWM tasks with performance trends similar to older cohorts. These same animals were then tested in BWM, demonstrating reliable egocentric navigation and sensitivity to scopolamine-induced deficits. Reversing the sequence, BWM at weaning and MWM in adulthood was also feasible, with no impact on task acquisition or memory recall. Across both paradigms, scopolamine produced statistically significant impairments in learning and memory, validating the sensitivity of the assays. **Conclusions:** This study confirms that standardized protocols enable robust cross-laboratory reproducibility of MWM outcomes, reinforcing the reliability of behavioral data in safety evaluations. Furthermore, age-adapted dual testing strategies using MWM and BWM offer flexible, developmentally appropriate approaches for assessing spatial and egocentric navigation in rodents. These findings support the integration of multiple cognitive assays into regulatory studies, aligning with evolving expectations for comprehensive neurobehavioral assessment. The ability to reuse animals across paradigms while maintaining data quality enhances study efficiency and animal welfare, while expanding the scope of cognitive evaluation in toxicology.

PS 3219 The Functional Observational Battery in Rats - Results from a Positive Control Study to Support Crop Protection and Chemical Safety Studies

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Background and Purpose: Regulatory guidelines from agencies such as the US EPA and OECD require laboratories conducting neurotoxicity and developmental neurotoxicity studies to periodically validate their procedures using positive control substances. These studies confirm the sensitivity and reliability of the testing methods, equipment, and personnel, especially when key elements change. Guidelines including EPA OPPTS 870.6200, 870.6300, OECD 424, OECD 443, and OECD Guidance Document No. 43 emphasize the need to detect both increases and decreases in behavioral performance. Positive control studies also help demonstrate technician expertise and inter-observer reliability. This study aimed to validate the Functional Observational Battery (FOB) for regulatory neurotoxicity assessments and to confirm its suitability for detecting chemically induced behavioral changes across rat strains. **Methods:** The FOB is a structured assessment of an animal's appearance, behavior and responses to stimuli, performed in a sequence from least to most interactive. It includes a home cage observation, a handheld assessment, an arena evaluation and a series of elicited and quantitative tests such as pupil response, landing footsplay and grip strength. Female Sprague Dawley (SD) and Han Wistar (HW) rats were administered either 3 mg/kg D-Amphetamine or 4/4.3 mg/kg Di-isopropylfluorophosphate (DFP) one hour prior to FOB testing. Animals were at least 42 days old at dosing. These substances were selected for their known neuro-behavioral effects and ability to produce observable changes in behavior and physiology. Observations were recorded using standardized scoring criteria to ensure consistency and inter-observer reliability. Animal care and use was conducted in alignment with animal welfare regulatory requirements in an AAALAC accredited facility and AWERB approved. **Results:** Rats treated with D-Amphetamine exhibited an increased incidence of rearing, fast respiration and an absence of eyelid closure in the home cage. Handheld assessments showed an increase in exophthalmos and increased avoidance behavior when removed from the cage. Arena evaluations showed abnormal high-stepping gait, piloerection, elevated tail posture and increased levels of alertness alongside increased ambulation and rearing counts. Elicited tests revealed excessively dilated pupils, slow or incomplete pupil constriction in response to light and impaired righting reflex. These effects were consistent across both SD and HW strains. Rats treated with DFP exhibited an increased incidence of lying down, jaw chomping, gaping mouth movements and tremors in the home cage and felt cold when held. Arena evaluations showed flattened or hunched posture, gait abnormalities including flattened, high stepping, slow and deliberate, splayed, and ataxic. Alertness was reduced as were the ambulation and rearing counts. Abnormal

motor movements such as jaw chomping and excessive licking were observed and tremors continued. Elicited tests showed a reduced response to stimuli, pupil abnormalities, low body weight and temperature, and increased landing footsplay. These findings were more pronounced in HW rats but observed in both strains. **Conclusions:** Following administration with positive control chemicals, the FOB procedure effectively detected both increases and decreases in behavioral performance. The results confirm the sensitivity of the FOB and its suitability for regulatory neurotoxicity and developmental neurotoxicity studies. The use of standardized scoring and trained technicians supports inter-observer reliability and consistency across strains. These findings underscore the importance of periodic validation using positive controls to maintain the integrity and reliability of behavioral assessments in safety evaluations.

PS 3220 Exploratory Assessment of Preconception Phthalate Exposure and its Impacts on Pregnancy and Offspring Health Outcomes in Mice

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Background and Purpose: Infertility impacts approximately 10-15% of couples globally and is attributed to environmental exposures disturbing endocrine function. Among endocrine-disrupting chemicals (EDCs), *phthalates* are particularly concerning for their widespread use in consumer products, including cosmetics and feminine hygiene items, and their ability to leach into the environment, resulting in near-universal human exposure. Studies link phthalates with menstrual irregularities, anovulation, and adverse pregnancy outcomes, yet mostly focusing on *gestational* or *postnatal* exposure periods. The *preconception* window, spanning puberty through early adulthood, represents an understudied period when endocrine homeostasis governs oocyte maturation, estrous cyclicity, and uterine receptivity. Preconception perturbations can alter maternal physiology before pregnancy, thereby influencing implantation, placentation, and offspring health. We hypothesized that preconception exposure to a human-relevant phthalate mixture would perturb hormone-dependent processes such as estrous cyclicity and implantation, leading to altered placental architecture, endocrine signaling, and offspring metabolic programming. **Methods:** Female CD-1 mice were orally dosed daily from PND 30-60 with either corn oil or a six-component phthalate mixture (BzBP, DiBP, DiNP, DBP, DEHP, DEP) at 200 µg/kg bw/day, a human-relevant dose based on biomonitoring data. Following a 24-h washout, females were paired with untreated males; sperm plug detection marked embryonic day (E) 0.5. Dams were assigned to three experimental cohorts: (1) implantation (E4.5, via Chicago Blue dye injection), (2) placental/fetal development (E14.5, fetal body weight and placental histology, morphometrics, RNA-seq), and (3) offspring outcomes (PND 60, body weight, reproductive cycling, and liver transcriptomics). A biomarker of hypothalamic-pituitary-gonadal (HPG) axis function was provided monitoring daily maternal estrous cyclicity. Placentas underwent hematoxylin and eosin staining and blinded quantitative ImageJ analysis. Total RNA from E14.5 placentas and PND 60 offspring livers (both sexes) was sequenced on the Illumina platform. Differential expression was analyzed with DESeq2 and edgeR, and GO/KEGG enrichment identified disrupted pathways. **Results:** Preconception exposure modestly altered estrous cyclicity, prolonged preestrus, shortened metestrus, without affecting implantation or litter size. At E14.5, fetuses displayed increased bodyweights accompanied by expansion of the placental junctional zone in males ($\eta^2 = 0.57$). Placental RNA-seq revealed 518 DEGs in females (mostly down-regulated) versus 9 in males. Female placentas showed suppression of immune and steroid-metabolic genes (*SerpinA6*, *Gstm1*, *Cyp21a1*, *Tph1*) and enrichment of complement/coagulation, and lipid homeostasis pathways. Male placentas exhibited limited transcriptional changes but expanded trophoblast layers. In adult offspring, maternal exposure induced sex-specific hepatic reprogramming: females showed down-regulation of *Cyp7a1*, *Mthfr*, and *H2-DMB1* (impaired bile acid and one-carbon metabolism), whereas males up-regulated *Elf4*, *Tnfrsf22* and down-regulated *Adm2*, *Ly6a*, consistent with reduced stress adaptability. Growth trajectories inverted from fetal overgrowth to adult growth restriction, aligning with developmental-origins "thrifty phenotype" programming. **Conclusions:** Preconception phthalate mixture exposure produces subtle but biologically meaningful endocrine disruption, modifies placental structure and transcriptomes, and reprograms offspring metabolic gene networks in a sex-specific manner. These findings identify the preconception period as a critical, previously overlooked window of EDC susceptibility with implications for reproductive success, placental resilience, and intergenerational health. Future work will quantify hormone and metabolite profiles to define the placenta-liver signaling axis underlying these outcomes.

PS 3221 Investigating the molecular mechanism of reproductive dysfunction caused by transgenerational BPA exposure

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Background and Purpose: Toxicants can alter the epigenome and cause changes in gene expression. Changes in the epigenome caused by ancestral exposure to toxicants can exhibit transgenerational inheritance, indicating molecular effects on the germline and meiosis. The nematode, *C. elegans*, is a valuable research model

to study the transgenerational molecular and epigenetic effects of toxicants due to its fast reproduction cycle (3 days), utility in toxicological research, well-established distribution of histone marks, and broad conservation of chromatin regulation with humans. Bisphenol A (BPA) is a reproductive toxin associated with recurrent miscarriages in humans and male and female infertility. Ancestral BPA exposure in *C. elegans* causes a transgenerational reduction in repressive histone modifications and an increase in germline apoptosis and embryonic lethality. However, the molecular mechanism of how BPA causes reproductive dysfunction and which processes during meiosis are impaired is not fully understood. **Methods:** Worms were ancestrally exposed to BPA (100 µM) in liquid culture for 48 hours from the L4 stage. After BPA exposure, worms were maintained on plates through the F3 generation without any additional BPA exposure. F3 germlines were used for immunofluorescence to characterize defects in meiotic recombination and synaptonemal complex formation. Genetic analyses were done to assess whether the transgenerational increase in germline apoptosis can be rescued by inactivation of the recombination checkpoint (*spo-11*) and/or synapsis checkpoint (*pch-2*) by counting the number of apoptotic nuclei per germline from ancestral BPA exposure. **Results:** Ancestral BPA exposure causes a significant change in the frequency of crossovers in F3 germlines resulting in nuclei with fewer or excess crossovers than the typical six crossovers that form in *C. elegans* nuclei. Genetic ablation of the synapsis checkpoint after ancestral BPA exposure rescues apoptosis (mean 3.27 apoptotic nuclei) to similar levels as control *pch-2* worms (mean 2.67 apoptotic nuclei), indicating defects in synaptonemal complex formation. We are currently investigating the presence and extent of synapsis defects and whether synapsis defects colocalize with epigenetic aberrations of repressive histone marks. **Conclusions:** Our work can inform our molecular understanding on how BPA can have lasting reproductive effects on generations that were not directly exposed and could provide insight into how other related bisphenols could affect fertility.

PS 3222 Acute Exposure to Perfluorooctanoic Acid (PFOA) Disrupts Steroidogenesis and Mitochondrial Function in Granulosa Cells

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Background and Purpose: Perfluorooctanoic acid (PFOA) is a legacy Per- and polyfluoroalkyl substances (PFAS) classified as a long-chain perfluoroalkyl acid and historically employed in various industrial and commercial applications. Due to its bioaccumulative nature arising from the chemical structural characteristics, PFOA has been linked to various toxicological effects, including liver and kidney toxicity. Moreover, recent studies indicate that PFOA exposure contributes to reproductive dysfunction such as hormonal imbalance, reduced birth weight, infertility, and immune toxicity in fetuses. Recent studies further indicate that PFOA exposure induces ovarian dysfunction through disruptions in folliculogenesis, steroidogenesis, and oocyte quality *in vivo*. Despite these findings, the cellular pathways through which PFOA exerts its effects remain insufficiently characterized. Follicular development begins with the primordial follicle containing an oocyte surrounded by a single layer of flattened granulosa cells. As maturation progresses, granulosa cells proliferate and form multiple layers to create the secondary follicle. During this stage, theca cells emerge and cooperate with granulosa cells to synthesize steroid hormones that are essential for follicular growth. Beyond steroidogenesis, granulosa cells supply metabolic substrates and paracrine signals regulating oocyte growth and meiotic competence while maintaining the follicular microenvironment through gap junctions and growth factor secretion. In the preantral and antral stages, granulosa cells produce estrogen and facilitate bidirectional communication between the oocyte and theca cells, thereby supporting oocyte maturation, antrum formation, and overall follicular integrity. Given these key roles, assessing PFOA induced toxicity in granulosa cells can serve as an appropriate model to investigate how PFOA exposure affects female reproductive health. This study investigated how PFOA affects granulosa cell function as a potential mechanism underlying female reproductive toxicity. Specifically, this study tested the hypothesis that PFOA exposure impairs granulosa cell viability and function by inducing oxidative stress, disrupting steroidogenesis, and causing mitochondrial dysfunction. **Methods:** Granulosa cells were collected from antral follicles isolated from ovaries of six-week-old CD-1 mice and cultured *in vitro*. The cells were treated with PFOA at concentrations of 1, 10, 25, 50 and 100 µg/mL for cell viability assays and 50 and 100 µg/mL for oxidative stress and gene expression analyses. Dimethyl sulfoxide (DMSO) was used as the vehicle control for all treatments, which were conducted for up to 72 hours. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, while reactive oxygen species (ROS) levels and gene expression related to steroidogenesis and mitochondrial function were analyzed by ROS assay and quantitative polymerase chain reaction (qPCR). **Results:** PFOA exposure significantly reduced granulosa cell viability in a dose-dependent manner compared to DMSO vehicle control. It also increased the level of reactive oxygen species and suppressed *Sod1* antioxidant expression. PFOA also decreased *Cyp11a1* and increased *Cyp19a1* expression, indicating that PFOA may alter steroid hormone synthesis. In addition, PFOA exposure increased *Aifm1* and decreased *Ppargc1a* expression, suggesting that PFOA exposure may induce mitochondrial dysfunction and apoptosis activation. **Conclusions:** These results suggest that the PFOA impairs granulosa cell function by inducing oxidative stress, disrupting steroid hormone synthesis, and impairing

mitochondrial function. These cellular alterations may contribute to PFOA-induced ovarian dysfunction and reduce female fertility. Supported by NIH R01 ES034112, T32 ES007326, and T32 HD108075

PS 3223 Differences in placental gene expression by maternal demoralization

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Background and Purpose: Poor maternal mental health has previously been associated with adverse childhood health - including neurodevelopment, metabolic, and immunological issues - through impaired fetal development. The placenta oversees fetal development through several biological pathways and is influenced by maternal health. Emerging evidence has shown that maternal stress can alter placental gene expression, but prior studies primarily focus on changes in expression of individual genes which can limit the mechanistic insight from these findings. Studying the co-expression of genes in the placenta can potentially highlight biological pathways that are implicated with poor maternal mental health. The objective of this study is to explore differences in placental gene networks related to maternal demoralization. **Methods:** Pregnant individuals were enrolled into the Siblings-Hermanos cohort in New York City, and during their prenatal visit they completed a demoralization questionnaire (PERI-D scale). Demoralization has been previously characterized by nonspecific psychological distress and disempowerment. A subset of the cohort had placental biopsies collected (n=89), and sixty-eight of those participants also had maternal demoralization data. RNA was extracted from placental biopsies, and RNA sequencing was conducted to generate transcriptome-wide gene expression levels. Using weighted gene co-expression network analysis (WGCNA), genes were grouped into distinct modules based on the clustering of their expression profiles. Multivariable regression analyses were performed to assess associations between maternal demoralization and module eigengene values. Additionally, we assessed correlations between gene module membership and gene significance with maternal demoralization scores. **Results:** We found that maternal demoralization was positively associated with membership in one of the twenty-two identified modules. The correlation of gene module membership and demoralization gene significance was moderately positive ($r=0.530$, $p=2.32 \times 10^{-15}$). The genes most strongly correlated with demoralization were involved in cellular structure, proliferation, energy production, immune signaling, and other homeostatic processes. **Conclusions:** Maternal demoralization during pregnancy could have implications on placental development, morphology, and signaling. Due to its role in coordinating fetal development through nutrient, hormonal, immune, and inflammatory pathways, the placenta is an important structure to understand how risk factors during pregnancy can lead to adverse outcomes in childhood. These findings provide insight into possible pathways through which maternal demoralization may affect placental physiology and potentially impact fetal development. Future, larger studies can further investigate the relationship between maternal mental health, placental molecular profiles, and associated postnatal outcomes.

PS 3224 Influence of *in utero* and/or lactational exposure to the antidepressant venlafaxine: late repercussions on reproductive and behavioral parameters in male Wistar rats.

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Background and Purpose: Clinical depression is commonly found in women of child-bearing age, especially during pregnancy and postpartum. Venlafaxine, classified as a Serotonin Norepinephrine Reuptake Inhibitor (SNRI), is one of the main options for the treatment of pre and postnatal depression. As serotonin plays an important role in central nervous system development, *in utero* and lactational exposure to SNRIs may affect hormone-dependent processes such as the hypothalamic sex differentiation process. Additional data comparing exposure to this drug at different critical periods of development are needed. The objective of this study was to evaluate the effects resulting from maternal exposure during pregnancy and/or lactation to the antidepressant venlafaxine on sexual development and possible late repercussions on reproductive and behavioral parameters in male rats. **Methods:** Female Wistar rats were treated daily, via gavage, with distilled water or venlafaxine (4.5 mg/kg/day) during pregnancy and lactation (CGL and VENGL) or only during lactation (CL and VENL). On the 5th day of lactation, the dams underwent maternal behavior assessment. Male offspring were evaluated at different ages (postnatal day - PND-21, 50 and 90) using the following parameters: evaluation of sexual development, play behavior, fertility testing, sexual behavior, quantification of Sexually Dimorphic Nucleus of the Preoptic Area (SDN-POA) volume, organ weight, sperm parameters, reactivity of vas deferens and hormonal dosages. **Results:** Females treated with venlafaxine during lactation had a lower weight gain compared to those treated during gestation and lactation. In the male offspring, exposure to venlafaxine in both periods increased anogenital distance at PND1, with an increase in offspring weight in VENGL. On PND 50, animals exposed during gestation and lactation had lower serum

testosterone concentrations. In adulthood, male offspring showed a decrease in liver weight (VENGL), an increase in testis and vas deferens weight (VENGL and VENL), a greater epididymal weight (VENGL) and a smaller SDN-POA volume (VENGL). In the reactivity of the vas deferens, the animals exposed to venlafaxine showed a lower potency of response to the stimulus, as well as a lower maximum response. **Conclusions:** Maternal exposure to venlafaxine during pregnancy and/or lactation altered male offspring development, affecting sexual differentiation, hormone levels, reproductive organ morphology and function. These findings suggest that early-life exposure to venlafaxine may disrupt hormone-dependent developmental processes with lasting reproductive effects.

PS 3225 Novel detection of legacy and emerging per- and polyfluoroalkyl substances (PFAS) in the follicular fluid of *in vitro* fertilization (IVF) patients from North Carolina

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Background and Purpose: North Carolina (NC) has per- and polyfluoroalkyl substances (PFAS) contamination, including the Cape Fear region, as shown by detection in drinking water and serum. However, little is known about PFAS accumulation in human follicular fluid (FF), a critical microenvironment for oocyte maturation. Prior work has shown that PFAS can traverse the blood-follicle barrier, yet no studies have evaluated FF PFAS levels in NC, a region with complex exposure mixtures. Given the key role of FF in oocyte maturation and ovarian hormone regulation, measuring PFAS in this compartment is necessary to better define potential reproductive risks among exposed individuals. The objective of this study was to quantify PFAS in the FF of a cohort of women undergoing *in vitro* fertilization (IVF) in NC. **Methods:** Using targeted PFAS analysis, we measured 23 analytes in the FF collected from 86 IVF patients. At oocyte retrieval, FF from multiple follicles was combined into a pooled sample for each patient, centrifuged at 500 x g for 15 minutes and supernatant stored at -80° until analysis. Patient demographics and basic clinical data were collected. Legacy compounds, alternatives, and emerging ether compounds were measured using UPLC-MS/MS. Detection frequencies and concentrations were evaluated across chemical classes, and cumulative PFAS load was compared between patients with and without an infertility diagnosis and between types of infertility diagnoses using a Kruskal-Wallis rank sum test. Additional correlation analyses were used to compare PFAS load and patient characteristics, as well as to assess associations between analyte detection. **Results:** PFAS were detected in all FF samples. Perfluoroalkyl sulfonic acids (PFSA), particularly PFHxS and PFOS, were measured at the highest concentrations (2.36 and 1.28 ng/mL). Legacy PFCA and PFSA were detected more frequently than emerging alternatives. Notably, hexafluoropropylene oxide dimer acid (GenX) was detected in 88% of samples at relatively low concentrations despite being infrequently reported in human serum. Across patients, 3-16 analytes were present within a concentration range of 0.5-12.8 ng/mL. Cumulative PFAS load did not differ significantly between patients, with and without an infertility diagnosis or between types of diagnoses, although substantial inter-individual variability was observed. Several of the highest detected PFAS analytes were significantly correlated with one another, including PFOS with PFOA, PFNA and PFDA and GenX with PFDS, PFHpS, PFDA and 4:2 FTS. **Conclusions:** This is the first study to characterize PFAS in FF in NC and demonstrates that both legacy and alternative PFAS, including PFDS, PFTrA, and GenX, can cross the blood-follicle barrier and accumulate in FF. These findings expand current understanding of PFAS exposures in reproductive microenvironments and highlight the presence of several compounds not previously reported in NC serum. The detection of multiple PFAS classes in this biologically sensitive compartment supports the need for further evaluation of ovarian PFAS transfer, potential implications for oocyte development, endocrine function, and fertility outcomes. Although we did not detect statistically differences by infertility diagnoses, our findings are important with increasing reports of various PFAS effects on ovarian and reproductive target sites.

PS 3226 Reproductive System Effects in Male C57Bl/6 Mice Exposed to Diesel Exhaust Particulate (DEP) and/or Desert Sand Dust (DSD)

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Background and Purpose: Warfighters on deployment are often exposed to airborne particulate matter (PM) such as diesel exhaust particulate (DEP) from engine emissions and/or desert sand dust (DSD) that is carried by the wind or kicked up by vehicles—often at levels exceeding exposure rates of the average population. These particulates can enter the lungs when breathed in by the service members, and over time these exposures have been linked to several detrimental health outcomes including respiratory, cardiovascular, and neurodegenerative diseases. Cascading effects beyond the lungs have been known to occur, however DEP and/or DSD effects on the reproductive system are scarcely researched. Therefore, this study investigates several elements of the reproductive systems of male C57Bl/6 mice exposed to DEP, DSD, or a combination of DEP+DSD. **Methods:** Over a 60-day study period, 7-8-week-old male and female C57Bl/6 mice were exposed twice a

week to either sterile saline (control) or a low, medium, or high dose of DEP, DSD, or DEP+DSD suspended in 0.9% sterile saline. The mice were anesthetized with 3-4% isoflurane, prior to exposure via nasal instillation. Mice were euthanized with Euthasol® followed by exsanguination 14-16 hours after their last exposure. Sperm analysis was performed on male mice immediately upon harvest of the epididymis. Sperm was prepared for analysis by slicing open the cauda in warmed M199 buffer and incubated for 5 minutes. The resulting sperm solution was pipetted onto a 2-chamber 100 µm slide and analyzed by the Hamilton ThorneIVOS® II system using the Animal Motility program. Endpoints collected included sperm count, motile cells, progressive cells, percent motile cells, percent progressive cells, and sperm per gram cauda. **Results:** Epididymides were frequently red and inflamed among the low combination exposure group, with occurrences happening less frequently among other groups such as the control, low DSD, medium combination, and high combination. Average sperm counts were significantly lower in the low combination exposure group compared to the medium combination and medium DSD exposure groups; while average motile cell counts and percentage were significantly lower in the low combination group compared to the medium DSD and control groups; and average progressive cell counts were significantly lower in the low combination group compared to the medium DEP and control groups. Other notable differences were found for progressive cell counts and percentage including significantly lower averages for the high DEP, low DSD, high DSD, low combination, and high combination exposure groups relative to the control group. No significant differences were found among any exposure groups for sperm per cauda weight. These findings may suggest interesting toxic effects exhibited by inflamed epididymides correlating with decreased sperm quality/production in the low combination exposure group, as well as potential compound-related effects attributed more to DSD and combination exposures than DEP exposures. **Conclusions:** Further investigation and analyses are currently ongoing; however, these data suggest the combined effect of DEP and DSD have some level of reproductive toxicity in male C57Bl/6 mice. This was evident in the percentage of progressive cells, where five of the ten dose groups had significantly lower average progressive cell percentages relative to the control group. Progressive cells are spermatozoa that achieve forward motion, which facilitates fertility by movement of the sperm towards female eggs during reproduction—without this contact, fertilization cannot occur. Also, several mouse epididymides presented as red and inflamed, most commonly in the low combination exposure group, which is also the group that tended to have the lowest averages for each sperm endpoint compared to the other exposure groups. This suggests some relationship between inflammation and sperm quality/production as a consequence of DEP/DSD reproductive toxicity. Studies testing the reproductive toxic effects of DEP and DSD are limited, and these results support the need for more research to determine if these observed effects can be attributed to compound-related toxicity. DISTRIBUTION A. Approved for public release: distribution unlimited.

PS 3227 Pituitary Hormone Alterations and Inflammatory Responses Associated with Chronic Phthalate Exposure in Female Reproductive Aging

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Background and Purpose: Exposure to phthalates, a group of ubiquitous endocrine-disrupting chemicals used in plastics and consumer products, has been associated with a decline in female reproductive health in both human and animal studies. Chronic exposure to phthalates disrupts hypothalamic-pituitary-gonadal signaling, but the mechanism behind this disruption is yet unknown. Focusing on the pituitary gland, we hypothesized that exposure to common phthalates, di(2-ethylhexyl) phthalate (DEHP) and diisononyl phthalate (DiNP), increases pituitary gonadotropins, aryl hydrocarbon receptor (AHR) signaling, and inflammatory markers in aging female mice. **Methods:** To investigate this, adult female CD-1 mice were orally dosed with corn oil or increasing concentrations of DEHP and DiNP for 9 months and 15 months. Pituitary tissues were collected immediately after dosing and analyzed by quantitative PCR (qPCR), cytokine array analysis, and immunohistochemistry. **Results:** Our results showed that chronic phthalate exposure over 9 months did not affect gonadotropin mRNA levels compared to controls. However, exposure to DEHP alone for 9 months showed a borderline increase in *Ahr* mRNA levels, while exposure to both DEHP and DiNP significantly elevated the mRNA expression of the AHR transcriptional target, *Cyp1a1*, compared to controls. Unlike the 9-month phthalate exposure, continuous phthalate dosing for 15 months increased the mRNA levels of gonadotropin and pituitary reproductive hormones compared to controls. DEHP exposure resulted in a borderline increase in *Fshb*, *Lhb*, *Cga*, *Esr1*, and *Gnrhr* mRNA levels, and DiNP exposure increased *Lhb*, *Cga*, *Esr1*, and *Pgr* mRNA levels, compared to controls. Immunohistochemistry revealed that after 9 months, exposure to DiNP alone reduced the number of LH- and FSH-positive cells; however, no changes were observed at 15 months following exposure to either DEHP or DiNP. Additionally, 9 and 15-month phthalate exposure did not alter the mRNA expression of proinflammatory cytokines—*Il1b*, *Il18*, *Tnfa*, but cytokine array analysis showed that the protein levels of other inflammatory markers associated with endothelial cell inflammation were altered. **Conclusions:** Together, these findings suggest that long-term phthalate exposure alters the expression of pituitary hormones and inflammatory markers, which could impact the process of reproductive aging. Supported by NIH R01 ES034112 and a Toxicology Scholar Fellowship from the University of Illinois Urbana-Champaign.

PS 3228 Analyzing the Effects of Charged Oxygen Particle Radiation on the Ovaries of Female Mice

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Background and Purpose: As human space exploration pushes beyond Earth's magnetosphere, astronauts face increased exposure to galactic cosmic radiation (GCR). These particles originate from explosions involving supernovae, black holes, and other cosmic events. GCR consists mostly of protons (~85%) and helium nuclei (~14%). Approximately 1% of GCR consists of high charge and energy (HZE) particles, which are unstable ions that travel at high speeds. HZE particles have high linear energy transfer (LET), which causes significant DNA damage. Our previous research has shown that exposure to iron or oxygen (O) radiation causes ovarian damage by reducing the ovarian reserve of oocytes, which are enclosed in ovarian follicles, just one week after exposure. While prior work effectively established that O ions can cause ovarian failure, the long-term histopathological effects of this exposure remain unknown. Understanding all aspects of the biological effects of GCR is increasingly vital, especially for female astronauts, who are underrepresented in research. We hypothesized that ovaries from mice exposed to 50 centigray (cGy) of O ions would have fewer ovarian follicles at all stages (primordial, primary, secondary, and antral), as well as higher tumor prevalence of smooth muscle and theca cell origin compared to controls exposed to 0 cGy of O ions. **Methods:** Female B6C3F1 mice were exposed to 0 or 50 cGy of O ions at three months of age. Ovaries were collected 14-15 months post-exposure, fixed in Bouin's fixative and washed, then stored in 70% ethanol until embedded in paraffin. Every fifth 5-micron paraffin section was stained with hematoxylin and eosin (H&E) for ovarian follicle counts. Follicles were categorized based on established morphological criteria. Corpora lutea and presence of oviductal ovulated eggs were also evaluated. Immunohistochemistry was conducted to identify tumor type using the markers actin, alpha 2, smooth muscle, aorta (α-SMA) for smooth muscle tumors, and cytochrome P450, family 17, subfamily a, polypeptide 1 (CYP17A1) for theca cell tumors. **Results:** Compared to controls, exposure to 50 cGy O ions caused significant and long-lasting ovarian damage, which was seen by an overall decrease of total healthy follicles ($p < 0.001$, Student's T-Test) and presence of tumors. Specifically, there was a significant difference between 0 and 50 cGy irradiated ovaries with primordial ($p = 0.016$, Mann-Whitney U Test), primary ($p = 0.008$), and secondary ($p = 0.008$) follicles. Antral follicles ($p = 0.31$), fresh corpora lutea ($p = 0.056$), and old corpora lutea ($p = 0.151$) did not differ significantly, which is consistent with age-related declines in the numbers and quality of these ovarian components in older mice. Although no smooth muscle tumors were observed, tumors of theca cell origin appeared in irradiated ovaries. **Conclusions:** This research contributes to safeguarding astronaut health during deep space missions and highlights the importance of addressing sex-specific risks as human space exploration expands. Understanding the biological impacts of space radiation on female reproductive health is essential, not just for astronaut safety, but also for the development of effective shielding technologies and medical countermeasures to enable safe human exploration of Mars and beyond.

PS 3228a Diisononyl phthalate exposure increases Cyp1a1 transcript localization in the antral follicle

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Background and Purpose: Phthalates are chemicals commonly used as plasticizers and known endocrine disrupting chemicals (EDCs). Humans are exposed to di(2-ethylhexyl) phthalate (DEHP) and its replacement diisononyl phthalate (DiNP) daily. Exposure to these chemicals over a person's lifetime is a concern because phthalate exposure can accelerate reproductive aging, causing early menopause. Recently, we have shown that phthalates alter aryl hydrocarbon receptor (AHR) signaling in ovarian cells *in vitro*. However, it is not known how phthalates affect AHR signaling in the ovary and throughout reproductive aging *in vivo*. The AHR is a transcription factor that plays a role in ovarian follicle development, function, and xenobiotic metabolism. Once a ligand is bound, the AHR can promote transcription of its downstream targets such as *Cyp1a1* and *Cyp1b1*. We hypothesized that DEHP and DiNP exposure alter the expression of genes in the AHR signaling pathway in the ovary throughout reproductive aging. **Methods:** We treated adult female CD-1 mice with varying doses (0 ppm, 0.15 ppm, 1.5 ppm, or 1500 ppm) of DEHP and DiNP in the diet for 1 and 6 months. The 0.15 ppm and 1.5 ppm doses correspond to human daily exposure and occupational exposure, respectively. We also included a high dose (1500 ppm) to examine dose-response effects. We collected ovaries at each time point and then examined expression of *Ahr*, *Cyp1a1*, and *Cyp1b1* using quantitative polymerase chain reaction (qPCR) for the whole ovary and localization of these transcripts in the antral follicles using RNAscope. **Results:** Since how transcripts in the AHR pathway change throughout reproductive aging has not been characterized, we first determined the expression and localization of transcripts for *Ahr*, *Cyp1a1*, and *Cyp1b1* by comparing the control samples collected at 1 and 6 months. At 6 months, we observed decreased expression in *Cyp1b1* in the whole ovary compared with 1 month samples, but did not observe changes in expression of *Ahr* or *Cyp1a1* over time. However, at 6 months, we observed an increase in *Cyp1a1* transcript, but not *Cyp1b1*, localized in the antral follicle compared to the 1-month time-point. Further, DEHP and DiNP did not alter expression of *Ahr* in the whole ovary at either

time point. DEHP (1500 ppm) increased expression of *Cyp1a1* only after 6 months of exposure compared to control in the whole ovary. DiNP did not alter *Cyp1a1* expression in the whole ovary. Conversely, DEHP did not alter *Cyp1a1* localization in the antral follicle at either time point. However, DiNP (1.5 ppm and 1500 ppm) increased the amount of *Cyp1a1* transcript localized in the antral follicle compared to controls after 6 months of exposure. We did not observe changes in transcript in the whole ovary or localized in the antral follicle for *Cyp1b1* after phthalate exposure at either time point. **Conclusions:** Overall, changes observed in the whole ovary do not reflect the changes observed in the antral follicle over time or with phthalate exposure. Within the antral follicle, DiNP exposure appeared to promote the greatest amount of changes in the *Cyp1a1* transcript in the antral follicle. As *Cyp1a1* plays a role in estrogen metabolism, the observed changes in *Cyp1a1* transcript may have greater implications regarding how DiNP affects estrogen metabolism in the antral follicle. Supported by NIH T32 ES007326, NIH R01 ES034112, the Wanda Haschek-Hock Travel Award, and the Department of Comparative Biosciences Travel Award.

PS 3229 Investigating the Involvement of Pyroptosis in Cutaneous Nitrogen Mustard Induced Toxicity

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Background and Purpose: Sulfur mustard (SM) is a vesicating agent used in chemical warfare. It most readily affects several organs including the exposed skin causing blistering, erythema, edema, and inflammatory consequences. It remains a potential chemical threat today, yet its complete effects on cellular toxicity mechanisms are poorly understood. Its laboratory safe alternative nitrogen mustard (NM) has been shown to induce apoptosis, ferroptosis, and necrosis, but other mechanisms like pyroptosis, a pro-inflammatory programmed cell death process, have not been investigated. The canonical pyroptosis mechanism is a gasdermin-D mediated pathway triggered by the activation of the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome. NLRP3 activation can occur via diverse stimuli including reactive oxygen species (ROS) generation which leads to activation of caspase-1 (casp-1) and activation of gasdermin-D N-terminal which forms membrane pores. Casp-1 also activates pro-inflammatory mediators interleukin-18 (IL-18) and interleukin-1 β (IL-1 β) which are subsequently released through the gasdermin-D pores along with other cellular contents. NM toxicity produces a pro-inflammatory response, oxidative stress, innate immune response, and long-term inflammatory lesions like psoriasis. Since pyroptosis is involved in such responses and may be a cell death pathway involved in NM toxicity, the purpose of this study was to identify if pyroptosis is a cell death mechanism induced by cutaneous NM exposure. **Methods:** To study pyroptosis following cutaneous NM exposure we used an *in vivo* model composed of male C57BL/6 mice (n=5), 7-9 weeks of age. They were exposed to 1 mg of NM or acetone vehicle via topical application to shaved dorsal skin, and groups were euthanized at various time points post-exposure (1, 3, and 8 days) with subsequent skin tissue collection and RNA extraction. We also used an *in vitro* model composed of the skin epidermal human HaCaT cells. Cells were exposed to 50 μ M NM for 2 hours before replacement with fresh media and incubation for an additional 22 hours. Following incubation, cells were collected and RNA was extracted. The skin tissue and HaCaT cells were analyzed for increased expression of pyroptotic genes (NLRP3, Casp-1, and IL-18) via qPCR. **Results:** In the *in vivo* model, our results showed a significant increase in relative fold gene expression of NLRP3, Casp-1, and IL-18 at 1 day and trends at 8 days post NM exposure. Data collection and analysis of these genes and mechanistic studies are underway in our *in vitro* model to confirm pyroptotic cell death following NM exposure. **Conclusions:** Increased expression of pyroptosis related genes 1 day post-exposure indicates that pyroptosis plays a role in acute NM induced skin injury. On going studies aim to further study and confirm the presence of pyroptosis via further molecular and protein analysis at other NM exposures and time points. We will further investigate pyroptosis as a potential target for therapeutic development to counter acute NM induced toxicity that can lead to long-term inflammatory consequences.

PS 3230 NOD1/UPR Signaling Axis in the Pathogenesis of Acute Kidney Injury Following Inhalation of Arsenic Gas Exposure

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Background and Purpose: Arsenic (military designation SA) gas is the extremely toxic from of inorganic arsenic. During World War II, arsenic was developed as a chemical weapon for its possible use in the battlefield. Fortunately, no evidence exists of its actual use in the combat zone. However, accidental occupational exposure of arsenic is known among industrial workers. The ease with which such a highly toxic gas is prepared may also attract terrorists for its intentional use in small-scale public meetings or concerts. Inhalation of arsenic causes severe red blood cell (RBC) destruction, leading to the release of hemoglobin and heme, which then accumulate in renal tubules and contribute to acute kidney injury (AKI). However, the exact underlying molecular mechanisms of its renal toxicity are poorly understood. Absence of an appropriate animal model that can recapitulate the exact pathogenesis of

arsenic gas is an important gap that needs to be addressed. In this study, we establish and validate a mouse model that manifests arsenic-induced hemolysis-mediated AKI. In addition, we also developed a surrogate model of hemolysis-induced AKI. This model demonstrates similarities with arsenic-exposed murine model and can be used for the screening of potential drug candidates for abrogating arsenic toxicity. **Methods:** A dose-dependent study of arsenic gas (10-25 ppm for 1 hour) was conducted using 10-week-old C57BL/6 mice under nose-only exposure conditions at MRIGlobal (Kansas City). In a parallel, another group of mice received phenylhydrazine (PHZ; 1.5 mg/10 g, intraperitoneally), a well-established hemolytic agent. Mice were euthanized at 24 and 72 h post-exposure and blood, serum, and kidney samples were collected to evaluate hematology and AKI biomarkers of the kidneys. Furthermore, utilizing human proximal tubular kidney cells (HK-2), we investigated a novel molecular signaling axis underlying arsenic gas-induced hemolysis and its role in AKI. **Results:** Inhalation of arsenic gas significantly induces intravascular hemolysis as observed by dark red plasma and bloody urine in our mouse model. These observational changes were associated with a significant decline in RBC count, hemoglobin and hematocrit levels with concomitant increase in urinary heme content at 24 h. These changes accompanied marked increases in biomarkers of AKI, namely NGAL and KIM-1 in both urine and kidneys following arsenic exposure. A significant increase in levels of arsenic in blood and kidney tissue were also prominent. Histopathological analysis revealed tubule injury identified by brush border loss, cytoplasmic vacuolation and appearance of intracytoplasmic iron deposition. Parallel to arsenic-associated AKI, our PHZ treated surrogate model also exhibited similar pathotoxicological responses. In this PHZ model, kidney injury was characterized by hemolysis-induced upregulation of KIM-1 and NGAL in the urine and kidney, with reduced glomerular filtration rate (GFR), a functional marker for renal damage. We found that hemolysis-induced AKI in both models was linked with the activation of nucleotide-binding oligomerization domain 1 (NOD1) and unfolded protein response (UPR) signaling pathway. Western blot analysis demonstrated a significant upregulation of NOD1/2, pRIPK2 and UPR regulated PERK/ATF4/CHOP proteins in the kidney of both arsenic and PHZ exposed mice. In addition, there was a substantial increase in levels of serum and kidney cytokines. Utilizing human proximal tubular kidney cells (HK-2) *in vitro*, we tested the protective effects of ML130 and GSK2656157, a specific inhibitor of NOD1 and PERK, respectively. We found that blocking NOD1 or PERK significantly attenuated expression of inflammatory cytokine (IL-6, IL-8) and cell death markers (cleaved caspase-3 and DR4) along with reduced expression of hemin-induced KIM-1, NOD1 and PERK signaling proteins. **Conclusions:** Our findings reveal that NOD1/UPR signaling pathway axis underpins hemolysis-induced AKI and may serve as a potential therapeutic target for treating arsenic toxicity and perhaps other hemolytic diseases.

PS 3231 Phenylarsine Oxide Disrupts Mitochondrial Function and Redox Signaling in Human Keratinocytes

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Background and Purpose: Phenylarsine oxide (PAO), a structural analog of the chemical warfare agent Lewisite, is a highly toxic organoarsenic compound and a potential threat to both civilian and military populations. Human skin is particularly vulnerable to PAO. Depending on the dose and exposure duration, it can cause inflammation, edema, and blistering. As a trivalent arsenical, PAO exerts its toxicity primarily through covalent binding to sulfhydryl groups on cysteine residues, thereby disrupting protein structure and function and promoting oxidative stress. These molecular events can alter cellular redox homeostasis, impair metabolic activity, and trigger programmed cell death. In this study, we analyzed the effects of PAO on mitochondrial function and redox signaling in human keratinocytes. **Methods:** Human keratinocytes (HaCaT cells) were maintained in DMEM supplemented with 10% fetal bovine serum. Cells were treated with 0.1-0.4 μ M PAO or vehicle control in serum-free DMEM prepared immediately before use. Protein expression was assessed by western blotting and mitochondrial respiration and glycolysis using an Agilent Seahorse. Production of reactive oxygen species was measured by flow cytometry using dichloro-dihydro-fluorescein diacetate (DCFH-DA). HaCaT bioenergetics, Nrf2 signaling, and ROS production were analyzed up to 4 hr post PAO treatment. Protein expression was assessed 24 hr post PAO treatment. **Results:** PAO treatment inhibited mitochondrial oxidative phosphorylation in HaCaT cells in a concentration- and time-dependent manner, reducing basal, ATP-linked, and maximal respiration; glycolytic activity was also impaired as reflected by reduced basal glycolysis, glycolytic capacity, and glycolytic reserve, indicating that PAO disrupts overall cellular bioenergetics. Western blot analysis revealed that PAO downregulated SIRT1 and SIRT3, key regulators of mitochondrial function and antioxidant defense. Expression of the antioxidants SOD2, catalase, and GPx4 was also reduced by PAO. This correlated with a concentration-dependent increase in intracellular ROS, confirming oxidative stress. PAO also promoted nuclear localization of Nrf2 and upregulated its downstream target HO-1 in a concentration- and time-dependent manner, indicating activation of the Nrf2-mediated antioxidant response. Additionally, PAO increased LC3-II and p62 levels, suggesting induction of autophagy in response to sustained oxidative and metabolic stress. **Conclusions:** Together, these results indicate that

PAO disrupts keratinocyte bioenergetics by impairing both mitochondrial respiration and glycolysis, while simultaneously promoting oxidative stress and activating adaptive antioxidant and autophagic responses. This coordinated dysregulation of metabolic and redox pathways likely underlies the cytotoxic effects of PAO in human skin cells and highlights potential targets for mitigating its toxic impact. This work was supported by National Institute of Environmental Health Sciences (grant/award numbers: P30ES05022 and National Institute of Arthritis and Musculoskeletal and Skin Diseases (grant/award number: U54AR055073).

PS 3232 The Effect of Chemical Threat Agents on Corneal Nerve Injury and Related Biomarkers

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Background and Purpose: Chloropicrin (CP) is a potent chemical threat agent (CTA) resulting in severe ocular injury. The eye is the most sensitive organ to CP-induced injury, especially the cornea, due to its tear film acting as a reservoir and anterior location. Exposures can lead to photophobia, dry eye, corneal ulcerations, scarring, and blindness. In recent years, CP has been deployed in warfare in Russia and used as a fumigant for agriculture posing as an additional risk for injury. However, there are still no effective treatments, only symptomatic management. Phosgene Oxime (dichloroform oxime; CX), is another highly toxic vesicant/irritant CTA and penetrates tissues faster than other CTAs with high mortality. We have recently published that CP and CX ocular exposures lead to inflammation and cell death in the cornea. However, very little is known regarding CP and CX toxicity on corneal nerve tissue. Ocular nerves, especially in the cornea, are vital to maintain tissue homeostasis, regeneration, and restore sight. They release key mediators that interplay with immune cells to provide a healthy ocular microenvironment. In this study, we aim to investigate the corneal nerve morphology, function, and related biomarkers of nerve injury post CP and/or CX ocular exposure at time-points vital for injury and regeneration. This in turn will provide further insight into the underlying mechanisms of CP and CX-induced ocular injury and potential targets to develop treatments. **Methods:** CX-exposure was induced in male and female WT (C57BL/6) mice (5-9 weeks) by placing 10 μ l (liquid) for 15 seconds (short duration) and 30 seconds (long duration) using a vapor cap placed on the eye with filter paper. CP-exposure was carried out in male BALB/c mice (5-9 weeks) by placing 2 μ l (liquid) in DMSO at either 2.5% (low dose) or 10% (high dose) for 1 minute. For controls, sham and DMSO were used, respectively. Clinical assessment for corneal sensitivity using a Cochet Bonnet Aesthesiometer (centimeters, cm) was performed. Eyes were collected for corneal flatmount or paraffin-embedded sectioning, staining (B3 Tubulin), and analysis for nerve density (mean fluorescent intensity, MFI), substance P (SP) MFI, nerve length (um), and number of nerve branches. The mRNA was extracted from control and CP-exposed eyes followed by cDNA synthesis and quantitative PCR to determine nerve injury related gene expression (*Tubb3*, *Sp*, *Cgrpr*, *Ngf*). The delta-delta CT method was used to calculate relative gene expression fold change compared to the control housekeeping gene. **Results:** The results demonstrate significantly reduced nerve density at 30 seconds post CX-exposure compared to controls at 24 hr. At day 1 post CP-exposure, there was observed greater expression of substance P (SP) for nerve function and more co-localization with varicosities for 2.5% CP compared to controls and 10% CP. At day 7 post CP-exposure, significantly greater nerve branching and length was observed for 2.5% CP compared to 10% CP, indicating that 10% CP induces greater nerve damage over time. Additionally, corneal sensitivity was significantly reduced for both CP doses, yet 2.5% CP showed recovery by 7 days compared to 10% CP. Currently, the gene expressions of nerve injury related biomarkers are being analyzed. **Conclusions:** These findings strongly suggest CTA exposure, CP and CX, induce corneal nerve damage and higher doses reduce the ability for nerve regeneration and function. These effects may explain the prolonged ocular tissue damage seen post CP and CX-exposure. Additionally, the nervous system and its related nerve injury biomarkers are a promising mechanism to identify therapeutic targets to treat CTAs.

PS 3233 Colony stimulating factor 1 receptor (CSF1R) inhibition mitigates the development of spontaneous recurrent seizures and alleviates neuropathology in adult female rats acutely intoxicated with diisopropylfluorophosphate (DFP)

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Background and Purpose: Acute intoxication with organophosphate (OP) cholinesterase inhibitors can trigger a cholinergic toxidrome that includes seizures that rapidly progress to life-threatening *status epilepticus*. Individuals who survive the acute cholinergic crisis are susceptible to long-term adverse neurological effects, including spontaneous recurrent seizures (SRS) even when standard of care treatment for acute OP poisoning is administered within an optimal therapeutic window. Neuroinflammation is widely posited to contribute to SRS associated with acute OP intoxication; however, attempts to modify neuroinflammatory responses following OP-induced SE have yielded equivocal therapeutic results. However, it is not clear that the inflammatory status of the innate immune cells in the brain changes with

time post-exposure, suggesting that the timing of anti-inflammatory agents is critical. **Methods:** We assessed whether pharmacological deletion of microglia using the CSF1R inhibitor PLX5622 (PLX) administered at varying times post-exposure modified SRS development in a rat model of acute intoxication with the OP diisopropylfluorophosphate (DFP). Adult female Sprague Dawley rats were instrumented for cortical electroencephalography (EEG) and allowed 10 d recovery. Animals were administered DFP (4 mg/kg, s.c.) followed by atropine sulfate (2 mg/kg, im) and 2-PAM (25 mg/kg, im) given 1 min post-DFP, and midazolam (0.65 mg/kg, im) administered at 40 and 50 min post-DFP. Seizure behavior during the first 4 h post-DFP was scored using a modified Racine scale and video-EEG recordings. Animals that exhibited robust SE were randomly assigned to receive vehicle (VEH, 5% DMSO in 20% Kolliphor EL/80% 0.9% saline) or PLX (50 mg/kg, ip, twice daily) from 1-3 or 4-6 days post-exposure (DPE). Animals were euthanized at 28 DPE to collect brain tissues for quantitative immunohistochemical (qIHC) analysis. The amygdala, somatosensory and piriform cortices, hippocampus, and thalamus were immunostained for (1) IBA1 and iNOS or arginase-1, biomarkers of pro- and anti-inflammatory microglia, respectively; (2) GFAP with C3 and S100A10, biomarkers for pro- and anti-inflammatory astrocytes, respectively; and (3) NeuN, a biomarker of mature neurons. **Results:** PLX administration from 1-3 DPE, but not 4-6 DPE, prevented the development of SRS over 28 DPE. Animals that received PLX5622 from 4-6 DPE developed SRS at the same rate with comparable average seizure duration as VEH controls. qIHC indicated that PLX treatment from 1-3 DPE or 4-6 DPE decreased iNOS+ microglia specifically in the piriform cortex ($p < 0.05$), but PLX treatment from 4-6 DPE increased the density of arginase-1+ microglia ($p < 0.05$). PLX did not influence overall astrocyte density; however, DFP animals treated with PLX from 4-6 DPE trended towards a higher density of S100A10+ astrocytes compared to DFP animals treated with PLX from 1-3 DPE. Lastly, treatment with PLX 1-3 DPE increased neuronal cell density at 28 DPE in multiple brain regions when compared to VEH controls or DFP animals treated with PLX from 4-6 DPE. **Conclusions:** Collectively, our results revealed that CSF1R inhibition at specific times following acute OP intoxication may modify epileptogenesis. While PLX treatment doesn't significantly alter the overall density of microglia and astrocytes at 28 DPE, it altered microglial and astrocytic inflammatory responses and was neuroprotective. In conclusion, our data suggest that microglia depletion is a potential therapeutic strategy for mitigating SRS following acute OP-induced SE but that timing of administration is a critical determinant of therapeutic efficacy. This work is supported by the NIH CounterACT Program (U54 NS127758).

PS 3234 Mast Cells Shape Early Pulmonary Inflammation and Regulate the Abundance and Localization of Dendritic Cells after Chloropicrin Exposure

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Background and Purpose: Chemical threat agents remain a significant pulmonary hazard, yet the mechanisms by which they trigger inflammation and modulate immune networks are poorly defined. Chloropicrin (CP; trichloronitromethane) is a highly toxic pulmonary irritant that disrupts the alveolar-capillary barrier, promotes neutrophilic infiltration, and may lead to long-term lung remodeling. Mast cells, strategically positioned near airway epithelium and vasculature, rapidly release cytokines, proteases, and bioactive lipids upon injury, influencing both innate and adaptive immunity. We hypothesized that mast cells are central mediators of CP-induced lung injury, orchestrating neutrophil recruitment, dendritic cell (DC) dynamics, macrophage polarization, and oxylipin metabolism leading to an exacerbation of injury when present. **Methods:** Wild-type C57BL/6J, c-Kit-dependent mast cell-deficient $\text{Kit}^{\text{W-sh}}$, and c-Kit-independent mast cell-deficient $\text{Cpa3-Cre}; \text{Mcl1}^{\text{fl/fl}}$ mice were exposed via oropharyngeal aspiration to 0.1-20 mg/kg CP. Lung injury was assessed via histology (H&E), bronchoalveolar lavage (BAL) cellular differentials, immunohistochemistry, and high-parameter flow cytometry to quantify T cells, neutrophils, cDC1/cDC2 subsets, alveolar (AM) and interstitial macrophages (IM), and cKit+ cells. Immune cell localization relative to the airway epithelium was measured. Semi-targeted UHPLC-MS lipidomics quantified oxylipins in BAL fluid. Statistical analyses employed two-way ANOVA with post hoc testing. **Results:** Wild-type mice displayed pronounced alveolar injury, edema, and inflammatory cell infiltration, whereas $\text{Kit}^{\text{W-sh}}$ mice exhibited preserved alveolar architecture and reduced neutrophilic influx. BAL analysis revealed elevated total cell counts in both strains post-CP exposure, but significantly lower neutrophil percentages in $\text{Kit}^{\text{W-sh}}$ mice (1 mg/kg: 31% vs 15%; 20 mg/kg: 61% vs 46%). cDC1 and cDC2 densities decreased in C57BL/6 lungs (12.7→2.3 and 7.7→4.3 cells/mm²) but increased in $\text{Kit}^{\text{W-sh}}$ mice (4.3→23.7 and 2.0→92.7 cells/mm²). Following CP exposure, cDC1 cells moved closer to the lung epithelium in wild-type mice (~61%) while cDC2 moved closer to the epithelium in $\text{Kit}^{\text{W-sh}}$ (~35%), highlighting MC-dependent spatial redistribution. $\text{Kit}^{\text{W-sh}}$ mice displayed attenuated T cell responses, increased cDC1/cDC2 populations, and preserved Ly6C+ monocytes compared to wild-type mice, indicating a shift toward tissue repair and diminished acute inflammation. Lipidomic profiling revealed near-absent α -linolenic acid and suppressed linolenic acid pathway metabolites (GLA, DHLA, AA) in $\text{Kit}^{\text{W-sh}}$ mice, with diminished EPA/DHA and leukotriene B4/E4 production. Pro-resolving mediators, including 15-keto-PGE₂ and (S)-H₂PE₂, were significantly elevated in wild-type BALF but blunted in mast cell-deficient mice, demonstrating that MCs coordinate both pro-inflammatory and resolution-phase lipid mediator responses. Validation in c-Kit-independent $\text{CPA3-Cre}; \text{Mcl1}^{\text{fl/fl}}$ mice recapitulated these findings, confirming MC-specific effects. **Conclusions:** Mast cells are central regulators of CP-induced

pulmonary injury, promoting neutrophil and T cell recruitment, exacerbating epithelial damage, and suppressing reparative dendritic cell and Ly6C⁺ monocyte/macrophage populations. They coordinate oxylipin metabolism, integrating pro-inflammatory and pro-resolving lipid signaling, which is critical for initiating and resolving lung inflammation. These findings identify mast cells as key drivers of acute chemical-induced lung injury and highlight their potential as therapeutic targets for mitigating inflammation and preventing long-term remodeling.

PS 3235 Comparison of Established and Experimental Skin Decontaminants Against the Nerve Agent VX in Rat Skin and a Three-Dimensional Bioartificial Human Skin Model

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Background and Purpose: The Chemical Weapons Convention prohibits the development, production, stockpiling, and use of chemical weapons. Nevertheless, nerve agent exposure remains a persistent threat. Given the limited efficacy of currently approved antidotes, effective skin decontamination following percutaneous exposure is critical to prevent systemic uptake. In this study, rat skin (shaved, without connective and adipose tissue) and a full-thickness, three-dimensional bioartificial human skin model were used to evaluate the efficacy of various decontamination protocols on the dermal penetration and diffusion of the nerve agent VX using Franz diffusion cells. **Methods:** Decontamination was initiated 5 or 30 minutes after exposure to VX (10% v/v; 10 µL applied to 1.5 cm²) at 35°C. Samples were collected every 30 minutes for 5 hours (mean, n=5). The decontamination efficacy of Reactive Skin Decontamination Lotion (RSDL), potassium salt of acetohydroxamic acid (AHAK; 880 mg/mL, 250 µL), sodium hypochlorite (NaOCl; 2%, 250 µL), and tap water (250 µL) was assessed. RSDL was applied using fully soaked sponge disks (0.8 cm diameter) with 10 circular motions in alternating directions, left for 2 minutes, and then removed with gauze. NaOCl and water were applied for 2 minutes and similarly removed. AHAK was applied for the full experimental duration (5 hours) according to a previously published protocol (Bloch-Shilderman et al., *Arch. Toxicol.* 2023, 97:2771-2783) to neutralize agent within the dermal depot. VX penetration into the acceptor chamber was quantified using a modified Ellman enzymatic assay, and results were expressed as percent reduction of inhibitory activity relative to untreated controls. **Results:** RSDL, an FDA-approved skin decontaminant, achieved substantial efficacy in rat skin (99.7% after 5 min; 95.0% after 30 min) and comparable results in the human model (96.2% and 85.5%, respectively). NaOCl (2%) reduced VX penetration by 94.0% and 79.0% in rat skin and by 92.5% and 81.0% in human skin at 5 and 30 minutes, respectively, without causing visible tissue damage. In contrast, 10% NaOCl caused macroscopic damage to the human model. Tap water showed moderate efficacy after 5 minutes (84.8% in rat; 91.2% in human skin) but poor performance at 30 minutes (0.8% and 49.6%). AHAK achieved excellent decontamination in both models (rat: 99.5% and 97.9%; human: 99.9% and 99.6% at 5 and 30 min, respectively). Moreover, prophylactic AHAK application, whether removed or left in place before VX exposure, provided near-complete protection (99.9%) in the human skin model. **Conclusions:** In summary, both models demonstrated consistent trends in VX penetration and decontamination efficacy. Hydrophilic agents (water, NaOCl 2%) were less effective, particularly when decontamination was delayed. RSDL and the experimental compound AHAK showed superior performance, with AHAK exhibiting comparable efficacy to RSDL. These findings highlight AHAK as a promising, cost-effective candidate for both post-exposure skin decontamination and prophylactic protection against VX.

PS 3236 Evaluation of novel oximes as antidotes for an organophosphate requiring bioactivation in rats.

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Background and Purpose: Organophosphates (OP) are a group of synthetic chemicals that include nerve agents and some insecticides. The mechanism of toxic action for OPs is the inhibition of the enzyme, acetylcholinesterase (AChE). Many of the insecticidal OPs must be bioactivated, via cytochrome P450s (CYPs), to an active metabolite(s) to inhibit AChE. One such OP is phorate, an insecticide that can be bioactivated to multiple anticholinesterase metabolites. Phorate is very toxic with acute exposures in rats resulting in a delay in toxic signs compared to OPs of similar structure. Once initiated, the progression of toxic signs is very rapid. Standard of care (SOC) for OP intoxication includes atropine (antimuscarinic) and an oxime to reactivate OP inhibited AChE. The currently approved oxime (pralidoxime, 2-PAM) can reactivate OP inhibited AChE but has very limited ability to cross the blood-brain barrier (BBB) and reactivate OP inhibited brain AChE. Our laboratory has synthesized and tested a series of novel pyridinium oximes (US Patent 9,227,937) that have previously been shown to cross the BBB, reactivate OP inhibited brain AChE and enhance survival following lethal exposures to OPs. These oximes have longer half-lives (several hours) than 2-PAM (about an hour) and can reactivate OP-inhibited plasma butyrylcholinesterase (BChE), converting it to a pseudo-catalytic OP scavenger. In

this project, we evaluated some of our oximes to provide greater survival efficacy than 2-PAM to lethal doses of phorate. **Methods:** Phorate and its major metabolites were tested *in vitro* for potency as inhibitors of rat brain AChE using a spectrophotometric assay. Bioactivation of phorate to its anticholinesterase metabolites was measured spectrophotometrically *in vitro* using both rat and human hepatic microsomes. A lethal dose (LD₅₀) *in vivo* phorate rat model was established to determine the therapeutic window for evaluating oxime efficacy. Male and female rats were challenged with phorate (LD₅₀) in a biocompatible solvent (multisol) followed by administration of novel oxime (IM) 146 µmol/kg (human 2-PAM molar equivalent dosage, HED) or 2-PAM. Atropine [0.65 mg/kg atropine (HED) in saline IM] and novel oxime in a biocompatible vehicle, 90% water and 10% benzyl alcohol were administered at the time of initiation of seizure-like behavior for phorate challenged rats. Brain and blood were collected at the time of death or in 24-hour survivors and brain AChE and serum BChE activities were determined spectrophotometrically. To evaluate the role of BChE, rats were administered Iso-OMPA, a specific BChE inhibitor, 1 mg/kg IM one hour prior to phorate challenge and compared to rats not administered Iso-OMPA. **Results:** *In vitro* potency (AChE IC₅₀s) of phorate-oxon metabolites was much greater (nanomolar) than the parent compound (molar). Phorate incubation with hepatic microsomes produced anticholinesterase activity that was NADPH-dependent (CYP bioactivation). An LD₅₀ challenge of phorate (12 mg/kg) in both male and female atropinized rats resulted in death occurring at about 3 hours for female rats and about 4.5 hours for male rats. Novel oximes were selected for efficacy (24-hour survival) testing based on *in vitro* AChE reactivation and *in vivo* pharmacokinetics (longer half-life in the blood, compared to 2-PAM). Oxime efficacy studies following a lethal phorate challenge resulted in greater than 70% survival compared to about 60% for 2-PAM. Limited results from the Iso-OMPA pre-treated rats indicate that survival is decreased in rats with non-functional BChE. **Conclusions:** Phorate exposure results in a delay in the cholinergic toxidrome, compared to many other OPs, making it a challenging compound to develop countermeasures against it. We have observed promising *in vivo* therapeutic efficacy of our novel oximes against phorate toxicity when administered at the signs of severe poisoning. Survival efficacy in the *in vivo* experiments seem to be enhanced with the novel oxime's longer half-life. The ability of the novel oximes to reactivate OP-inhibited BChE plays a significant role in increased survival following phorate challenge, as evidenced by improved survival in rats exposed to phorate with functional BChE. These data are important in the continued development of these novel oximes as a treatment for OP poisoning. Supported by NIH R21NS131159-01 and U01 NS123255-03.

PS 3237 Soluble epoxide hydrolase as a therapeutic target for mitigating the adverse neurological consequences of acute organophosphate intoxication

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Background and Purpose: Acute intoxication with OP cholinesterase inhibitors, which include pesticides and nerve agents, can cause a cholinergic crisis that rapidly progresses to status epilepticus. Survivors can develop long-term neurological consequences, including persistent brain injury, epilepsy and cognitive impairment. Current standard of care (atropine, oxime and a benzodiazepine) increases survival, but does not effectively protect against chronic adverse neurological outcomes, highlighting the urgent need for more effective therapeutic strategies. We are investigating the pharmacological inhibition of sEH, which catalyzes the conversion of the anti-inflammatory lipid epoxyeicosatrienoic acid (EET) to pro-inflammatory lipid derivatives. Using a rat model of acute intoxication with the OP diisopropylfluorophosphate (DFP), we previously showed that EET is reduced in the brain of male rats during the first 3 d post-DFP, coincident with increased levels of pro-inflammatory lipids. Here, we tested whether administration of the sEH inhibitor 1-(1-propionylpiperidin-4-yl)-3-(4-trifluoromethoxy) phenyl) urea (TPPU) improved neurological outcomes. **Methods:** Adult female Sprague Dawley rats were randomly divided into groups for immunohistochemical (IHC) or electroencephalographic (EEG) analyses and within these, randomly assigned to vehicle (VEH) or TPPU (DFP+VEH vs. DFP+TPPU) groups. For EEG analyses, animals were implanted with telemetry devices 10 d prior to experimentation. In all experiments, animals were intoxicated with DFP (4 mg/kg, sc) followed 1 min later with atropine sulfate (2 mg/kg, im) and 2-pralidoxime (25 mg/kg, im) then two sequential doses of midazolam (0.65 mg/kg, im) at 40 and 50 min post-DFP. TPPU (1 mg/kg, ip) or an equal volume of VEH (20% PEG400, ip) were given once daily from 1 - 7 d post-exposure (DPE). Animals implanted with EEG telemeters were continuously monitored up to 36 DPE to quantify seizure frequency, and underwent behavioral tests (open field, fear conditioning) starting 1 mo post-DFP. Non-surgerized animals were euthanized at 3, 7 and 21 DPE to collect brain tissue for IHC analyses. Tissue sections were immunostained for GFAP, an astrocytic biomarker, or stained with Fluoro-Jade-C to visualize degenerating neurons. IHC data were analyzed using two-way ANOVA and mixed effects modeling; seizure data, repeated measures ANOVA; behavior data, unpaired t-test and two-way ANOVA. **Results:** DFP triggered acute status epilepticus as determined electrographically or behaviorally. There were no significant group differences in the % area of GFAP immunoreactivity in the dentate gyrus or piriform cortex nor in number of FJC-immunopositive cells in the somatosensory and piriform cortices, amygdala, thalamus and hippocampus at any timepoint (p < 0.05; n=6-10 animals/group). No significant group differences were detected in the number of seizures

up to 3 weeks post-DFF, the velocity or distance moved in the open field test, or the average motion index in the fear conditioning task ($p < 0.05$; $n=8-9$ animals/group). **Conclusions:** These data do not support sEH as a therapeutic target in female rats acutely intoxicated with DFP. However, whether this is because the lipid profile after OP intoxication differs in the female vs. male brain or whether a different therapeutic window or dose of TPPU would be efficacious remains to be determined. Supported by the NIH CounterACT program (U54 NS127758). MM was supported by the Northern California Achievement Rewards for College Scientists (ARCS); BD by a predoctoral fellowship (T32GM136559).

PS 3238 Exploration of Mechanisms Driving Nitrogen Mustard-Induced Injury in Mouse Skin

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Background and Purpose: Sulfur mustard (SM) is the most widely used chemical warfare agent and belongs to the vesicant, or blister, agent class. SM causes debilitating blisters and inflammation in the acute phase of injury with psoriasis, fibrosis, and chronic inflammation developing in victims years later. SM was used extensively during World War 1 and its use was confirmed in the more recent Syrian Civil War. SM's ease of manufacture and its potency as a weapon make it highly attractive to terrorist groups. The threat of SM is exacerbated by our lack of effective targeted therapies. The development of such therapies is impaired by our lack of understanding of the mechanisms driving the toxic effects of SM, especially the long-term effects. We carried out a study characterizing the long-term dermal injury induced by nitrogen mustard (NM, an analog of SM) that heavily implicated mast cell activity and chemokine (C-C motif) ligand/receptor 2 (CCL2/CCR2) signaling in NM-induced dermal injury. Preliminary studies were then conducted to assess the role of these two potential drivers of NM-induced injury. **Methods:** Male C57BL/6 mice (age 5-6 weeks) were exposed on their dorsal skin to 0.5 mg NM dissolved in 100 μ L acetone or acetone alone. Mice were euthanized and skin samples collected at 1-, 7-, 14-, 29-, and 84-days post-exposure. The preliminary studies examining mast cells and CCL2 signaling utilized male C57BL/6 (WT), mast cell-deficient B6.Cg-Kit^{W-sh}/HnJrJaeBsmJ (MCD), and CCR2 knock-out B6.129S4-Ccr2tm1lf/J (CCR2KO) mice of the same age and given the same treatment as in the long-term study. Mice in the MCD study were sacrificed at day 14 post-exposure and mice in the CCR2KO study were sacrificed at day 63 post-exposure. Clinical analyses were made throughout the study and skin samples were flash frozen for PCR analysis or fixed for sectioning and histological analyses. **Results:** NM induced significant and persistent mast cell activation and upregulation of CCL2/CCR2 expression in C57BL/6 mice. CCR2KO mice had less severe weight loss, reduced mast cell activity, and reduced expression of inflammatory markers compared to WT mice following NM exposure. MCD mice had improved survivability and reduced expression of CCL2 and other inflammatory markers compared to WT mice following NM exposure. **Conclusions:** These observations indicate significant roles for mast cell activity and CCL2/CCR2 signaling, and the interactions between these pathways, in mustard agent-induced dermal injury. Further studies and analyses will need to be performed to better clarify the role of these pathways in mustard agent-induced injury and how these pathways synergize with each other to produce said injury. These studies will influence future studies exploring the efficacy of therapies targeting these pathways in counteracting mustard agent toxicity.

PS 3239 Characterization of Circulating miRNAs in Rodents after Pulmonary Exposure to Nitrogen Mustard

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Background and Purpose: Acute exposure to ionizing radiation (IR) or chemical warfare agents including mustard vesicants such as sulfur mustard and nitrogen mustard (NM) can lead to chronic lung disease, resulting in long-term morbidity and mortality months to years after the initial insult. Both IR and NM exert their harmful effects primarily through DNA damage. Notably, the DNA-damaging effects of NM were recognized as "radiomimetic" 75 years ago, highlighting their mechanistic similarity to radiation-induced injury. Both IR and NM cause DNA single- and double-strand breaks; NM alkylates DNA, causing inter- and intra-strand crosslinking. DNA damage from both agents leads to cell cycle arrest, apoptosis, oxidative stress, and inflammation. We observed conserved miRNA responses in plasma from mice, non-human primates and humans following exposure to IR leading to pulmonary fibrosis. Dysregulation of miRNAs included increases in miR-34 family members and decreases in miR-155-5p and miR-199 family members. Changes in these miRNAs were linked to pathways associated with fibrosis, within the first 30 days post exposure. In the present studies, we analyzed the effects of NM exposure on plasma miRNAs with the overall goal of identifying a common repertoire of miRNAs that could be used to pre-symptomatically predict the onset of pulmo-

nary fibrosis. **Methods:** Male Wistar rats (225-250 g) were treated intratracheally with NM (0.125 mg/kg) or PBS control using a 22-gauge gavage needle (1.25 mm ball diameter). Animals were euthanized 1, 3, 7, 14, or 28 days later and plasma collected ($n = 3-6$ per time point). Small RNAs were extracted, sequencing libraries prepared, and mature miRNA sequences quantified using next-generation sequencing. Longitudinal miRNA profiles for PBS and NM-treated animals were fit to a regression spline model using 3 degrees of freedom (df) that assumes miRNA expression changes smoothly over time. A moderated F-test was conducted on each miRNA on 3 df to detect differences between treatment and control groups. miRNA with FDR-corrected p-values < 0.05 were considered significant. **Results:** The top four plasma miRNAs altered by NM exposure (miR-126a-3p, miR-199a-5p, miR-155-5p, and miR-34c-5p) corresponded to those identified in plasma after IR exposure. These miRNAs regulate signaling pathways linked to fibrogenesis. Plasma levels of these miRNAs increased rapidly following NM exposure reaching a peak at 3 d; this was followed by a decrease at 14 d. By 28 d post-NM, miR-126a-3p, miR-199a-5p, and miR-155-5p were at control levels, while miR-34c-5p remained reduced. **Conclusions:** The relative abundance of miRNAs in plasma is increasingly recognized as a reflection of tissue-specific physiology. Importantly, changes in circulating miRNA profiles have been shown to indicate organ damage prior to the onset of clinical symptoms, offering a window for early detection and timely medical intervention. Our findings that two well characterized pulmonary toxicants known to cause fibrosis, NM and IR, induce similar preclinical changes in circulating miRNAs suggest that selective groups of miRNAs may be useful as predictive biomarkers for the development of this chronic lung disease.

PS 3240 Verapamil Protects Neurons Via NADPH Oxidase Inhibition in A Rat Model of Organophosphate DFP-Induced Status Epilepticus

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Background and Purpose: Exposure to organophosphate (OP) compounds at high doses leads to status epilepticus (SE) and rapid death. Current standard-of-care therapy aimed at controlling cholinergic response and SE has improved mortality outcomes. However, OP-SE survival is associated with long-term neurological morbidities, including recurrent seizures and memory deficits. Targeting neuronal injury pathways and inflammatory mechanisms has produced beneficial outcomes in rat models of OP-SE. Verapamil (VPM) is a calcium channel blocker used clinically to manage blood pressure. VPM has been reported to reduce cell death and ameliorate inflammation in animal models of CNS injuries. In this study, we tested whether adjunct treatment with VPM could exert neuroprotective and anti-inflammatory effects following OP toxicity. **Methods:** Rats (SD; male and female; 250-300g) were injected with DFP (4 mg/kg, S.C.). One minute later, atropine sulfate (0.5 mg/kg, IM) and 2-PAM (25 mg/kg, IM) were injected. Rats immediately developed SE, which was then controlled with midazolam (1.78 mg/kg, IM) at 1 h post-SE onset. Rats were then treated with VPM (10 mg/kg, IM) *b.i.d.* for 3 days. On the fourth day, rats were perfused, and brains were post-fixed for sectioning. A separate cohort of rats was euthanized and brains freshly dissected, and brain regions were flash frozen and stored at -80°C. Neuronal injury was assessed using the FJC staining. Activation of microglia and astrocytes, and NADPH oxidase expression, were detected by immunohistochemistry using their respective markers IBA1, GFAP, and gp91phox. Cytokine expression was assessed using antibody-specific western blotting. **Results:** DFP exposure produced a rapid onset of SE. VPM combined with atropine, 2-PAM, and midazolam therapy was well tolerated, and no significant increase in mortality was observed compared with rats treated with the standard-of-care alone. Extensive FJC+ staining indicative of dying neurons was seen in multiple brain regions from DFP-SE rats, which was markedly reduced in VPM-treated DFP rats. Significant numbers of IBA1+ and GFAP+ cells were observed in DFP-treated rats, which were reduced upon VPM treatment. Western blotting studies showed a significant (7-fold) increase in the pro-inflammatory cytokine IL-6 and a significant (4-fold) decrease in the anti-inflammatory cytokine IL-4 expression in DFP rats compared to the control condition. Quantification of gp91phox and IBA1 colocalized microglial cells showed significant increases in % area colocalized in DFP-SE rats compared to SAL-SAL control. Upon VPM treatment, significant reductions in % area colocalized for gp91phox were noted in multiple brain regions (Two-way ANOVA (sex x treatment) followed by post-hoc Sidak test. $*p < 0.05$, $n = 6-8$ /group/condition). **Conclusions:** VPM delivered via the IM route post-OP-SE exerted significant neuroprotective and anti-neuroinflammatory effects compared with standard-of-care alone. VPM treatment significantly reduced the rise in IL-6 levels and restored IL-4 to control levels in DFP-VPM rats compared to DFP-SAL. VPM treatment was also associated with a significant reduction in the microglial gp91phox expression. Given NADPH oxidase's role in driving inflammatory response via oxidative stress, VPM exerts a potent anti-inflammatory effect by inhibiting NADPH oxidase in OP-SE. We anticipate that these protective effects of VPM would improve long-term neurological outcomes following DFP-SE.

PS 3241 Nitro-oleic Acid Mitigates Chlorine-Induced Lung Injury in Mice by Suppressing Pro-inflammatory Activity of Macrophages and Neutrophils

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Background and Purpose: Chlorine (Cl₂) is a highly reactive gas which upon inhalation triggers extensive airway injury, including bronchiolar epithelial cell damage, disruption of epithelial barrier integrity, oxidative stress, accumulation of inflammatory cells within the lung, and impaired pulmonary mechanics. Nitrated fatty acids, such as nitro-oleic acid (OA-NO₂), are formed by the reaction of reactive nitrogen species and unsaturated fatty acids. Earlier studies demonstrated that nitrated fatty acids exert anti-inflammatory activity. In this study, we investigated the potential of OA-NO₂ to mitigate Cl₂-induced lung injury, oxidative stress and inflammation. **Methods:** Spontaneously breathing male C57BL/6 mice were exposed to air or Cl₂ (300 ppm, 25 min) in a whole-body exposure chamber; mice were treated i.v. with 50 µg OA-NO₂ or vehicle control 30-45 min later. Bronchoalveolar lavage (BAL) fluid and cells and lung tissue were collected 24 h later and analyzed for markers of injury, oxidative stress, and inflammatory cell activation. **Results:** Exposure of mice to Cl₂ resulted in peribronchial edema and inflammation, and necrosis of the bronchiolar epithelium. This was associated with an accumulation of bronchiolar epithelial cell debris in the alveoli, and upregulation of oxidative stress markers including heme oxygenase (HO)-1 and Ym-1. Cl₂ inhalation also increased levels of cells, IgM, total protein, and fibrinogen in BAL fluid indicating alveolar epithelial barrier dysfunction. BAL levels of surfactant protein (SP-D), soluble receptor for glycation end products (sRAGE) and matrix metalloproteinase (MMP)-9 also increased, consistent with pulmonary inflammation. Structural and inflammatory changes in the lung following Cl₂ inhalation were linked to functional alterations including a loss of elastic recoil properties, as reflected by a leftward shift in the pressure volume (PV) loops and significant increases in airway resistance and elastance in response to methacholine (12 mg/ml). Flow cytometric analysis of CD45⁺ lung cells showed significant increases in immature (CD11b⁺Ly6G⁺CD11c⁺ Ly6C^{hi}) and mature (CD11b⁺Ly6G⁺CD11c⁺ Ly6C^{lo}) pro-inflammatory macrophages, and CD11b⁺Ly6G⁺CD11c⁺ neutrophils in the lungs following Cl₂ exposure, with no effect on anti-inflammatory (CD11b⁺Ly6G⁺CD11c⁺ Ly6C^{lo}) macrophages, while CD11b⁺Ly6G⁺Ly6C⁺CD11c⁺Siglec F⁺ resident macrophages were reduced. This was correlated with increases in numbers of alveolar macrophages expressing cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS), markers of pro-inflammatory activation. Treatment of mice with OA-NO₂ mitigated Cl₂-induced peribronchial edema and inflammation, along with macrophage expression of HO-1 and Ym-1; alterations in pulmonary mechanics were also blunted. Numbers of immature pro-inflammatory macrophages and neutrophils were also reduced by OA-NO₂, a response associated with reduced expression of COX-2 and iNOS. Conversely, numbers of mature anti-inflammatory and resident macrophages were increased after OA-NO₂ administration to Cl₂ treated mice; macrophage expression of CD206 also increased. We also identified a population of immunosuppressive CD11b⁺Ly6G⁺CD11c⁺ neutrophils in the lungs of mice treated with Cl₂ and OA-NO₂. **Conclusions:** These findings demonstrate that OA-NO₂ reduces lung injury and oxidative stress following Cl₂ inhalation. We speculate that this is due to suppression of pro-inflammatory macrophages and neutrophils and activation of anti-inflammatory macrophages. Supported by NIH Grants U54AR055073 and P30ES005022.

PS 3242 Sulfur Mustard Alters Fatty Acid Synthase Expression During Rabbit Meibocyte Differentiation

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Background and Purpose: Sulfur mustard (SM; bis(2-chloroethyl)sulfide) is a cytotoxic vesicant known to induce chronic ocular injury. Characteristic long-term responses of the eye to SM include persistent inflammation, corneal opacity, and instability of the tear film. This is attributed, in part, to impaired tear film formation resulting from the dysregulation of conjunctival mucin secretion and meibomian gland (meibum) production, an oily secretion produced by differentiated meibocytes. Meibomian glands are a type of holocrine exocrine gland located along the tarsal plate of the eyelids; they play an essential role in maintaining corneal surface integrity and preventing tear film evaporation. Herein, we used the New Zealand white rabbit corneal exposure model to investigate the effects of SM on meibomian gland differentiation and lipid production. **Methods:** Experiments with rabbits and SM were conducted at MRIGlobal (Kansas City, MO) in an AAALAC-accredited facility, under protocols approved by the Institutional Animal Care and Use Committee (IACUC). The central cornea of the right eye of New Zealand white rabbits was exposed to 0.4 µL neat SM (5 mg; ~30 nmol) for 5 min and then flushed with sterile saline for 2 min. Left eyes served as controls and received only saline. Rabbits were euthanized 28 days later, eyelids excised, trimmed, fixed in paraformaldehyde/sucrose, and embedded in paraffin. Five-micron tissue sections were stained with hematoxylin and eosin (H&E) or Masson's trichrome for histopathology. For immunohistochemistry, sections were incubated with antibodies against fatty acid synthase (FAS), proliferating cell nuclear antigen (PCNA), and keratin 1 or IgG controls. Antibody binding was visualized using DAB Peroxidase Substrate. Tissue images were acquired using an Olympus

VS120 Virtual Microscopy System. **Results:** H&E and trichrome staining revealed distinct stages of meibocyte differentiation within the acini of the meibomian glands. Meibocytes in the basal region of the acini were proliferative and progressively enlarged as they accumulated lipid-rich material. As differentiation advanced, mature meibocytes underwent disintegration and released their contents into the glandular lumen, consistent with holocrine secretion. Rupturing cells were observed as they migrated toward the ductal opening. FAS was upregulated in lipid laden immature meibocytes, while downregulation was evident in mature and ruptured cells. In SM-exposed rabbit eyelids, expression of FAS, was markedly decreased, indicating disrupted lipid synthesis during meibocyte differentiation. PCNA expression was primarily confined to basal meibocytes at the gland periphery. SM increased PCNA expression of basal meibocytes, which is essential for the replacement of differentiated meibocytes. Keratin 1 was localized around individual meibocytes in control glands and glands from SM-treated rabbits, but was lost as the cells matured, consistent with differentiation-associated remodeling of the cytoskeleton. **Conclusions:** The tear film is essential for maintaining the integrity of the ocular surface, and its disruption leads to corneal epithelial damage, erosion, and dry eye, conditions known to result following SM exposure. Our findings demonstrate that SM alters meibomian gland architecture, specifically affecting meibocyte acini and reducing FAS expression, a key enzyme involved in lipid synthesis. Increased proliferation and keratinization of meibocytes were also observed following SM. These changes in FAS expression and meibomian gland secretion impair lipid composition and tear film stability. Together, these data indicate that SM-induced meibocyte dysfunction contributes to chronic ocular surface injury, underscoring the need to target meibocyte preservation and repair in the development of effective SM countermeasures.

PS 3243 Levels of novel oxime sufficient to counteract brain AChE inhibition by a sarin surrogate neither induce gene expression of nor inhibit metabolism by the human P450 isozymes CYP1A2, CYP2B6, and CYP3A4

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Background and Purpose: To counteract inhibition of brain AChE by organophosphate nerve agents or insecticides, our lab synthesized novel pyridinium oximes and has experimental evidence that some cross the BBB and reactivate rat brain AChE previously inhibited by a sarin surrogate (nitrophenyl isopropyl methylphosphonate: NIMP) or the active metabolite of the insecticide parathion (paraoxon). There is also evidence of attenuation of NIMP-induced seizure-like behavior and of brain neuropathology. Because of these positive results, several of our novel oximes have been down-selected for drug development. Oxime 20 is our lead candidate. FDA guidance recommends pre-clinical testing of lead drug candidates for potential interactions with the CYP 450 isozymes responsible for the majority of drug metabolism in humans. Inhibition or induction of CYP3A4, CYP2B6, CYP1A2 frequently results in drug-drug interactions (DDI) which influence therapeutic efficacy. **Methods:** Induction of CYP3A4, CYP2B6, and CYP1A2 gene expression was measured in induction qualified Gibco™ human plateable hepatocytes treated as per the manufacturer's instructions. Each well of a 24 well, collagen-coated tissue culture plate received 350,000 hepatocytes which were then grown for 24 hours at 37°C, 5% CO₂. The next day cells were exposed to either prototypical inducers [2 µM 3-methylcholanthrene (CYP1A), 1 mM phenobarbital (CYP2B), 10 µM rifampicin (CYP3A)], Oxime 20 from 1 nM to 1 µM, or the vehicle control (DMSO) and returned to the incubator for 48 hours. After 48 hours, hepatocytes were washed twice with iced HBSS which was then replaced with RLT Plus Lysis Buffer from the Qiagen RNeasy Plus Mini Kit. The cell lysates were stored at -70°C prior to RNA isolation using the same Qiagen kit. Gene expression was analyzed with RT-qPCR on the Agilent AriaMx system, using IDT PrimeTime assays and One-Step RT-qPCR Master Mix. Expression levels of CYP3A4, CYP2B6, and CYP1A2 mRNA were normalized to the housekeeping gene *Rplp1* and assessed using the ΔΔCT method. Inhibition of metabolism by CYP3A4, CYP2B6, CYP1A2 was studied using the Vivid® CYP450 screening kit from Life Technologies™ following the manufacturer's methods. The kits use CYP450 Baculosomes® Plus Reagents which are microsomes prepared from insect cells expressing a single human P450 isozyme. Different Vivid® substrates are metabolized by different CYP450 enzymes into fluorescent metabolites. Drugs are analyzed by their capacity to inhibit the fluorescent signal. Oxime 20 was combined with the CYP450 Baculosomes®, glucose-6-phosphate and glucose 6-phosphate dehydrogenase. After pre-incubation, the background fluorescence was measured. The enzymatic reaction was started with a mix of NADP+ and Vivid® substrate. The plate was read for 60 minutes on a Molecular Devices Spectramax M5 (EX=415 and EM=460) in Kinetic Assay Mode at 1-minute intervals. **Results:** Oxime 20 exposure at 1 nM, 10 nM, 100 nM and 1 µM, caused no statistically significant induction of CYP3A4, CYP2B6, and CYP1A2 gene expression compared to the DMSO vehicle (*p* ≥ 0.15). The prototypical inducers of CYP3A4, CYP2B6, and CYP1A2 did cause statistically significant induction compared to vehicle (*p* < 0.001). A minimal inhibition of CYP1A2 and CYP3A4 metabolism was observed at Oxime 20 concentrations below 50 µM. At this same concentration, however, CYP2B6 metabolism was more than 80% inhibited compared to the positive control. **Conclusions:** The lack of gene expression induction by Oxime 20 suggests a low risk of DDI due to increased metabolism by CYP1A2, CYP2B6 or CYP3A4. Our earlier pharmacokinetic study (Backer et al., 2022) indicated the maximum amount of Oxime 20 in the rat brain was 397.84 pM. This suggests that the biologically relevant concentration of Oxime 20 is much less than the 50 µM which caused 80% inhibition of CYP2B6 metabolism. Although effects at therapeutic

levels are minimal, given the significant inhibition of CYP 2B6, Oxime 20 may present a risk of interacting with drugs metabolized by this enzyme. This study supports Oxime 20's low DDI risk at therapeutic levels but necessitates further studies to assess its clinical impact, especially as a combination therapy.

PS 3244 Idebeneone Treatment Reduces Nitrogen Mustard Induced Mitochondrial Dysfunction in Lung Epithelial Cells

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Background and Purpose: Sulfur and nitrogen mustards are bifunctional alkylating agents and vesicants developed in the early 20th century for use as chemical warfare agents. One prominent route of exposure is through inhalation where damage occurs in the lungs from DNA alkylation and reactive oxygen species generation (ROS). In this study we aimed to utilize the antioxidant quinone compound idebenone to reduce the ROS stress and toxicity from nitrogen mustard exposures. **Methods:** We utilized both primary and immortalized bronchial epithelial cells (HBEC and BEAS-2B cells respectively). Mitosox, Cellroxx and peroxiredoxin 1 and 3 westerns were used to determine the presence of oxidative stress in both lung epithelial cell models. Seahorse cell mito stress assay was used to determine mitochondrial dysfunction. We also utilized MTS and Calcein-AM to determine cell viability after pre-treatment of Idebeneone and exposure of nitrogen mustard. Cells were pretreated with idebenone at concentrations between 1uM and 50uM followed by treatment of nitrogen mustard at concentrations between 1uM and 100uM. **Results:** We observed a significant reduction in basal respiration, maximal respiration, and ATP production 6hrs following nitrogen mustard exposure in a dose dependent manner in both lung epithelial cell models. Pre-treatment with 10uM and 30uM of idebenone was able to recover the observed decrease in maximal respiration 6hrs following nitrogen mustard treatment. **Conclusions:** Idebeneone is an antioxidant drug which may be effective in reducing the toxicity of nitrogen mustard exposure by preventing the mitochondrial dysfunction in lung epithelial cells.

PS 3245 Sulfur Mustard Disrupts Epidermal and Dermal Structures in Hairless Guinea Pig Skin

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Background and Purpose: Sulfur mustard (SM; bis(2-chloroethyl) sulfide) is a highly reactive bifunctional alkylating agent and potent vesicant that causes severe damage to the skin. Following dermal exposure, SM induces erythema, inflammation, epidermal erosions, and blistering. In this study, we investigated the temporal progression of SM injury in the hairless guinea pig, a translationally relevant animal model for studying SM-induced cutaneous injury. Hairless guinea pig skin closely resembles human skin, possessing a thick epidermis and a well-developed dermis containing superficial microvessels and secondary appendages. Proteins essential for skin homeostasis were visualized following injury including elastin, a marker of skin elasticity, myeloperoxidase (MPO), a marker of inflammation, and keratin 5 (K5), an epithelial scaffolding protein. The objective of this work was to characterize the pathogenesis of SM-induced skin injury and the subsequent wound-healing responses in the hairless guinea pig. **Methods:** Exposures to SM were conducted using a vapor cap model at MRIGlobal (Kansas City, MO) under an IACUC-approved protocol. Air control or saturated SM vapor caps were applied to the dorsal flanks of 6-8 wk-old Hartley hairless guinea pigs for 20 min. Forty-eight hours post-exposure, SM-exposed sites were debrided daily for 4d using saline gauze soaks. Animals were euthanized 2d post-SM (prior to debridement) and at 20d and 66d post-SM exposure. Full-thickness skin biopsies from control and SM-treated animals were collected and processed for histopathology using hematoxylin and eosin (H&E) and Masson's trichrome staining. Elastic fibers were visualized using orcein stain. Immunohistochemistry was performed using antibodies against MPO and K5, with matched IgG controls. **Results:** The skin of control hairless guinea pigs displayed a well-developed, multi-layered epidermis overlying a dermis composed of an interwoven collagen network with abundant, branching elastic fibers. Two days post-SM skin was characterized by epidermal necrosis and dermo-epidermal clefts containing numerous necrotic heterophils. In the dermis, perivascular infiltrates and extravasated red blood cells surrounding degenerate vascular endothelial cells were observed. By 20d post-SM, moderate epidermal acanthosis and perivascular vacuolization were noted in the stratum granulosum and stratum spinosum. Elastic fibers within the edematous superficial dermis were fragmented and agglomerated. By 66d post-SM, there was a clear demarcation between the superficial and deep dermis, with the superficial dermis containing dense, highly compacted collagen fibers running parallel to the epidermis, which was devoid of elastic fibers and secondary appendages. MPO, a marker of azurophilic granules in neutrophils, was not expressed within the epidermis of control skin. Two days post-SM, MPO positive degenerate heterophils were observed within the dermo-epidermal cleft and throughout the necrotic epidermis. Epidermal MPO expression had decreased by 20d post-SM and was not evident 66d post-SM. K5, a protein essential for the structural integrity of epithelial cell attachment to the basement membrane, was expressed in basal keratinocytes in control skin. In epithelial cells along the basement membrane,

K5 was downregulated 2d post-SM. Twenty days post-SM, K5 expression within the basal layer had returned, approaching control levels by 66d. **Conclusions:** SM exposure in hairless guinea pig skin produced a well-defined sequence of injury and repair. Early lesions were characterized by extensive epidermal necrosis, dermo-epidermal separation, and an acute neutrophilic infiltrate, accompanied by vascular damage in the dermis. By 20d post-SM, partial re-epithelialization and remodeling of the epidermis were evident, though dermal elastic fibers remained fragmented and collagen organization was altered. At 66d, the skin exhibited structural remodeling marked by dense, compact collagen deposition with a loss of elastic fibers and adnexal structures, indicative of scar formation. The transient expression of MPO and restoration of K5 confirmed a shift from acute inflammation to tissue repair and re-establishment of epidermal integrity. Overall, these findings demonstrate a progressive transition from acute injury to chronic dermal remodeling following SM exposure.

PS 3246 Dexamethasone treatment alleviates lewisite-induced delayed proteomic dysregulations, inflammation, and fibrosis in the rabbit cornea in vivo

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Background and Purpose: Lewisite (Lew) emerged as a chemical warfare agent (CWA) during World War I and since then, it has been produced in large quantities and stockpiled. Eyes, particularly corneas, are extremely vulnerable to Lew exposure. However, in-depth analysis of corneal injury mechanism following Lew exposure has not been undertaken. Of interest are the delayed effects of Lew exposure on corneas. Moreover, British anti-Lew is the only approved therapeutic available, but it predominantly acts systemically, has several side effects, and is not recommended for ocular use. Thus, targeted ocular therapeutics that can be used in mass casualty situations are an immediate need. In this regard, dexamethasone (Dex), an FDA-approved steroidal anti-inflammatory, was used for treatment of ocular injuries following exposure to mustard vesicating CWA. Thus, in the present study, we assessed Dex efficacy for treatment of Lew induced corneal injuries at the delayed injury timepoint, leveraging high-throughput proteomic analysis, using an *in vivo* rabbit model. **Methods:** Male white rabbits (≤ 3 months old; 2-3 kg) were used for the study and procedures performed were approved by IACUC. Groups ($n=4$ /group) included: (1) control (2) Lew exposed (Lew); (3) Lew exposed and Dex treated (Lew+Dex). Lew vapor exposure was performed to achieve targeted mild to moderate corneal injuries ~ 0.2 mg/L; 300 ml/min; 8 min). Dex (0.1%; Bausch and Lomb) was delivered, starting 2 h post exposure, and thereafter every 8 h for 28 days. Samples were harvested at day 28 and divided into two fractions: 1) flash frozen and stored at -80°C until proteomic analysis (LC-MS/MS); and 2) formalin fixed paraffin-embedded, and sectioned (5 μm) for histopathological (H&E and Masson's trichrome staining) and molecular expression analysis (immunofluorescence). Statistical analysis was performed using R (ver 4.2.3; one-way ANOVA with pairwise post-hoc testing; unadjusted $p<0.05$; FDR <0.01) to identify the significantly dysregulated proteins between study groups. Pathway enrichment analysis of differentially expressed proteins was performed using Ingenuity Pathway Analysis (IPA, Qiagen). **Results:** Histopathological examination indicated that Lew exposure induced epithelial degradation, immune cell infiltration, and stromal collagen degradation. Molecular assessments revealed increased expression of cathepsin C and cell division cycle 42 (CDC42) in the epithelium and stroma, as well as increased expression of vimentin only in the stroma. Dex treatment prevented epithelial and stromal degradation. Furthermore, reversal of cathepsin C, CDC42, and vimentin elevation was observed with Dex treatment, thus preventing the prolonged inflammatory response observed in the untreated corneas. Proteomics analysis showed that the expression profiles for most proteins from control and Lew+Dex groups were similar but differed from the Lew group, with 1,163 proteins dysregulated in the three groups. Lew exposure led to the change in the expression profile of 1,045 proteins (Lew vs Control $p<0.05$), of which 575 were downregulated and 470 proteins were upregulated. Of the downregulated proteins, 479 were upregulated after Dex treatment (Lew+Dex vs Lew $p<0.05$). Further, of the upregulated proteins, 360 were downregulated after Dex treatment (Lew+Dex vs. Lew $p<0.05$). Dex treatment completely or partially reversed Lew-induced expression changes $\sim 80\%$ proteins. Top 20 canonical pathways were categorized into 5 groups based on biological functions: category 1, immune response and inflammation associated pathways; category 2, cytoskeletal remodeling and cell junction dynamics pathways; category 3, ECM organization and structural integrity mechanisms; category 4, intracellular transport, trafficking, and protein processing pathways; and category 5, pathways indirectly contributing to Lew injury and Dex efficacy. **Conclusions:** Lew-induced modulation on day 28 indicates persistent toxicity and delayed injury. Dex effectively reversed these molecular alterations, thus preventing activation of all top dysregulated pathways, with significant reversal in associated protein expressions. Dex is a potential therapeutic candidate that can be used as a countermeasure for treatment of Lew-induced corneal injuries. (Funding: U01 EY030405)

PS 3247 Post-exposure inhibition of IL-1 β signaling delays the onset of spontaneous recurrent seizures (SRS) and improves cognitive impairment in adult male Sprague-Dawley rats acutely intoxicated with the organophosphate (OP), diisopropylfluorophosphate (DFP)

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Background and Purpose: Acute intoxication with organophosphorus (OP) cholinesterase inhibitors can cause life-threatening status epilepticus (SE), respiratory paralysis and death. When administered promptly following intoxication, standard of care therapeutics improve survival, but do not prevent long-term neurological sequelae, including spontaneous recurrent seizures (SRS) and cognitive deficits. Clinical and preclinical studies implicate the pro-inflammatory cytokine interleukin-1 β (IL-1 β) in epileptogenic processes and cognitive decline. We previously demonstrated that IL-1 β mRNA gene expression is upregulated immediately following acute DFP intoxication and during the epileptogenic period. Therefore, to determine the contribution of IL-1 β signaling in the pathogenesis of the long-term neurological sequelae resulting from acute DFP intoxication, we quantified the effects of administering Anakinra, an IL-1 β receptor antagonist, beginning 1 h post-exposure and continuing for 10 d post exposure (DPE) on electroencephalographic (EEG) activity in the brain and cognitive behavior. **Methods:** Adult male Sprague-Dawley rats were surgically implanted with cortical screws and an EEG telemetry unit 10 d prior to acute DFP intoxication. DFP (4.0 mg/kg, sc), or an equal volume of vehicle (PBS, sc) was administered followed 1 min later by atropine sulfate (2.0 mg/kg, im) and 2-PAM (25 mg/kg, im) (n=10 VEH / 12 Anakinra). Animals were administered midazolam (0.65 mg/kg, im) at 40- and 50-min post-DFP. At 60-min post exposure animals received Anakinra (33.3mg/kg, sc) or vehicle (saline) with repeated administration every 8 h for 10 d. Continuous EEG with time-locked video was recorded for 30 d (n=7 saline / 10 Anakinra). Animals underwent behavioral assays to evaluate cognitive function from 30-36 DPE (n=7 saline / 10-12 Anakinra). Animals were euthanized and brain tissue collected at 36 DPE. **Results:** Anakinra delayed the onset of SRS events during administration and reduced total convulsive seizure burden during the study period. During the novel object recognition task, DFP animals administered Anakinra had a significant increase in the number of interactions with a novel object compared to familiar objects; compared to DFP animals who were treated with saline that interacted with the familiar object and novel object comparably. DFP animals from both treatment groups underwent contextual fear conditioning, learned the task and performed at a comparable level during the context and cue tasks as indicated by significant decreases in the average motion index. **Conclusions:** Administration of Anakinra following acute DFP intoxication delayed the onset of SRS and moderately improved cognitive improvement compared to DFP-intoxicated animals who received saline. These findings are consistent with the hypothesis that IL-1 β plays a role in the pathogenesis of SRS and cognitive dysfunction associated with acute OP intoxication. These data provide evidence supporting the use of Anakinra as an adjunct therapy to standard of care to modify the long-term comorbidities associated with acute DFP intoxication. Supported by the NIH CounterACT Program (U54 NS127758) and predoctoral fellowships to Chelsea Unkel (T32 GM135741) and Brittany Davis (T32 GM136559).

PS 3248 Systemic and Pulmonary Effects of Acute Chlorine Exposure in a Preclinical Rat Model

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Background and Purpose: Chlorine is a highly reactive and toxic gas produced in large quantities in industry. Accidental exposures occur frequently, highlighting a need for effective countermeasures. Animal models provide important insights into the injury mechanisms and serve as preclinical models for drug development and advancement. Here we describe a rat model of chlorine inhalation using near lethal dose (LD₅₀) concentrations to characterize the physiological and systemic effects of exposure and to establish a platform for longitudinal therapeutic assessment. **Methods:** Sprague Dawley rats were exposed to 500 or 600 ppm of chlorine for 45 min and transferred to room air. Pulse oximetry was performed every 2h till 8h and then at 24h after exposure. At the predetermined timepoints (3, 6, and 24h), body weights were recorded and animals were euthanized for arterial blood collection from the descending aorta for arterial blood gas (ABG) measurements. Before bronchoalveolar lavage fluid (BALF) collection, the right upper lung (RUL) was clamped and excised for determination of lung wet-to-dry weight ratios. Lungs were then perfused, collected, and frozen. Additional endpoints included heart weight, spleen weight, complete blood cell count (CBC), BALF total cell counts, protein and lactate dehydrogenase (LDH) concentration in BALF supernatant. Cardiac injury markers, N-terminal pro-brain natriuretic peptide (NT-proBNP) was measured in the plasma. Separate animals were processed for histological evaluations. **Results:** A single acute chlorine (500 or 600 ppm) exposure caused about 50% mortality and excessive respiratory distress in survivors. Exposed animals exhibited increased clinical scores, reduced heart rate and decreased tissue oxygenation. ABG analysis revealed hypoxemia accompanied by respiratory and metabolic acidosis (or compensated respiratory acidosis) at all time points. Systemic inflammation was indicated by decrease in monocyte and lymphocyte counts along with increased eosinophils and neutrophils

in the blood. Lung injury and edema were confirmed by significantly increased BALF protein, LDH concentrations, higher lung wet-to-dry weight ratios, and increase in total BALF cell counts at all time points measured. Histopathological evaluation of lung hematoxylin and eosin (H&E) sections revealed marked injury to the airway and alveolar regions. Cardiac involvement was evident from increased heart-to-body weight ratios, increased plasma NT-proBNP, and ultrastructural damage observed by electron microscopy. **Conclusions:** This study establishes a robust preclinical rat model that reproduces the systemic and physiological effects of acute chlorine exposure. Using two near LD₅₀ concentrations we demonstrate severe respiratory distress, systemic inflammation, cardiopulmonary damage and altered pulmonary physiology in the exposed animals. This model provides a valuable platform that can guide future mechanistic studies and evaluation of novel therapeutics.

PS 3249 Phosgene inhalation in swine: reproducible ARDS phenotype and systems proteomics to guide countermeasure discovery

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Background and Purpose: Phosgene is one of the highly produced industrial gases and is an essential intermediate in polymer, pharmaceutical, and metallurgical synthesis. However, phosgene is highly toxic when inhaled. Phosgene inhalation causes a delayed onset of symptoms starting 8 hours post-inhalation. While phosgene inhalation-induced lung injury is consistent with acute respiratory distress syndrome (ARDS), pulmonary edema is the pathognomonic symptom. Due to its high propensity for toxicity, it was deployed as a chemical warfare agent during World War I. Currently, there are no mechanistic-based medical countermeasures for treating phosgene inhalation injuries. The treatment is mainly supportive and symptomatic. As the medical countermeasures are approved through the FDA's animal rule, there is a knowledge gap in non-rodent animal models that recapitulate human phosgene inhalation injuries and detailed proteomic profiling to identify potential drug targets and forensic diagnostic biomarkers. **Methods:** Sedated domestic Yorkshire swine were exposed to phosgene (40 ppm for 20 minutes) via a nose cone. Animals were recovered from sedation and observed in their home pens frequently. Animals were re-sedated at 8 or 24 hours post-phosgene exposure. Oxygenation, respiratory physiological, and pulmonary function testing parameters were recorded. At the end of the study, animals were euthanized, and lung tissues were collected. Lung tissues were processed for unbiased global proteomics using previously established mass spectrometry methods, and multiplex protein array assays were also performed to identify potential drug biomarkers and targets. **Results:** Phosgene inhalation resulted in hypoxemia and worsening of respiratory physiological parameters such as reduced compliance dynamics, elevated airway resistance and peak inspiratory pressure. Pulmonary function testing showed decreased tolerance to increasing concentrations of nebulized methacholine. Lung histopathology revealed neutrophilic alveolitis, hyaline membranes, and increased septal thickness consistent with ARDS. Unbiased global proteomics studies in phosgene-exposed lung tissues revealed >8,000 protein groups with good precision and accuracy based on quality control pool samples and internal spike-in standards. Prominent among the differentially-expressed proteins were plasma-derived and acute-phase proteins. Upregulated proteomic signatures included phospholipid efflux, lipoprotein metabolism, TGF β activation, and coagulation/complement pathways, while cilia-associated and epithelial barrier proteins were decreased. Extracellular matrix remodeling with altered collagen isoforms was evident. Multiplex protein array assays corroborated inflammatory changes. **Conclusions:** This acute porcine model recapitulates key features of human phosgene inhalation injury and is suitable for countermeasure testing. Differentially expressed proteome changes may serve as potential diagnostic biomarkers and drug targets. Extended observation and dose-response studies are underway.

PS 3250 Cytotoxicity screening in primary human epidermal keratinocytes and dermal fibroblasts

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Background and Purpose: The skin is the first protective barrier in the human body against damage caused by UV light and chemical substances as well as protecting internal structures against microorganisms and mechanical trauma. It is composed of three layers: the epidermis, containing keratinocytes as the most frequent cell type, the dermis, which consists mainly of connective tissue and fibroblasts, and the hypodermis, the deepest layer which is made up of adipose and connective tissue. Following damage, injury, or during the normal turnover of cells, the appropriate proliferation response of both fibroblasts and keratinocytes is essential for the regeneration and repair process to occur effectively. Delays in this process can lead to chronic wounds and an increased potential for bacterial colonization, while excessive and uncontrolled proliferation can lead to benign or malignant neoplasms. Despite the common clinical use of various antimicrobial agents to prevent delays in skin healing and the standard use of chemotherapy agents to treat skin cancers, these compounds can have adverse effects on healthy skin cell viability. Evaluation

of these cytotoxic effects during the drug discovery process is crucial to understanding how to achieve a balance between adequate clinically relevant outcomes and minimizing toxicity or off-target effects that could damage skin or prevent wound healing. Here we present a workflow for cytotoxicity assays targeting normal human epidermal keratinocytes (NHEK) and normal human dermal fibroblasts (NHDF). We validated this workflow by exposing both cell types to common antimicrobial or chemotherapy agents. **Methods:** Lonza NHEK and NHDF cells (n=3 donors for each cell type) were plated at 3500-5000 cells per cm² and cultured in KGM®-Gold or FGM®-2 medium, respectively. NHEK cells were exposed to eight concentrations of Doxorubicin (DOX; 0.1-2 µM) and Chlorhexidine (CHEX; 0.25-10 µM), while NHDF cells were exposed to eight concentrations of Camptothecin (CAMP; 0.001-2 µM) and Chloroxylenol (CHXY; 25-500 µM). DOX and CAMP are commonly used chemotherapy agents, while CHEX and CHXY are anti-microbials often employed in wound healing protocols and as antiseptics. Cells were exposed across acute (24 hours) or chronic (120 hours) timescales. Cells were cultured until 40-60% confluence before initiating exposure at the typical period of maximal proliferation. For chronic exposure, cells were cultured until reaching 10-20% confluence. This relatively low density was chosen to prevent over-confluence of cells by the experiment endpoint that itself could impact cell health. Cytotoxicity was measured by using total cellular ATP relative to untreated control cells as a proxy for viability, generating dose response curves and IC₅₀ values. **Results:** Cytotoxic effects were observed across all experiments. Across an acute timescale (24 hours), both NHEKs and NHDFs saw drop-offs in ATP activity, but only the highest dose of Chlorhexidine caused complete lethality (0% ATP activity). Across a chronic timescale (120 hours), lethal effects were observed for each compound tested at the highest concentrations. There was relatively low variation across donors in IC₅₀ values. Dose-response curves for all donors followed the same trajectories for each experiment and IC₅₀ values fell within 1.5 SD of the mean. In addition, mean IC₅₀ values for each compound tested were within range of those previously reported in the literature. Donor behavior was also consistent between acute and chronic timescales, as donors with higher IC₅₀ values at 120 hours also had higher IC₅₀ values at 24 hours. **Conclusions:** Our data shows that this cytotoxicity workflow generates robust and reproducible cytotoxicity results with calculated IC₅₀ values in line with previously published literature. The trajectory of the dose-response curves was near-identical across donors for each compound tested with overall minimal variation in IC₅₀ values between donors. This assay provides an excellent system for cytotoxicity screening of both epidermal keratinocytes and dermal fibroblasts, two cell types critical to the structure and function of human skin, capturing physiologically relevant data that accounts for the variability in patient populations while providing consistent, reliable results.

PS 3251 A retrospective study on the use of Local Lymph Node Assay EC3 values to derive doses for the Confirmation of No Induction in Humans test

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Background and Purpose: Assessment of skin sensitization potential is a critical component of fragrance ingredient safety evaluation, as exposure to sensitizing substances can lead to allergic contact dermatitis. Weight-of-evidence analysis integrating *in silico*, *in vitro*, and *in vivo* data may be performed to determine a compound's sensitizing potential. This includes the use of predictive models, experimental data, historical animal and confirmatory human datasets. However, only historical animal studies are considered due to the ban on animal testing for cosmetics. Once a material is determined to be sensitizing, then quantitative risk assessment is required, which begins with determining the No Expected Sensitization Induction Level (NESIL). A key aspect to deriving a NESIL involves estimating the quantitative potency of a compound - historically achieved by using data from the Local Lymph Node Assay (LLNA) as the gold standard. Confirmatory human studies, also known as Confirmation of No Induction in Humans tests (CNIH) are conducted to confirm the NESIL. Previous studies have demonstrated that safe doses in human were closely aligned with LLNA EC3 values, showing a strong correlation between NESILs and LLNA EC3s without the need for additional uncertainty factors. This study builds on prior findings by expanding the dataset and reinforcing the relationship between LLNA EC3 values and CNIH-derived NESILs. Importantly, this retrospective analysis provides insight into how EC3 values can be leveraged when validating new *in vitro* methods, supporting the transition toward NAMs. **Methods:** Using expert judgement, historical LLNA studies, as well as CNIH studies were evaluated to ensure their sufficiency. They were then assessed and set to a potency category based on the LLNA EC3 or CNIH NESIL. If these categories were in discordance, all relevant data were evaluated through expert judgement. **Results:** Based on a preliminary assessment of an expanded dataset, 199 materials, the analysis confirms that LLNA EC3 values generally provide protective estimates for NESILs with only a few well-recognized exceptions. **Conclusions:** This study confirms the usefulness of the correlation between the historical LLNAs and the dosing strategy for CNIH tests. The analysis enables more accurate application of existing animal data in predictive models, which in turn supports the development of more human-relevant non-animal methods.

PS 3252 Outside the validated range: Performing a Weight of Evidence (WoE) assessment of the skin sensitization potential for fragrance chemicals positive at high concentrations in the Local Lymph Node Assay (LLNA)

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Background and Purpose: The Local Lymph Node Assay (LLNA) has been the gold standard for skin sensitization assessment for two decades. During its validation, most chemicals were tested at a maximum concentration of 25%, which showed high predictivity versus guinea pig and human data. However, when the OECD guideline was established, testing up to 100% was introduced as a mandatory requirement despite this range never undergoing formal validation. Even accepted solvents and negative standards have yielded false positives under this unvalidated practice. As highlighted in an OECD curation exercise, the specificity of the LLNA versus human data has dropped significantly. The main concern is the assay's tendency for false positives, particularly with irritants. LLNA-positive results at high concentrations may reflect irritation rather than true sensitization. For chemicals positive within this 25-100% range, a Weight-of-Evidence (WoE) approach may be more appropriate for a final conclusion on the sensitization hazard. High-concentration LLNA results may reflect irritant-driven proliferation rather than true sensitization, reinforcing the need for human-relevant, non-animal approaches such as OECD 497. **Methods:** *In vitro*, *in silico*, human, and guinea pig data were compiled for 101 LLNA-positive chemicals. Of these, 69 had at least one LLNA EC3 ≥25%, 19 had EC3 values between 19.4-24.8%, and 13 had mixed positive and negative LLNA data. For each material, corresponding *in vitro*, *in silico*, and Confirmation of no Induction in Humans (CNIH) data were compared, and the Expert Panel for Fragrance Safety chemists classified parent and metabolite reactivity to support the overall WoE evaluation. **Results:** 53 materials had negative CNIH data sufficient to establish a No Expected Sensitization Induction Level (NESIL). 8 were concluded as non-sensitizers through the Research Institute for Fragrance Materials (RIFM) safety assessment process. Using the OECD 497 two-out-of-three (2o3) defined approach, 48 of 101 materials (47.5%) were non-sensitizers, 33 (32.7%) sensitizers, and 20 (19.8%) inconclusive. Overall, 49 were determined to be non-sensitizers based on WoE, 27 as sensitizers, and 25 as inconclusive. Among the non-sensitizers, 67% were LLNA-positive at ≥25%, suggesting many LLNA positives at high concentrations do not translate to human-relevant sensitization. For 53 materials with both LLNA and CNIH potency data, three consistent patterns were observed: (1) close alignment, (2) LLNA overprediction (human NOEL >> mouse EC3), and (3) conservatively low human NOELs indicating high sensitivity. Expert chemists classified 53.4% of parent molecules as non-reactive and 33.7% as reactive, aligning with *in vitro* results for most cases. **Conclusions:** A significant fraction of these chemicals likely yield false-positive results in the LLNA. A comprehensive WoE assessment provides a more granular understanding of sensitization hazard and risk than reliance on LLNA results alone. High-dose LLNA testing may overestimate sensitization hazard, and integrating human and *in vitro* evidence provides a more accurate, biologically relevant foundation for modern risk assessment.

PS 3253 Skin sensitization risk assessment using a machine learning model that considers aggregate exposure to cosmetic products

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Background and Purpose: Chemical-induced contact allergy is a medical issue of considerable concern to consumers, clinicians, industries, and regulatory authorities. Chemicals such as fragrance materials are used in a wide variety of consumer products at varying levels, leading to a wide range of exposure. The previously published quantitative risk assessment (QRA) approach uses both potency prediction and exposure data to assess the risk of skin sensitization to fragrance materials. However, the first version of the QRA method does not incorporate aggregate exposure from multiple product use and requires *in vivo* experimental data, including a murine local lymph node assay. Therefore, in this study, we predicted the potency of target chemicals using *in vitro* test results and a machine learning model. Furthermore, to assess the risk of various commonly used chemicals, aggregate exposure was considered. **Methods:** Salicylic acid and methylisothiazolinone were selected as commonly used skin sensitizers. The no expected sensitization induction levels (NESILs) of these chemicals were calculated using a previously developed *in silico* potency prediction model based on XGBoost, a machine learning algorithm. The acceptable exposure level (AEL) was calculated using both the NESIL and applied safety assessment factor, the latter of which is derived from the product type and inter-individual variability. The consumer exposure level (CEL) was calculated on the basis of intended and foreseeable conditions of use. To predict consumer aggregate exposure, we use the probabilistic Creme RIFM aggregate exposure model. Finally, the maximum acceptable concentration level for each product was calculated as the upper concen-

tration limit (UCL) from the exposure amount at which AEL = CEL. **Results:** For both compounds, lower UCL values were obtained when aggregate exposure was taken into account, suggesting that this is a more appropriate risk assessment approach for these commonly used chemicals. For example, the UCL for salicylic acid in hair sprays was 11.77% when aggregate exposure was not considered and 6.83% (aggregate adjusting) when it was, whereas the respective values for methylisothiazolinone were 0.34% and 0.20% (aggregate adjusting). For both chemicals, the NESIL was calculated using the non-animal testing approach. Furthermore, the UCLs calculated with our machine learning model were similar to the measured values obtained from animal experiments. For example, the aggregate adjusting UCL for salicylic acid in bar soap was 2.54% in conventional animal testing and 8.42% when using the non-animal testing approach, whereas the respective values for methylisothiazolinone were 0.40% and 0.24%. **Conclusions:** In conclusion, this quantitative skin sensitization risk assessment model with aggregate exposure considered is a useful approach for the non-animal safety evaluation of chemicals. To put this method into practical use, verification through accumulated case studies using a large number of chemicals is required.

PS 3254 Investigation of the applicability of a new consensus *in silico* model for predicting skin sensitization using combined alternative approaches methods

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Background and Purpose: Skin sensitization is a complex occupational health issue. Historically, it has been assessed with animal tests, which has motivated the development of new approach methodologies and integrated, multi-method assessments due to animal-welfare considerations. To address these limitations, We constructed a consensus model based on three different independent skin sensitization key event (KE)/adverse outcome (AO) s from a rule-based model, a local lymph node assay (LLNA) statistics-based model, and a guinea pig maximization test (GPMT) statistics-based model. Building on this, we developed a rule and statistics-based model of replacement for KE1 (RSRKE1) and performed integrated testing strategy-defined approach (ITS-DA) combining RSRKE1 (KE1), h-CLAT (KE3), and the *in silico* consensus model. Our objective was to build a new ITS-DA for sensitization hazard prediction and benchmark it against the OECD Defined Approaches for Skin Sensitization (DASS) dataset and established *in silico* tools (Derek Nexus and the OECD QSAR Toolbox). **Methods:** We developed a consensus skin sensitization model (Murakami et al., 2025) that integrates a rule-based reactivity component with statistics-based models trained on LLNA and GPMT data and evaluated it within an OECD-aligned ITS-DA spanning KE1 and KE3. Two ITS configurations were assessed: v1 (DPRA + h-CLAT + Derek Nexus) and v2 (DPRA + h-CLAT + QSAR Toolbox). A summed score from KE1 (0-3), KE3 (0-3), and *in silico* (0-1) was used for hazard and Globally Harmonized System (GHS) potency assignments (0-1: not classified; 2-5: Category 1B; 6-7: Category 1A). Across OECD DASS chemicals, we compared KE1 scoring via DPRA versus ADRA and benchmarked *in silico* components using Derek Nexus, QSAR Toolbox, and consensus decision rules—OR, VOTE, and AND. To replace KE1 testing, we introduced RSRKE1, a rule-based surrogate that detects protein reactive structural alerts and assigns 0-3 points via a DPRA/ADRA-calibrated ensemble. We then constructed ITS variants combining RSRKE1, h-CLAT, and the most sensitive the consensus (OR) model and compared their performance with ITS v1/v2 and with versions substituting ADRA for DPRA, using the total battery score for hazard identification and potency subcategorization. **Results:** Replacing Derek Nexus and the QSAR Toolbox with the consensus (OR) model improved performance. On human data, the consensus (OR) model achieved the highest hazard-identification sensitivity (96.4%) and balanced accuracy (70.4%) and delivered the best potency performance for weak sensitizers (GHS Category 1B; 83.9% correct; 71.2% accuracy). When KE1 was scored with RSRKE1 (0-3 points) alongside h-CLAT and the consensus (OR) model, sensitivity increased for both LLNA and human datasets. Balanced accuracy reached 70.4% (exceeding ITS v1: 68.6% and ITS v2: 69.4%), and potency classification correctly identified over 80% of weak sensitizers with accuracy comparable to ITS v1/v2 and superior to DPRA/ADRA for weak substances. **Conclusions:** We developed an *in silico* consensus model that achieved balanced accuracies of 77% (LLNA) and approximately 70% (human), matching or outperforming existing methods. Leveraging its rule-based component, we created RSRKE1 as a higher-accuracy KE1 replacement and showed that an ITS-DA combining RSRKE1 (KE1), h-CLAT (KE3), and the consensus (OR) model delivered sensitivities of about 80% (hazard) and about 70% (potency) for LLNA and about 70% for human data, comparable to current ITS-DAs. These results indicate that the consensus model is suitable for *in silico* ITS-DAs and that RSRKE1, together with a KE3 alternative, can obviate the need for *in vitro* KE1 assays.

PS 3255 Me-Too Validation of the Epi2SensA Method Using EpiDerm™ Model for Skin Sensitization Testing Under OECD TG442D

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Background and Purpose: The Adverse Outcome Pathway (AOP) for skin sensitization has driven the development and publication of OECD Test Guidelines 442C, D, and E, enabling animal-free assessment of skin sensitizing potential of chemicals. In 2023, JaCVAM validated the epidermal sensitization assay (EpiSensA), an *in vitro* method addressing the Key event 2 of the AOP by measuring gene expression of four mechanistically-relevant keratinocyte markers in reconstructed human epidermis (RhE) following chemical exposure. The purpose of this study was to validate Epi2SensA, a similar method, using EpiDerm™ (EPI-200) as an alternative RhE model. Like EpiSensA, Epi2SensA will be used in conjunction with other *in chemico* and *in vitro* tests in the OECD TG442 series as part of an IATA in the AOP framework to identify the sensitization potential of substances for the classification and labeling of skin sensitization hazards. Integration of Epi2SensA in the OECD TG442D will provide laboratories with multiple RhE supplier options and ensure worldwide supply chain reliability. **Methods:** The use of EpiDerm in Epi2SensA required the following adaptation of the EpiSensA protocol: a) increased application volume (10 µL vs 5 µL), b) reduced exposure time (1 hour vs 6 hours), c) modified killed controls using basolateral Triton-X application, and d) increased positive control concentration (1.56% vs 0.78% clotrimazole). The me-too validation study was conducted in 2024-2025 across four laboratories, three naïve laboratories (BRT, USA; Eurofins, Germany; FDSC, Japan) and Mattek as the lead laboratory. The validation study was designed according to OECD Guidance 34 principles and to the Epi2SensA performance standards published by the OECD. After a training phase, the laboratories tested the 20 reference substances in a coded manner, 8 in one run and 12 in three independent runs to assess: a) the between laboratory reproducibility (BLR), b) within laboratory reproducibility (WLR) and c) predictivity. Mattek, Eurofins and BRT tested all reference substances while FDSC tested only eight. After selection of the solvent and dose finding to assess cytotoxicity, gene expression of four biomarkers (ATF3, GCLM, DNAJB4, IL-8) was measured by RT-PCR following exposure of EpiDerm™ tissues to four concentrations of the coded substances. At the end of the experimental phase, the identity of the test materials was decoded and statistical analysis of the results was conducted to assess WLR, BLR, and predictive capacity using both original VRM prediction model and modified criteria. **Results:** Using the original VRM criteria, the average WLR was 63.9%, BLR was 70%, with 66.6% specificity, 90.5% sensitivity, and 83.3% accuracy. Modified criteria adapting the cell viability threshold (60% vs 80%) and prediction model (requiring ≥2 positive genes vs 1) significantly improved performance. With the modified criteria, the average WLR reached 83.3% and BLR achieved 85%, both exceeding the minimum performance standard (PS) of 80% for WLR and BLR. The predictive capacity was 88.9% specificity, 88.1% sensitivity, and 88.3% accuracy which exceeded the minimum PS values of 65%, 85%, and 85%, respectively. The independent peer review of the validation concluded that the Epi2SensA method's overall performance meets the VRM performance standards and demonstrates sufficient robustness. **Conclusions:** The Epi2SensA me-too validation successfully demonstrated that EpiDerm™ can serve as an alternative RhE tissue model for skin sensitization testing under OECD TG442D. Method adaptations specific to EpiDerm™ characteristics, including modified assay parameters, acceptance criteria, and the prediction model, resulted in robust performance meeting the performance standard requirements. Independent scientific peer review of the me-too validation concluded that the Epi2SensA assay is sufficiently robust for OECD submission and potential integration into TG 442.

PS 3256 Interpreting *In Vitro* Permeation Testing Layer-Deposition Data Using a Mechanistic Skin Model in MoBi

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Background and Purpose: Predicting the bioavailability of a chemical following topical application or exposure is essential for assessing the risk and efficacy of dermatological products. *In vivo* studies using preclinical species are often constrained by cost, complexity, and limited translational relevance. As a result, new approach methodologies (NAMs), including *in vitro* and *in silico* tools, are increasingly relied upon in toxicological evaluations. *In vitro* permeation testing (IVPT) with excised human skin can be combined with mechanistic skin permeation models to extrapolate these data toward *in vivo* predictions. However, refining the implementation and understanding the limitations of such models is necessary before they can be widely adopted for regulatory purposes. In this study, IVPT data were compared with simulated results generated using a skin permeation model implemented in MoBi, part of the Open Systems Pharmacology suite. Additionally, layer-deposition data from both experimental and simulated datasets were analyzed to extrapolate the *in vivo* bioavailability of the test compounds. **Methods:** IVPT was performed on four test articles,

caffeine (CAS No. 58-08-2, MW 194.19 g/mol, LogK_{ow} -0.07), salicylic acid (CAS No. 69-72-7, MW 138.12 g/mol, LogK_{ow} 2.26), testosterone (CAS No. 58-22-0, MW 288.43 g/mol, LogK_{ow} 3.32), and mannitol (CAS No. 69-65-8, MW 182.17 g/mol, LogK_{ow} -3.10). Excised human skin (LifeNet Health; cryopreserved, cadaver, leg or back, 500 µm dermatomed) was mounted on Franz diffusion cells (PermeGear; 0.64 cm² active area) connected to a heated water circulator system. Skin temperature and barrier integrity were measured by infrared thermometer and transepidermal water loss (Delfin VapoMeter), respectively. A finite dose (10 µL/cm²) of test article (1% w/v in buffer) was applied to the apical skin surface, and receptor fluid (DPBS) collections were performed at 0, 0.5, 1, 2, 3, 4, 6, and 24 hours post-dosing. Apical wash, stratum corneum tape strips (three per sample), and tissue were collected following the final (24 hour) receptor fluid sampling. All samples were extracted and analyzed by liquid chromatography tandem mass spectroscopy (LC-MS/MS). *In silico* predictions of chemical dermal absorption were collected in parallel. Test article physicochemical properties and test system parameters were input into the MoBi (version 12.0.434) implementation of the (Dancik et al. Adv Drug Deliv Rev 2013) skin permeation model (<https://github.com/Open-Systems-Pharmacology/Skin-permeation-model>) and simulated cumulative permeation, flux, and layer deposition data were generated. **Results:** The Mean ± SEM 24-hour cumulative absorption observed in the *in vitro* permeation test ranked from lowest to highest as follows: mannitol (no detectable absorption), testosterone (2.53 ± 0.20 µg/cm²), salicylic acid (7.70 ± 3.93 µg/cm²), caffeine (14.84 ± 5.30 µg/cm²). The simulated mean 24-hour cumulative absorption, alternatively, ranked from lowest to highest as follows: mannitol (2.07x10⁻²⁹ µg/cm²), caffeine (0.87 µg/cm²), testosterone (1.75 µg/cm²), salicylic acid (99.92 µg/cm²). For testosterone, the model layer deposition data predicted 96.69% of the applied dose would remain on the apical skin surface versus the experimentally observed 91.62 ± 3.40%. Simulated versus experimental comparisons of apical retention for the remaining test articles were as follows: caffeine = 41.94% vs 70.86% ± 5.32%, salicylic acid = 0.06% vs 3.02% ± 0.18%, mannitol = 57.98% vs 20.85% ± 10.24%. **Conclusions:** The mechanistic skin model was limited in its ability to accurately predict the cumulative absorption of the test compounds. The layer deposition data generated, however, was more accurate at correctly ranking the results observed experimentally. This study underscores the value of mechanistic skin permeation models in refining *in vivo* dermal absorption predictions. Integrating *in vitro* permeation testing data with computational models can strengthen predictive accuracy and increase confidence in risk assessments. Conversely, computational models can contextualize experimental findings, offering deeper insights into observed results. In both scenarios, employing multiple NAMs within a weight-of-evidence framework enhances the robustness and regulatory relevance of the assessment.

PS 3257 Next Generation Risk Assessment Case Study: A Quantitative Machine Learning Approach for Skin Sensitization in Oxidative Hair Dyes

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Background and Purpose: To establish a skin sensitization risk assessment method, it is important to conduct a case study on the next-generation risk assessment method (NGRA) using *in vitro* and *in silico* new approach methodologies (NAMs). Previously, we have developed an *in silico* model for the quantitative prediction of skin sensitization potency, combining *in vitro* assay data and chemical property information through a machine learning algorithm. In this study, we focused on oxidative dyes used in hair coloring products and aimed to perform a case study of skin sensitization risk assessment using *in silico*-predicted sensitization potency values, following the quantitative risk assessment approach. **Methods:** As oxidative hair dyes, we selected 2-aminophenol, which has strong sensitization potency, and resorcinol, which has moderate sensitization potency. The consumer exposure level (CEL) was calculated based on product usage amount, retention factor, scalp surface area, and formulation concentrations obtained from patent information. The acceptable exposure level (AEL) was calculated by converting the LLNA EC3 values of the oxidative hair dyes to no expected sensitization induction levels (NESIL) and dividing them by a sensitization assessment factor (SAF). In addition, LLNA EC3 values of the selected oxidative hair dyes were predicted using our original machine learning model (XGBoost), which integrates *in vitro* assay data (ADRA, KeratinoSens™, h-CLAT) and chemical structural descriptors. Finally, the margin of safety (MOS) was calculated by dividing the AEL value by the CEL value. **Results:** The LLNA EC3 values predicted by the machine learning model were 0.62 for 2-aminophenol and 9.76 for resorcinol. Meanwhile, the measured LLNA EC3 values were 0.45 for 2-aminophenol and 6.3 for resorcinol. These predicted values closely matched measured LLNA EC3 data, confirming that the model reproduced sensitization potency adequately. The MOS values calculated based on these predictions were 0.03 (2-aminophenol) and 0.38 (resorcinol), while the MOS values calculated based on actual measurements were 0.02 (2-aminophenol) and 0.24 (resorcinol). The assessed exposure scenarios indicated that the risk associated with resorcinol was lower than that of 2-aminophenol. It should be noted that the MOS values calculated in these scenarios represented

conservative estimates. **Conclusions:** In this study, we attempted a quantitative risk assessment case study of oxidative hair dyes with different skin sensitization potencies. Since the risk assessment in this case depends on the prediction accuracy of the LLNA EC3 values, careful interpretation is required. Nevertheless, the results suggest that this approach could contribute to the implementation of NGRA using *in vitro* and *in silico* NAMs.

PS 3258 Skin sensitization assessment of recycled plastics for consumer product based on dermal sensitization threshold and *in vitro* testing methods

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Background and Purpose: In recent years, the use of recycled materials is being promoted worldwide. However, unlike virgin materials, recycled plastics can contain non-intentionally added substances. Therefore, when recycled plastics are used in consumer products, it is important to consider the potential migration of hazardous substances from packaging and to conduct careful safety assessments. In 2024, the CosPaTox consortium created guidance on the safety assessment of recycled plastics. In the assessment of skin sensitization, because it is difficult to assess each migration individually, application of exposure-based waiving using a dermal sensitization threshold (DST) has been proposed. Although there are three structure-based DSTs: Non-reactive DST, Reactive DST, and HPC DST (Safford et al., 2015, 2011, Nishijo et al., 2020), it has not been thoroughly examined which DST should be applied. Therefore, in this study we examined what kinds of skin sensitizers could migrate from recycled plastics and how to select appropriate DST value. In addition, because assessment using structure-based DST can be difficult for products with high consumer exposure level, we also conducted case studies assessing skin sensitization using *in vitro* testing. In the case studies, we evaluated the potential of applying *in vitro* data-based DST (Nishijo et al., 2022) and benchmarking assessment. **Methods:** To estimate the potential contamination of HPC substances in recycled plastics, Derek Nexus v2.8.0 predictions were performed for substances in CosPaTox substance list (2024). When "Skin sensitization HPC" alert was fired, the substance was classified as an HPC. For *in vitro* tests, migrations from the virgin container and recycled container of a product package were prepared and concentrated 200-fold before testing. The migrations were prepared at 60°C for 10 days using 95% ethanol as the simulant. Migrations above 10 ppb were then analyzed by GC/MS. KeratinoSens™ and h-CLAT were conducted in accordance with OECD Test Guidelines. The *in vitro* data-based DST was calculated by using KeratinoSens™ and h-CLAT data. **Results:** Based on the Derek predictions on the CosPaTox substance list, several substances whose structures match the HPC rules were identified. Although there were very few HPC substances, if a more precise assessment is required, it was considered preferable to apply Reactive DST after carefully reviewing the available information to confirm that the risk of HPC contamination is low. Therefore, we considered it would be desirable to apply Reactive DST if the following two conditions are satisfied: (i) No HPC substances above the HPC DST are detected by instrumental analysis (e.g. GC/MS), and (ii) There is little concern about impurity contamination from the manufacturing process (equipment qualification and/or Surrogate contaminant testing results). Regarding *in vitro* tests, KeratinoSens™ was negative, whereas h-CLAT showed a positive response in CD54 for the recycled container. Butylated hydroxytoluene was considered to be the cause of the positive response, but it is commonly used as an antioxidant in plastic containers and is of low sensitization concern. As a result, the acceptable exposure level calculated based on *in vitro* data-based DST was higher than the Reactive DST for the recycled container examined in this study, indicating that *in vitro* data-based DST is useful when assessment using structure-based DSTs is difficult. Additionally, equivalent responses were observed in both KeratinoSens™ and h-CLAT with the virgin container. Therefore, from a benchmarking assessment perspective, it was considered that the skin sensitization risk of the recycled container is comparable to or lower than that of the virgin container. **Conclusions:** Because there may be concerns that migrations from recycled containers contain HPC substances, it is necessary, in a more precise assessment, to assess the potential for HPC contamination when applying Reactive DST. The results of instrumental analysis and information on the manufacturing process can be used to assess the potential of HPC contamination. When assessment by exposure-based waiving using the structure-based DSTs is difficult, it was found that using *in vitro* data-based DST or benchmarking assessment can be effective.

PS 3259 Multi-Endpoint Characterization of ArtSkin®-RhE for Detecting Retinoic Acid-Induced Skin Irritation and Barrier Dysfunction

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Background and Purpose: With the global transition toward non-animal alternatives in cosmetic and pharmaceutical safety assessment, it has become increasingly important not only to ensure the reliability and reproducibility of reconstructed human epidermis (RhE) models, but also to diversify the evaluation endpoints. In this

study, we aimed to characterize our newly developed RhE model, ArtSkin®-RhE, and assess its applicability for evaluating retinoic acid (RA)-induced skin irritation and toxicity. Unlike strong irritants such as SDS or SLS described in OECD TG 439, RA induces barrier dysfunction and inflammatory responses while causing only minimal reductions in cell viability. Such characteristics are not easily detected by TG 439, which relies primarily on cell viability as the single endpoint for irritation classification. Therefore, this study sought to validate the stability of the ArtSkin®-RhE model and to develop a multi-endpoint irritation assessment approach capable of detecting more subtle and mechanistic skin responses across a concentration-dependent RA exposure. **Methods:** ArtSkin®-RhE was constructed using an air-liquid interface (ALI)-based keratinocyte differentiation protocol. For quality control (QC), MTT-based viability in negative control (NC) tissues, IC₅₀ values derived from SDS exposure, and histological confirmation of a multi-layered epidermal structure were evaluated. Model suitability for irritation testing was verified by determining accuracy, sensitivity, and specificity using the 20 Performance Standard (PS) reference chemicals described in TG 439. RA was applied at five concentrations (10 µM-10 mM). Irritation and toxicity responses were assessed through: MTT assay for cell viability, ICC for functional and irritation-related protein markers, qRT-PCR for differentiation- and inflammation-related genes, TEER for barrier integrity, ELISA for inflammatory cytokine secretion, H&E staining for histological evaluation. **Results:** ArtSkin®-RhE fulfilled all QC and performance standard requirements of TG 439 and demonstrated reproducible responses comparable to commercial RhE models under both NC and PC conditions. Although RA exposure resulted in minimal reductions in viability even at higher concentrations, multi-endpoint analyses (qRT-PCR, ICC, TEER, ELISA) revealed concentration-dependent alterations in barrier function and irritation/inflammatory markers. Additional analyses are ongoing to further validate the consistency and significance of RA-induced inflammatory responses. These findings suggest that ArtSkin®-RhE, when paired with multi-endpoint evaluation, is capable of detecting mild, functional perturbations that may not be captured by the TG 439 viability-only approach. **Conclusions:** ArtSkin®-RhE exhibited structural and functional performance consistent with TG 439 requirements, and the multi-endpoint assessment strategy enabled sensitive detection of concentration-dependent RA responses. This study highlights the potential of ArtSkin®-RhE as a reliable human-based platform for evaluating skin irritation and toxicity, particularly for substances that induce subtle or chronic barrier-related effects that are not fully identified through traditional TG 439 criteria. The results support the broader applicability of multi-endpoint RhE-based assays in mechanistic research and diverse non-animal skin toxicity evaluations.

PS 3260 Metabolomics analysis of human keratinocyte response to chemical allergens

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Background and Purpose: Allergic contact dermatitis (ACD) is a common skin disease caused by drugs and environmental chemicals acting as “chemical allergens”. Keratinocytes (KCs) are the main component of the skin’s epidermal barrier. They play a crucial role in initiating and developing inflammatory responses by releasing pro-inflammatory cytokines into the skin microenvironment. However, the mechanisms by which KCs integrate chemical stress remain incompletely understood. In this study, we investigated the impact of three well-known chemical allergens - hydroxycitronellal (HC), cinnamaldehyde (CINA) and methylisothiazolinone (MIT) - on the metabolome of human primary KCs using a metabolomics approach. **Methods:** Normal human primary epidermal KCs (HPEKp) were exposed to HC, CINA or MIT at low and high concentrations (100 and 500 µM for HC, 25 and 75 µM for CINA, and 10 and 40 µM for MIT). Exposure durations were 1h, 6h, and 24h, with cytotoxicity remaining below 30% in all cases. Intracellular metabolite extraction was performed in culture dishes with a cold H₂O/MeOH mixture (30:70, v/v). The metabolomes of cells treated with chemical allergens or vehicle controls (n = 12 per group) were analyzed using C18- and HILIC-based liquid chromatography coupled with high-resolution Q-Orbitrap mass spectrometry (LC-HRMS). Data processing was achieved using the R package XCMS and Workflow4Metabolomics Galaxy platform tools. Metabolite annotation was performed using an in-house spectral database. In parallel, a panel of pro-inflammatory cytokines (TNFα, IL-1α, IL-1β, IL-18, and CXCL8) was quantified in the extracellular medium using a Meso Scale Discovery (MSD) multiplex chemiluminescent immunoassay (n = 3 per group). **Results:** The metabolomics analysis identified 180 metabolites with high confidence levels (levels 1 or 2 according to the criteria of the Metabolomics Society). To assess differences between allergen-treated cells (HC500, CINA75 and MIT40) and vehicle controls (medium and 0.1% DMSO), multivariate analysis using partial least squares discriminant analysis (PLS-DA) was performed at each time point for the 180 metabolites. On the PLS-DA score plot of the first two components, the HC500-treated cells were clearly separated from the other allergen-treated cells and vehicle controls at all time points. CINA75-treated cells clustered apart from vehicle controls after 6h and 24h of exposure, while MIT40-treated cells showed separation only at 1h. Univariate analysis (Wilcoxon test with Benjamini-Hochberg correction) revealed that 109, 106, and 34 out of the 180 identified metabolites showed significant differences (p-value ≤ 0.05) between the HC500, CINA75- and MIT40-treated cells and their respective vehicle control at one or more time points. A comparison between differential metab-

olites at low and high exposure concentrations of each chemical allergen indicated that more metabolites were altered at higher concentrations, suggesting concentration-dependent effects of the chemical allergens on the KC metabolome. Notably, glutathione (GSH) was the only metabolite affected by all three chemical allergens, regardless of concentration. The significant metabolites in the univariate analysis were subsequently used for pathway analysis, which revealed several pathways commonly altered by HC, CINA and MIT, including glutathione metabolism, amino acid metabolism, glycerophospholipid metabolism, pentose phosphate pathway, and pyrimidine metabolism. Moreover, distinct inflammatory cytokine patterns were observed among the allergen-treated cells. **Conclusions:** This study represents the first comprehensive evaluation of metabolome remodeling and cytokine secretion induced by HC, CINA and MIT in human primary KCs. Significant metabolic changes were observed in allergen-treated cells showing time-, treatment- and concentration-dependent variations. Although the three chemical allergens induced distinct modifications in the abundance of intracellular metabolites in terms of direction and intensity, they also commonly affected several key metabolic pathways. These findings provide a strong foundation for further investigations into the role of metabolic reprogramming in the inflammatory response of KCs to chemical allergens in ACD.

PS 3261 Assessment of skin barrier function: A comparison of transepidermal water loss (TEWL) and skin absorption studies

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Background and Purpose: *In vitro* human skin absorption studies are essential for the safety evaluation of chemicals, crop protection products, consumer healthcare items, and cosmetics. According to the OECD 428 guideline, the barrier function integrity of skin samples studies must be verified. Commonly used barrier integrity assays include transepidermal water loss (TEWL), transepithelial electrical resistance, and chemical absorption tests. This study investigates whether TEWL alone is sufficient to detect skin barrier impairment following exposure to irritants or mechanical damage. **Methods:** Human abdominal skin disks from three donors were processed within 24 hours post-surgery and exposed for 24 hours to one of the following two irritants: (i) a finished cosmetic product classified as strongly irritant by MTT assay, and (ii) 10% SDS solution. TEWL was measured using the Aquaflex closed-chamber system (Biox Systems). In parallel, skin absorption of 7 compounds (Methylprednisolone, Umbelliferone, Sulfamethazine, Clopidogrel, PABA, Benzydramine, Carbazeren) was quantified by LC-MS/MS after 2-hour exposure in Franz cells. Mechanical damage was induced using two methods: needle punctures and a derma-roller. Additionally, a retrospective analysis of >1000 TEWL measurements collected over four years was performed to assess variability and influencing factors. **Results:** TEWL was less sensitive than skin absorption measurements in detecting barrier impairment. Irritant cosmetic exposure did not significantly alter TEWL, yet skin absorption increased by 45 (for Methylprednisolone succinate) to 82% (Sulfamethazine and Umbelliferone) across all compounds compared to untreated skin. TEWL rose markedly only after strong irritant exposure (10% SDS) or severe mechanical damage, increasing from 13 to 52 g·m⁻²·h⁻¹ on average. Mild mechanical injuries failed to elevate TEWL despite a 50% increase in skin absorption. Historical TEWL data revealed an average of 10.5 g·m⁻²·h⁻¹ (range: 7-15 g·m⁻²·h⁻¹) with only 3% of samples exceeding the 15 g·m⁻²·h⁻¹ threshold and no significant correlation to age, BMI, or sex. **Conclusions:** Irritant exposure and mild mechanical damage significantly enhance skin permeability, but these changes are not consistently detected by TEWL. While TEWL provides a rapid preliminary screening of skin integrity, it should be complemented by additional methods such as skin absorption assays for reliable assessment of barrier function.

PS 3262 Human skin equivalents reveal multiple cell death modes in mustard agent-induced dermal injury

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Background and Purpose: Chemical warfare agents represent a major global health concern due to their high morbidity and mortality. Particularly, mustard agents pose a particularly serious public health threat, as they can be easily produced, cause severe injury and lack effective medical countermeasures. While apoptosis and necrosis are known mustard-induced cell death pathways, the involvement of other modes of cell death, such as parthanatos, pyroptosis, necroptosis, and ferroptosis, remains largely unexplored. Understanding these diverse cell death modes could reveal new options for therapeutic intervention; however, progress has been limited by the lack of physiologically relevant human skin models. To address this limitation, we developed a three-dimensional human skin equivalent (HSE) that accurately replicates human skin biology and enables the study of vesicant-induced injury and cell death mechanisms. **Methods:** HSEs consisted of a stratified epidermis of keratinocytes overlying a dermal compartment containing fibroblasts, adipocytes, and endothelial cells. To evaluate their suitability for studying the toxic effects of vesicants, we established a novel method for exposing HSEs to sulfur and nitrogen mustard vapors, reproducing the likely route of exposure during a chemical attack. Histological and genotoxic markers of cellular injury were analyzed as well as the activation of specific cell

death pathways over time. **Results:** HSEs faithfully recapitulated key structural and functional characteristics of human skin, including epidermal stratification, stromal complexity, and spontaneous vasculogenesis. Upon exposure to mustard agents, HSEs exhibited dose-dependent vesication, an effect rarely observed in animal models, as well as DNA damage, as indicated by H2A.X staining. Time-course analyses revealed immunohistochemical evidence for the activation of multiple cell death pathways, including apoptosis, parthanatos, pyroptosis, necroptosis and ferroptosis. These findings were confirmed by immunohistochemistry, TUNEL staining, adenylate kinase release and qPCR analysis. **Conclusions:** Our findings support the use of HSEs as robust and physiologically relevant platform for studying skin pathogenesis of vesicant exposure. HSEs exhibited dose-dependent injury responses consistent with human pathology while revealing the involvement of diverse cell death pathways, offering potential targets for therapeutic development. By incorporating multiple cell types, these models provide a more authentic representation of the complex host responses to vesicant agents. Collectively, our results highlight HSEs as an innovative new approach methodology to reduce the use of animals for studying mechanisms of vesicant injury and screening medical countermeasures. It is anticipated that HSEs will make substantial contributions to the fundamental understanding and treatment of toxicological insults in human skin.

PS 3263 Doxorubicin-induced skin vascular permeability: a potential mechanism for cutaneous toxicities

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Background and Purpose: Doxorubicin is an anthracycline chemotherapy drug used in multiple cancers. To avoid cardiotoxicity, its long-circulating liposomal form (PEGylated doxorubicin, Doxil®) was approved in 1997. While Doxil avoids the heart tissue, it causes palmar-plantar erythrodysesthesia (PPE), a cutaneous toxicity characterized by pain, blistering, and erythema in pressure-prone areas (feet, palms, waist, and armpits). High-grade toxicity leads to suffering and drug discontinuation. As the mechanisms of accumulation of Doxil in the skin are not clear, this was the focus of our study. We previously observed that skin vasculature in the pressurized areas, such as feet, demonstrates leakiness and extravasation of liposomes. We hypothesize that initial extravasation of the drug in the skin increases subsequent drug accumulation. Therefore, this study investigates whether the accumulation of Doxil in the skin enhances permeability. **Methods:** We developed a mouse model of systemic or topical skin toxicity, where BALB/c mice were injected with Doxil (8 mg/kg) 3 times per week intravenously or Doxil/doxorubicin was injected intradermally (20 µg). The development of toxicity in mouse skin was characterized by redness, swelling, and nail necrosis in the hindlimb fingers. Following Doxil treatment, mice were injected with fluorescently labeled liposomes (Doxil formula), and their accumulation in the skin was quantified 24 h post liposome injection with fluorescence imaging and optoacoustic tomography. H&E staining was performed for histological assessment of toxicity, which was statistically analyzed using one-way ANOVA (with appropriate post-hoc testing; $p < 0.05$) for comparison among the three groups (control, IV Dox, ID Dox; $n = 3$ per group). **Results:** We found that the accumulation of doxorubicin in the skin increases vascular permeability. Thus, doxorubicin causes up to a 20-fold increase in the accumulation of fluorescent liposomes. Tissue levels of TNF- α , IL-1 β , and IL-6 did not increase after intradermal injection of doxorubicin ($n = 3$ per group), suggesting the absence of any strong inflammation. Histological analysis revealed that doxorubicin caused a modest increase in cell infiltration, with a 3.8-fold increase following intravenous delivery and a 1.57-fold increase following intradermal delivery. Vascular density showed a 1.51-fold increase for intradermal administration and no significant change with intravenous administration. Epidermal thickening was observed, with a 1.95-fold increase in the intravenous group and a 1.33-fold increase in the intradermal group. Stratum corneum integrity was also affected, showing a 2.42-fold increase in degradation and a 4.01-fold increase in detachment following intravenous administration, compared to 1.21-fold and 1.82-fold increases in degradation and detachment, respectively, in the intradermal group. **Conclusions:** We propose that skin damage amplifies Doxil accumulation, initiating a detrimental "vicious" cycle that progressively worsens tissue damage and symptoms in affected patients. This result suggests new nano formulations are needed for the prediction and prevention of cutaneous toxicities. Funding for this work was provided by the National Institutes of Health R01 CA289447, as well as the Skaggs Scholar grant to DS and SG.

PS 3264 Exercises in Applying Chemical Class Skin Sensitization Hazard Determinations

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Background and Purpose: Direct and prolonged exposure to certain chemicals in consumer products can potentially produce skin sensitization reactions. To minimize the risk of skin sensitization for consumers, hazard assessments can be utilized to classify chemicals that a consumer may come into contact with in products either as non-sensitizers or sensitizers with varying degrees of potency. A typical hazard assessment involves a literature search and evaluation of available toxicological test data. However, in some cases, the chemical of concern has not been tested for skin sensitization potential and read-across to structural analogs may be necessary. This can be true particularly for chemicals that are impurities, breakdown products, or are only tentatively identified in laboratory analysis. Assessing such chemicals can be time intensive, particularly if there is a large dataset of chemicals to evaluate. The objective of our research was to identify and create general hazard determinations for well-defined classes of chemicals with shared structures, which will reduce the effort required to evaluate newly detected chemicals lacking chemical-specific testing data. The results of the first four class evaluations are presented here. **Methods:** We reviewed an in-house database of more than 7,500 skin sensitization chemical assessments for classes of chemicals with similar structures to identify patterns in the conclusions of the assessments. Four groups of chemicals were chosen for investigation based on frequency of detections within consumer product extracts: alkanes, ethylene glycols, silanes and siloxanes, and aldehydes. Available data were tabulated for each previously evaluated member of the class. For the purposes of determining hazard for the chemical class, we relied on qualitative hazard conclusions (i.e., non-sensitizer, weak sensitizer, moderate sensitizer, or strong sensitizer). Based on the review, we developed definitions governing the allowable members of each chemical class. **Results:** Over 40 similarly structured members with hazard determinations were identified within each of these chemical classes. In all but a few cases, chemicals in the class shared the same skin sensitization classification. Skin sensitization hazard was concordant in greater than 97% of members in each class, with the exception of aldehydes. Among the aldehydes identified, saturated aliphatic aldehydes with a carbon chain length greater than 13 carbons were found to be non-sensitizing, while saturated aliphatic aldehydes with a smaller carbon chain length were sensitizing. In addition, unsaturated aliphatic aldehydes were found generally to be sensitizers. In the few instances where the hazard determination of one chemical differed from the rest of the chemical class based on experimental data, the results were not compelling. For example, cyclooctane (CAS 292-64-8) identified among the alkanes as a very weak sensitizer based on a positive mouse local lymph node assay (LLNA) in which an EC3 of 57.1% was derived. Such a high concentration is implausible in the consumer products of interest. The remaining 406 alkanes were all classified as non-sensitizers and this classification was judged suitable for the class. The remaining two chemical classes, ethylene glycols and silanes and siloxanes, were also considered to be non-skin sensitizers, as long as chemicals met the class definition. **Conclusions:** Applying hazard determinations for defined chemical classes to newly detected chemicals that fit within those classes reduces the time and resource burden of assessing large datasets of extracted chemicals. However, we found that, in some cases, a small number of chemicals had hazard determinations that differed from the majority of members in the same chemical class. Given these examples, we suggest that chemical class hazard determinations should only be applied to newly detected chemicals with no experimental skin sensitization data and no distinct features that deviate from the class definition. As illustrated by the aldehydes identified, it is important to carefully define the characteristics of a chemical class. Class-based determinations provide a weight-of-evidence (WoE) approach to the assessment of chemicals that lack experimental data, but should be conducted within a framework that defines and justifies the inclusion of new members.

PS 3265 Reapplication of the Type IV Hypersensitivity Quantitative Risk Assessment to Assess Ingredients Used on Canines

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Background and Purpose: A quantitative risk assessment (QRA) framework has been effectively developed and applied to evaluate the risk of human skin sensitization from contact allergens. So far, the QRA has not been applied in a canine risk assessment for establishing concentration limits of ingredients with Type IV hypersensitivity potential. Outlining this risk assessment is critical, as shampoos are known to cause allergic contact dermatitis (ACD) in canines, and rates of canine skin allergy insurance claims (both Type I and Type IV summed together) in the US are rising by ~10% each year. While rare, Miniature Schnauzers experiencing ACD can develop a severe and potentially fatal condition known as superficial suppurative necrolytic dermatitis. Additionally, humans have developed ACD after washing their dogs with dog shampoo. These facts underscore the importance of implementing a QRA framework to effectively assess canine personal care products for both human and dog skin sensitization potential. **Methods:** To address this gap, we conducted a

thorough literature review on canine Type IV hypersensitivity and identified potential differences which might demand an adaptation of the QRA. Historic induction of sensitization studies, elicitation patch testing reports, studies with repeated topical application of contact allergens, and skin sensitization reviews regarding dogs were all analyzed for this review article. **Results:** We summarize data from the literature demonstrating that all four key events in the skin sensitization adverse outcome pathway (1. Covalent Interaction with Skin Proteins, 2. Keratinocyte Stress Response, 3. Dendritic Cell Activation, and 4. T-Cell Proliferation) are present in dogs as they are in humans, guinea pigs, and mice. The hazard potential of sensitizers for canines is compared with the established sensitization QRA framework for humans. While there are only 5 chemicals with induction of sensitization data in dogs, only 3 were able to produce positive responses while all 5 have sensitized mice, guinea pigs and humans at equivalent or reduced concentrations as those tested on dogs. For example, cutaneous application of the strong sensitizer para-nitrosodimethylaniline up to 30% was unable to sensitize dogs, but concentrations as low as 2.5% and 0.1% can sensitize mice and guinea pigs respectively. This data further supports why mice and guinea pigs were selected as the model organisms for skin sensitization due to their heightened sensitivity for this endpoint compared to other animals. The topical repeat dose dataset consists of 13 contact allergens applied to dogs. All show equivalent or reduced potency compared to mice, guinea pigs, or humans. The limited collective suggests that dogs are not more susceptible to sensitizers than humans; therefore, safety-defined concentration limits from Type IV hypersensitivity risk assessments for humans can be re-applied to canines so long as their unique exposure considerations are factored in. **Conclusions:** Using the principles in this review, one can evaluate chemical sensitizers to support the safe development of new topical formulations for canines. The result of applying our current best approaches for assessing ACD to canine products should protect both the pet and the owners.

PS 3266 Optimization of the Human Cell Line Activation Test (h-CLAT) for the Identification of Photo-Allergens

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Background and Purpose: Photosensitization refers to light-induced tissue effects upon exposure to photoreactive chemicals and ultraviolet (UV) or visible light. Two primary mechanisms are recognized: phototoxicity, a direct, non-immune reaction leading to cell damage, and photoallergy, an immune-mediated delayed hypersensitivity response that occurs when light converts a chemical into a sensitizing agent capable of inducing allergic contact dermatitis. Photoreactivity is an emerging area of interest in the field of *in vitro* toxicology and increasingly seeks to differentiate between photoirritants and photoallergens using new approach methodologies (NAMs). One strategy is to adapt existing skin sensitization assays, such as those described in OECD Test Guidelines 442C-E, by incorporating a light-exposure step. However, published information on the photo-modified human cell line activation test (h-CLAT) procedures is scarce. Herein describes the adaptation, optimization, and transfer of the photo-h-CLAT assay for identifying photo-induced allergens that activate dendritic cells. Following optimization, the assay's performance was evaluated using a set of ten reference chemicals with compelling evidence of photoallergic potential. **Methods:** The photo-h-CLAT was established based on previously reported protocols (Hino, 2008; Hoya, 2009) and further refined using procedures developed by a partnering laboratory. The assay combined the standard h-CLAT method (OECD TG 442E) with UVA irradiation at an intensity of 5 J/cm² to compare cellular responses in the presence or absence of light. Following exposure to a test chemical, THP-1 cells were harvested and stained with antibodies against cell surface markers, CD54 and CD86, and expression was quantified by flow cytometry. Photoallergic potential was determined by comparing the relative fluorescence intensity (RFI) under light versus dark conditions. Compounds were classified as positive if the light-to-dark RFI ratio was ≥ 2.0 for CD54 or ≥ 1.5 for CD86. Optimization steps included evaluating extended antibody incubation times, the use of phenol red-free medium instead of DPBS, and adjusted test chemical exposure times to improve assay consistency. The reference chemical set was selected based on well-characterized literature containing clinical data for photoallergic responses. **Results:** During assay transfer and optimization, three chemicals, chlorpromazine, (CPZ), 6-methylcoumarin, and ketoprofen, were initially tested to evaluate reproducibility and performance thresholds. CPZ was tested 25 times and consistently exceeded the 2.0 CD54 threshold at 1.4 $\mu\text{g/mL}$ under UVA exposure but did not reach the 1.5 CD86 threshold. These results confirmed CPZ as a reliable positive control for photoallergic activation of dendritic cells. 6-Methylcoumarin exceeded the CD54 threshold (6.1 $\mu\text{g/mL}$) but not the CD86 threshold. Ketoprofen showed variable outcomes across four trials, some exceeding the CD54 threshold, others the CD86 threshold, and some negative for both. Following optimization, the full set of ten reference chemicals was evaluated. The adapted photo-h-CLAT correctly identified nine of ten compounds as photoallergens: 3,3',4',5-Tetrachlorosalicylanilide, CPZ, 6-Methylcoumarin, Bithionol, 4-Methyl-7-ethoxycoumarin, Musk Ambrette, Sulfanilamide, Lomefloxacin Hydrochloride, and Fencitol. One material, 7-Methoxycoumarin, tested negative despite literature evidence of photoallergenicity. The overall predictive accuracy was 90%, and optimization modifications did not affect the assay's classification outcomes. **Conclusions:** The optimized photo-h-CLAT successfully identified known photo-allergens through the incorporation of UVA exposure into the standard h-CLAT framework, demon-

strating its potential as an *in vitro* assay for assessing photo-allergic haptens. While a single false negative was predicted among ten reference chemicals, the overall results support the feasibility of the photo-h-CLAT for identifying chemicals that activate dendritic cells following light exposure. Continued evaluation using broader chemical sets will help further validate this method and strengthen its role among NAMs for photoallergy assessment.

PS 3267 Hyposkin as a human-based NAMs platform for local toxicity and formulation safety assessment

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Background and Purpose: In drug development, the appropriate design of the drug formulation is essential to ensure the successful commercialisation of a safe, stable, and effective product. Despite scientific advances, preclinical evaluation of local tolerance still relies largely on animal testing due to the lack of human-relevant *in vitro* alternatives accepted by regulatory agencies. Developing and validating translational, human-based New Approach Methodologies (NAMs) is therefore essential to improve predictive accuracy and reduce reliance on animal models. HypoSkin®, a biostabilized *ex vivo* human skin model developed by Genoskin, preserves the epidermis, dermis, and hypodermis, offering a physiologically relevant immunocompetent and injectable platform for assessing formulation-induced toxicity. **Methods:** Subcutaneous injections of various formulations of a test item were administered into HypoSkin® explants. The explants were derived from the skin of 3 human donors. To validate the *in vitro/in vivo* correlation of the model, control compounds were added including apomorphine, a known cytotoxic agent and a reference formulation previously associated with local toxicity in minipigs. Multiple complementary readouts were applied: macroscopic imaging, histopathological evaluation, TUNEL assay for apoptosis, LDH release assay for cytotoxicity, and an integrated control-based scoring system for comparative analysis across donors and replicates. **Results:** The HypoSkin® platform successfully identified toxic reference formulations, demonstrating sensitivity and validating an *in vitro-in vivo* correlation. Macroscopic imaging and histopathology consistently detected compound-induced alterations, confirming their robustness and reproducibility across donors. However, high variability in TUNEL results limited the interpretability, likely reflecting interindividual differences inherent to human skin. Similarly, LDH assays exhibited limited sensitivity to controls, highlighting the need for additional quantitative endpoints. Both assays were excluded from the final scoring system. These findings underscore both the advantages and practical challenges of human skin-based systems in early safety screening. **Conclusions:** This study validates HypoSkin® as a translational alternative to animal-based local tolerance assessment. Its ability to detect formulation-induced tissue alterations in a human model supports its potential use in preclinical safety evaluation. Continued optimization of assay design and endpoint selection will further enhance reproducibility, positioning the *ex vivo* skin model as a valuable NAMs candidate to support future regulatory acceptance in local toxicity assessment.

PS 3268 Microfluidic co-culture of iPSC-derived sensory neurons and human melanocytes demonstrates neuron-melanocyte interactions relevant to melanogenic pathways and neurocutaneous toxicology

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Background and Purpose: Melanocytes and peripheral sensory neurons both arise from neural crest cells during embryonic development, reflecting their shared lineage. As development progresses, Schwann cell precursors migrate along sensory axons and can differentiate into melanocytes within the skin. This process results in the formation of a specialized neurocutaneous interface—a site where neurons and melanocytes directly interact—which continues to function into adulthood. Investigating this physiological crosstalk is essential for understanding how neurons influence pigmentary processes and how the skin responds to neurotoxic insults. However, studying these interactions *in vitro* has been limited by the distinct media requirements of human induced pluripotent stem cell (iPSC)-derived sensory neurons and melanocytes, making their co-culture technically challenging. To address this limitation, this study used a compartmentalized microfluidic culture system. This model enables the controlled co-culture of iPSC-derived sensory neurons and melanocytes, facilitating the investigation of their reciprocal molecular influences—particularly on melanogenic signaling pathways. **Methods:** iPSC-derived neural crest cells were differentiated into sensory neurons within one microchannel compartment of DUALINK chips. Upon neuronal maturation and establishment of neuritic extensions through microchannels, primary human epidermal melanocytes were seeded in an adjacent compartment. Cultures were maintained under fluidically isolated conditions for 72 hours before termination. Melanocyte maturation and pigment-related markers were evaluated using RT-qPCR and immunocytochemistry. **Results:** Melanocytes exposed to extended sensory neurites exhibited pronounced morpho-

logical maturation and melanin accumulation compared to controls. RT-qPCR analysis revealed significant upregulation of TYR, MITF, PMEL, Rab27A, MYO5A, and MLPH expression, indicating enhanced melanosome biogenesis and trafficking. Immunostaining corroborated increased tyrosinase and MITF accumulation in neurite-associated melanocytes, suggesting that neuronal factors or physical contact modulate melanocyte activity *in vitro*. **Conclusions:** This microfluidic co-culture platform successfully reconstructed neurocutaneous interactions using human iPSC-derived cells. The observed neurite-dependent melanogenic activation highlights a mechanism by which neurotoxicants or skin-targeted drugs could alter pigmentation via neuronal influence. By enabling physiologically meaningful hetero-cellular interaction, the DUALINK system serves as an advanced platform to develop *in vitro* model for evaluating neurocutaneous toxic effects, screening for drug-induced pigment loss, and studying factors that promote melanocyte over activity.

PS 3269 Advanced Human-Based *In Vitro* Models for assessing Cardiac Toxicity and Arrhythmia Mechanisms

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Background and Purpose: Background/Purpose Cardiac toxicity and arrhythmias, particularly atrial fibrillation (AF), represent major contributors to morbidity and mortality worldwide. Preclinical evaluation of cardiac safety often depends on animal models that inadequately reflect human-specific electrophysiological mechanisms, thereby limiting translational predictivity. To overcome these limitations, we established an atrial fibrillation assay using human induced pluripotent stem cell (hiPSC)-derived atrial cardiomyocytes and a real-time impedance-based toxicity monitoring system, to enable quantitative assessment of electrophysiological function and cardiotoxic responses in a high-throughput, human-relevant context. **Methods:** hiPSC-derived atrial cardiomyocytes (Axol Biosciences Ltd.) were pre-cultured for 6 days in 96-well plates and subjected to electrical tachypacing at 2.5 Hz for 23 h using the CardioExcyte 96 system to induce an AF-like phenotype. Electrophysiological parameters, including extracellular field potential duration (FPD) and beat duration, were recorded before and after pharmacological intervention. In parallel, impedance-based assays using the AtlaZ system (6 × 96-well plates) employed Electric Cell-substrate Impedance Sensing (ECIS) across 0.1-100 kHz frequencies to monitor cell adhesion, proliferation, and cytotoxicity in real time. Human cardiomyocyte-like cells (H9C2) were exposed to a panel of cardiotoxic compounds, such as Vincristine, BMS-986094, and Doxorubicin, over acute (24-48 h) and chronic (up to 7 days) durations to quantify dose-dependent structural and functional effects. **Results:** In the hiPSC-AF model, hERG channel blocker dofetilide (1-10 nM) produced a clear, concentration-dependent prolongation of FPD by up to 48 ± 4% at 10 nM compared to baseline, along with a 37 ± 5% increase in beat duration, reflecting delayed repolarization and restored rhythmicity consistent with clinical QT-prolongation data. These findings validated the model's sensitivity to hERG-mediated pharmacodynamics. Impedance-based toxicity profiling revealed distinct compound-specific signatures. Vincristine induced a 25% reduction in Cell Signal after 48 h at ≥100 nM, indicating disrupted cell adhesion and viability. BMS-986094 caused a progressive 60% linear decay in impedance over 72 h at 10 μM, suggesting cumulative structural degradation and metabolic impairment. Doxorubicin triggered a concentration- and time-dependent decline in impedance (IC₅₀ ≈ 1.2 μM after 48 h), consistent with oxidative stress-induced cardiomyocyte damage. In contrast, ET-1, Nilotinib, and Erlotinib exhibited no significant changes (<5%) across all concentrations tested, confirming assay specificity. Multi-frequency impedance spectra provided additional insights: low-frequency data (≤2 kHz) reflected loss of barrier integrity and adhesion, while high-frequency signals (>40 kHz) captured changes in cell volume and proliferation dynamics. Long-term monitoring revealed early morphological alterations up to 24 h before cell death became evident microscopically, underscoring the method's predictive capacity for early-stage cardiotoxicity detection. **Conclusions:** Combining hiPSC-derived atrial electrophysiology with impedance-based cytotoxicity assays offers a comprehensive human-relevant approach to cardiac safety assessment. These systems provide quantitative, label-free, real-time data on functional and structural cardiomyocyte responses, capturing both acute and chronic toxicity mechanisms. The dofetilide response validates the electrophysiological platform for screening rhythm-modulating compounds, while impedance analyses accurately detect early toxic signatures of diverse agents. Together, these technologies represent a powerful framework for predictive cardiotoxicity testing, improving translational fidelity, reducing animal model dependence, and accelerating the development of safer therapeutics targeting cardiac function.

PS 3270 Human iPSC-Derived Mammary Gland Organoids for Non-Genotoxic Carcinogen Hazard Assessment

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Background and Purpose: Non-genotoxic carcinogens (NGTxCs), including endocrine-disrupting chemicals (EDCs) and oxidative stress-inducing agents, have been increasingly implicated in breast cancer initiation, particularly in individuals with

genetic susceptibility. Conventional breast cancer cell lines used for hazard assessment already carry oncogenic mutations, limiting their relevance for studying early carcinogenic events. To overcome these limitations, we established a physiologically and genetically adaptable human induced pluripotent stem cell (hiPSC)-derived mammary gland organoid model. This platform aims to provide a human-relevant, mechanistically informed New Approach Methodology (NAM) for studying early molecular and phenotypic events during mammary carcinogenesis. **Methods:** hiPSC-derived mammary organoids were generated through stepwise differentiation and characterized for structural and functional fidelity by immunostaining for lineage markers (KRT8, KRT14, SMA, TBX3, ESR1), hormone-responsive milk protein production, and mammary fat pad repopulation *in vivo*. To evaluate chemical responses, organoids were exposed for 24 hours to compounds with distinct modes of action (MOAs), including Aflatoxin B1, Bisphenol A (BPA), Phorbol 12,13-dibutyrate (PDBu), and Sulforaphane. Exposure responses were assessed using organoids expressing an endogenous Fucci cell cycle reporter and SRXN1 oxidative stress reporter, combined with transcriptomic profiling to capture molecular pathway activation. To model genetic susceptibility, TP53-knockout (p53KO) organoids were generated using CRISPR-Cas9. Dextran diffusion assays were performed to assess lumen filling, epithelial polarity, and barrier integrity. **Results:** HiPSC-derived mammary organoids exhibited distinct luminal and basal cell organization, Robust differentiation was achieved for wild-type, SRXN1-GFP, Fucci, and p53KO hiPSCs into mammary organoids, maintaining transcriptomic stability throughout differentiation and across exposures. Confocal imaging revealed concentration- and time-dependent cytotoxicity and reporter activity across several compounds. Transcriptomic analyses demonstrated compound- and concentration-specific responses, reflecting their distinct MOAs, and provided mechanistic insights into oxidative stress, and ERK pathway. p53KO organoids exhibited disorganized architecture and enhanced transcriptional sensitivity to chemical exposures, particularly in cell cycle-related pathways. Dextran diffusion assays showed measurable differences in lumen filling and epithelial polarity, supporting the use of these organoids for phenotypic carcinogenic endpoint assessment. **Conclusions:** This hiPSC-derived mammary organoid system provides a human-relevant, mechanistically rich platform for evaluating non-genotoxic carcinogenic hazards. Integration of live-cell reporters, confocal imaging, and transcriptomics enables quantitative, pathway-level assessment of chemical mode of action and genetic susceptibility. Ongoing work includes expanding the chemical library, introducing additional cancer driver mutations (BRCA1, PTEN, TP53). Together, these advances establish a New Approach Methodology (NAM) for mechanistic, human-based carcinogen hazard assessment. This project is financially supported by European Partnership for the Assessment of Risks from Chemicals (PARC) (This partnership has received funding from the European Union's Horizon Europe research and innovation programme under Grant Agreement No 101057014)

PS 3271 IPS-KOM : Immunocompetent Kidney Organoids as an Advanced *in vitro* Model for Immune-related Kidney Injury.

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Background and Purpose: Immune-mediated kidney injury is a major contributor to drug-induced nephrotoxicity. Although this mechanism has been extensively studied, modelling this adversity *in vitro* remains challenging due to the structural complexity of the kidney and the absence of immune components in most models. Recently, kidney organoids have emerged as a promising *in vitro* platform for studying kidney injury because they recapitulate key features of nephron architecture and function. However, their lack of immune cells limits their ability to relevantly reflect mechanisms of this injury. To address this limitation, we established a co-culture system of the immunocompetent kidney organoid model called IPS-KOM by incorporating kidney organoids with polarized macrophages, as the main immune cells orchestrating the progression of immune-mediated kidney injury. **Methods:** To generate the IPSKOM we incorporated kidney organoids differentiated from RFP-H2B hiPSC using the Takasato protocol and hiPSC-derived monocytes from the Orlova protocol. Upon the kidney organoid maturation, the organoids were sensitized with various cytokines known to be secreted during the course of kidney injury. Following, hiPSC-derived macrophages (M0, M1 and M2) are introduced to the sensitized organoids. This co-culture kidney organoids model was imaged live for 24 hours to investigate the invasion (temporal) dynamics. Furthermore, we applied a high-content image analysis pipeline to quantify the number of invading macrophages. In addition, we also investigate the mechanism of the immunogenic interaction between the kidney organoids and macrophages by performing immunostaining on the organoids as well as profiling the gene expression and secretome of the co-culture system. **Results:** Our findings showed an increase of macrophage invasion to the inflammatory sensitized kidney organoids including TNFalpha treatment which was associated with the upregulation of the NFkB target gene ICAM1. Interestingly, based on our quantification, different macrophage subtypes exhibit distinct activity upon different cytokines. Overall, our data suggests that the immunocompetent kidney organoids exhibit expected specific functionality reflecting immune-mediated kidney injury. Further investigations on the gene expression and secretome profiles confirmed the mechanisms of this immunogenic interaction. **Conclusions:** Our studies demonstrate a promising novel co-culture model for immunocompetent kidney organoid models. IPS-KOM represents a potential *in vitro* platform to study immune-mediated kidney

injury in a more physiologically relevant context. Ultimately, this model could serve as a holistic and high-throughput approach for disease modeling, chemical testing and safety assessment related to kidney injury.

PS 3272 p,p'-DDE Induces an Epidermal Phenotype and Perturbs Cell State and Stem Cell Markers in Mammospheres

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Background and Purpose: Breast cancer is the second leading cause of cancer deaths in U.S. women. Endocrine disruptors, such as the DDT metabolite DDE, are environmental factors linked to breast cancer risk. Emerging evidence shows that during carcinogenesis, cells can reacquire stem cell-like traits, losing their identity and exhibiting phenotypic plasticity. In this state, breast cells can transition from epithelial to mesenchymal (EMT) phenotypes or exist in a hybrid luminal/basal state, identified by their co-expression of keratin 8 and 14, a luminal and basal marker, respectively. Furthermore, breast cell populations that are CD44+/CD24- yield undifferentiated basal/mesenchymal cell properties and may indicate more favorable tumor initiation conditions. Discovering how environmental factors alter cell phenotype may be a critical step in preventing and treating breast cancer. Our objective was to use a mammosphere assay which provides a three-dimensional readout of breast cancer stem cell proliferation and self-renewal along with immunostaining for cell identity and stemness markers to test for alterations in stemness and phenotypic plasticity after introducing DDE. We performed RNA-seq analysis on the mammospheres to identify differentially expressed genes attributed to DDE exposure and then to assess mechanisms behind differential expression of identified genes, hypothesizing that DDE induces cellular plasticity via Wnt signaling. **Methods:** MCF10A cells were cultured in DDE-dosed mammosphere-forming media (0 μ M, 0.025 μ M, 0.25 μ M, 2.5 μ M, and 25 μ M) for seven days. The mammospheres were stained with the nuclear stain Hoechst 33342, immunostained with keratin 8, keratin 14, CD24, and CD44, and imaged. CellProfiler was used to quantify the count, area, and stain intensity of the mammospheres. This experiment was performed in triplicate and generalized linear regressions were used to measure effects of DDE on size, area, and intensity. A subset of mammospheres were collected and lysed for RNA sequencing. In the second round of RNA sequencing, primary mammospheres were formed in the following dosed media: 0 μ M, 25 μ M DDE, 2.5 μ M DDE, 3.75 μ M cardamonin, a known Wnt inhibitor, 0.9375 μ M cardamonin, and four mixtures of DDE and cardamonin at the same concentrations. We identified dose-dependent expression changes with BMDEExpress3. Benchmark concentration analyses were performed using the ToxicR Model Averaging analysis and the REACTOME pathway database was used for pathway enrichment analysis. **Results:** Keratin 8 and keratin 14 mean intensity significantly decreased in 25 μ M-treated DDE mammospheres compared to control ($p = 0.0001$ & $p = 0.008$). CD44 mean intensity significantly increased in mammospheres treated with DDE at concentrations of 2.5 μ M ($p = 0.012$) and 25 μ M ($p = 0.010$). In the first round of RNA-seq, mammospheres exposed to 25 μ M DDE had 578 genes upregulated and 628 genes downregulated out of 15,536 total genes compared to control. Keratinization and formation of the cornified envelope were biological pathways upregulated with a maximum fold change of 360.47 ($p = 2.44e-15$ & $3.09e-21$). In the second round of RNA sequencing, genes related to epidermal differentiation were upregulated in DDE as seen in the first round, confirming the reproducibility of this process. In the mixtures of DDE and cardamonin, many of the same genes related to keratinization were differentially expressed but at a modestly lower fold change. **Conclusions:** At 25 μ M DDE, keratin 8 and keratin 14 expression decreased, CD44 expression increased, and CD24 intensity did not change. This indicates a shift from an epithelial to mesenchymal phenotype. The RNA-seq data support these findings, where 25 μ M DDE treatment upregulated genes linked to keratinization and cornification, possibly linked to a squamous cell metaplastic phenotype. This phenotypic change was not fully rescued by cardamonin, a Wnt inhibitor, indicating that the effects of DDE on squamous metaplasia is likely through biological mechanisms besides altered Wnt signalling. This is currently being investigated. This study highlights the association between alterations in breast stem cell populations and exposure to environmental contaminants, showing that DDE can impact phenotypic plasticity, a Hallmark of Cancer, *in vitro*.

PS 3273 Flame Retardant Triphenyl Phosphate Induces Genetically Variable Exposure Responses in Mouse Pluripotent Stem Cells

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Background and Purpose: Despite advances in genomic technologies, the effects of genetic variation on responses to environmental toxicants remain largely uninvestigated, and this is one reason for the use of an uncertainty factor in chemical risk assessment. Genetic variation impacts the biological response to chemical and environmental exposures, contributing to differences in resistance or susceptibility. Forward genetic screens of genetically diverse cell populations can be used to identify the precise genetic variants that drive response variation, but such screens

are limited by the lack of available genetically diverse human derived cell panels. Cell lines from laboratory mouse genetic reference populations like the Diversity Outbred (DO) population are genetically diverse and powered for genetic mapping but also offer the significant advantage of *in vitro* / *in vivo* trait correlation and *in vivo* validation using genetically matched individuals. These diverse cell panels are especially beneficial for studying early developmental effects of chemical exposures. Triphenyl phosphate (TPHP) is an organophosphate flame retardant that has recently been linked to adverse developmental effects, including altered cell cycle and increased cell death in stem cell populations. Self-renewal and pluripotency are defining properties of stem cells during development; therefore, it is important to understand how chemicals like TPHP may alter these critical characteristics within a genetically diverse population. **Methods:** We created a panel of induced pluripotent stem cells (iPSCs) from more than 300 DO mice, as well as the 8 founder inbred strains that were used to create the DO population. Next, we exposed a subset of these lines to triphenyl phosphate (TPHP), an organophosphate flame retardant that has been linked to numerous adverse developmental effects, to identify cellular responses associated with developmental exposure risk. Beginning with the 8 DO founder strain iPSCs, we utilized high content imaging and flow cytometry after TPHP exposure to observe genetically variable changes to iPSC colony volume and cell cycle. We then expanded our analysis to characterize interline variation in gene expression in response to TPHP in 200 DO iPSC lines by RNA-seq and applied expression quantitative trait loci (eQTL) mapping to identify genomic loci that contribute to variation in gene expression following TPHP exposure. **Results:** Cell imaging and flow cytometry showed TPHP exposure increased cell death and reduced the G2 cell cycle population with significant interstrain variation in cell lines derived from NOD/ShiLtJ and A/J strain backgrounds. We mapped a genomic locus on chromosome 15 that is linked to expression variation in many genes and co-localized with a region of known importance for cell cycling. In addition, we observed that TPHP exposure reduces expression of important pluripotency genes like *Klf4* in mouse iPSCs. **Conclusions:** Using these data, we propose a potential cellular mechanism of TPHP-induced effects on pluripotent stem cells, where TPHP causes altered cell cycle and proliferation, in addition to increased cell death. Moving forward, we will continue to investigate the effects of TPHP on cell cycle and proliferation, which are crucial characteristics for the normal function of pluripotent stem cells. This information will further our understanding of the molecular and biological pathways that modulate triphenyl phosphate exposure response within developing tissues.

PS 3274 Isolation and Culture of Human Bone Marrow MSC Under Unbroken Physioxia for Better Cell Growth and Translatability of Studies

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Background and Purpose: Human bone marrow mesenchymal stromal/stem cells (MSC) are used for *in vitro* toxicologic assays, both in traditional 2D and in 3D organoid cultures for microphysiological systems. Publications by others showing that supraphysioxia room air adds artifact to *in vitro* assays have led to calls to protect cells from room air. We previously showed that commercially available MSC cultures proliferate faster and grow for more passages under physioxia (5% O₂) than under traditional conditions (18% O₂). We also showed that HIF-1 α , a key signaling molecule upstream of cell growth pathways, is modulated by room air exposure in MSC. Here we extend earlier findings by isolating the cells from cadaver bones and expanding them under constant physioxia, so that the cells are never exposed to supraphysioxia room air oxygen. The null hypothesis for this study is that there will be no difference in doubling times between cells exposed to, and those protected from, room air. **Methods:** Except for commercially sourced MSC cell lines, all cell isolation and culture operations were performed under closed and controlled conditions in Xvivo Systems (BioSpherix). Centrifuges, microscopes, and other laboratory equipment were housed inside the closed modules. Commercial human bone marrow MSC (Lonza) were cultured at constant 5% or 18% O₂ (room air incubator O₂ control) plus 5% CO₂ in T-flasks. Media were pre-equilibrated to the proper oxygen level. Commercially available MSC specialty media were tested for cell growth kinetics under constant physioxia. Cell counts were obtained with the Denovix CellDrop and manual trypan blue count. Cells were isolated from human cadaver pelvic bones, sourced from a licensed organ procurement organization, inside the Xvivo System for unbroken control of the cell environment. The primary bone marrow cell isolate was subjected to discontinuous centrifugal gradient separation and mononuclear cells were selected for adherence to plastic. To confirm MSC identity, flow cytometric analysis was done with a Cytek Aurora and the following anti-human antibody panel (Dead cell-Zombie Aqua, CD38-PACBLUE, HLA-DR-BV785, CD45-FITC, CD14-PerCP-Cy5.5, CD73-PE-Cy7, CD105-APC, CD90-APC-Cy7 (Biolegend), CD11b-BUV395 (BD), CD34-PE (Thermo)). The cells were cultured for 2-4 weeks in specialized media (Thermo) to differentiate them into adipocytes, chondrocytes, and osteocytes. The cultures were stained with Oil Red O, Alcian Blue, and Alizarin Red S, respectively, and assessed visually (qualitatively) for cell staining. **Results:** Flow cytometric analysis showed that the bulk of the cells isolated under full-time physioxia were negative for CD45, HLA-DR, CD14, CD11b, and CD38, and positive for CD73, CD90, and CD105. The cells cultured with adipocyte differentiation media stained positive with Oil Red O, the cells cultured with chondrocyte differentiation medium stained positive with Alcian Blue, and the cells cultured with osteocyte differentiation medium stained positive with Alizarin Red S. Together these findings suggest that the plastic-ad-

herent cells cultured from human bone marrow under fully controlled physiologic oxygen can be classified as MSC. At passage number 8, MSC isolated and cultured under traditional culture conditions averaged 128 $\pm$ 15.2 hrs doubling time (n=2 separate cultures). Isolated and cultured under unbroken physioxia (5% O₂), MSC at the same passage averaged 43.2 $\pm$ 1.2 hrs doubling time (n=3 separate cultures), less than half that of the cells grown under traditional conditions, disproving the null hypothesis. Under constant physioxia, different commercially available MSC culture media also affected cell doubling time and number of passages before cell senescence, with basic DMEM + 10% FBS performing well to passage 9. **Conclusions:** We concluded that human bone marrow MSC isolated and grown under traditional laboratory conditions exhibit slower growth at Passage 8 than those isolated and grown under constant physioxia. The artifact that is introduced by room air oxygen into MSC-based assays cultured under traditional conditions may be avoided by protecting MSC from room air. Protecting MSC from supraphysiologic oxygen even during cell isolation can help produce a more robust cell line that can help improve the reproducibility and translatability of MSC-based *in vitro* assays for toxicology.

PS 3275 Cannabidiol and Δ^9 -Tetrahydrocannabinol alters *in vitro* human hematopoiesis

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Background and Purpose: Cannabidiol (CBD) and Δ^9 -Tetrahydrocannabinol (THC), the primary active compounds in *Cannabis sativa*, are recognized for their therapeutic potential and immune-modulating effects. CBD is a non-euphoric compound widely used in over-the-counter products for purported benefits whereas THC is mostly used recreationally for its psychoactive properties. With increased legalization of cannabis and related products across many US states, CBD and high-THC containing cannabis products are commonly used by diverse population groups including pregnant women. The effects of CBD and THC on many aspects of human health are not well known and there is a critical need to understand their effects on different developing physiological systems. We studied the effects of CBD and THC on the early stages of human immune cell development from hematopoietic stem and progenitor cells (HSPCs) over 28 days. **Methods:** In a 96-well plate, 10,000 human umbilical cord-blood derived CD34⁺ HSPCs per well were plated in RPMI media supplemented with 5% human AB serum, cytokines (IL-6, IL-7) and growth factors (SCF, Flt3L), and cultured over a 28-day period. SCF, Flt3L, and IL-6 (each at 25 ng/mL) were added on day 0. CBD or THC were added at different concentrations on day 0 only. Concentrations used were 1, 3, 5, and 10 μ M. DMSO (0.02%) was used as the solvent. On day 7, half of the culture media was replaced with fresh media and SCF (25 ng/mL), Flt3L (25 ng/mL), and IL-7 (20 ng/mL) were added. Half of the culture media was then replaced every 7 days with cytokine-free fresh media. Cells were collected every 7 days for phenotypic analysis using flow cytometry. **Results:** We observed that both CBD and THC induced loss of CD34 expression on HSPCs in a concentration-dependent manner, suggesting faster differentiation of HSPCs to downstream progenitors. At multiple time-points, there was a trend towards increased proportions of monocyte populations with increasing concentrations of CBD and THC. On days 7 and 14, CBD induced a decrease in percentage of CD1c⁺ cells whereas THC had the opposite effect. Percentage of CD10⁺ lymphoid populations was also lower in CBD-treated cells at early timepoints when compared to vehicle-treated cells. However, THC did not elicit any appreciable differences in lymphoid cells. Megakaryocyte progenitors were unaffected by CBD and THC at all time-points. **Conclusions:** This study demonstrated, for the first time, in an *in vitro* model of human hematopoiesis, that CBD and THC deregulate hematopoietic differentiation towards specific cell populations, and identified the differential effect each compound has on hematopoietic lineage development. These results support the need for more investigation to determine the safety of cannabis and cannabinoid product use during pregnancy.

PS 3276 Electronic Cigarette Flavor Chemicals and Synthetic Coolants Disrupt Critical Processes in Human Embryonic Stem Cells Through TRP-Mediated Calcium Dysregulation

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Background and Purpose: Electronic cigarette (EC) use among reproductive-age individuals continues to increase, yet the developmental risks posed by flavor chemicals and synthetic coolants remain poorly defined. Menthol, vanillin, and WS-23 are frequently used at high concentrations in EC refill fluids and are known activators of transient receptor potential (TRP) channels involved in regulating intracellular calcium. Early embryogenesis and gastrulation occur when pregnancy is commonly unrecognized, disruption of calcium homeostasis or pluripotency during these stages may compromise normal development. This study used human embryonic stem cells (hESCs), which model the human epiblast, to determine how exposure-relevant concentrations of menthol, vanillin, and WS-23 affect cellular processes essential for early development, including viability, proliferation, colony growth, maintenance of pluripotency, and lineage specification. **Methods:** hESCs were exposed to menthol, vanillin, or WS-23 and evaluated across multiple endpoints. Calcium influx was quantified using Fluo-8 dye to assess activation of TRP channels known to be expressed in hESCs, as confirmed by immunocytochemistry. Time-lapse imaging over 72 hours

was analyzed using StemCellQC software to extract quantitative features describing colony size and morphology. Cell death was measured via activated caspase-3 immunostaining, and mitotic index was calculated to determine effects on cell division. To assess impacts on pluripotency and lineage bias, OCT4 expression and germ layer markers were evaluated after 72 hours. Preincubation with selective antagonists for TRPA1, TRPM8, and TRPV4 was used to determine whether observed effects were TRP-mediated. **Results:** All three EC chemicals activated distinct TRP channels and triggered sustained calcium influx. Menthol at nanomolar concentrations selectively activated TRPA1, leading to increased cell death and significant inhibition of colony expansion. Vanillin activated TRPV4 and caused significant cytotoxicity, characterized by reduced colony size and complete disintegration at exposure-relevant concentrations. Nanomolar WS-23 activated TRPM8, suppressing proliferation, impairing cell viability, and reducing colony integrity. Beyond growth and survival effects, vanillin and WS-23 downregulated OCT4 and induced premature SOX17 expression indicative of endodermal differentiation. These data demonstrated loss of pluripotency and altered lineage commitment. Across all endpoints, chemical-induced effects were prevented when cells were preincubated with the corresponding TRP antagonists, confirming that TRP-mediated calcium dysregulation was a primary mechanism underlying toxicity. **Conclusions:** Menthol, vanillin, and WS-23 each initiated TRP channel-dependent calcium influx and disrupted key processes required for maintaining the epiblast-like state of hESCs, including survival, proliferation, pluripotency, and proper lineage allocation. These findings identify a mechanistic basis by which flavor chemicals and coolants used in EC products may pose developmental risks during early pregnancy. Given the high concentrations of these chemicals in EC formulations and the potential for systemic exposure, these results underscore the need to consider flavor chemicals and coolants when evaluating the developmental toxicity of EC products.

PS 3277 New Approach Methodologies (NAMs): Assessing cardiotoxicity and neurotoxicity *in vitro* using human iPSC-derived cells

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Background and Purpose: New approach methodologies (NAMs) are gaining traction for drug toxicity screening. In recent years, human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) and hiPSC-derived neurons have found greater utility for *in vitro* toxicity screens. They are a scalable, physiologically relevant model system, providing an ethical alternative to animal testing. The current study aims to investigate the toxicity profiles of two novel, unknown compounds using commercially available hiPSC-derived excitatory cortical neurons and hiPSC-derived ventricular cardiomyocytes, validating the combined use of these NAM models for integrated safety screening. **Methods:** Toxicity assessment was performed using axoCells™ Human iPSC-derived Cortical Excitatory Neurons and axoCells™ Human iPSC-derived Ventricular Cardiomyocytes. Neural stem cells were differentiated and matured to cortical neurons over 21 days. The two test compounds were applied on day 15 at concentrations of 0.1 μ M, 1 μ M, and 10 μ M. Neurite outgrowth and branching were monitored using the IncuCyte S3 Live-Cell Analysis System over the course of 7 days, and cell viability was quantified at the experimental endpoint using the CellTiter-Glo® assay. hiPSC-CMs were cultured and matured for 7 days prior to compound exposure. Acute cardiotoxicity was evaluated using the CardioExcyte 96 platform to assess changes in field potential duration (FPD), with dofetilide serving as a positive control. Chronic cardiotoxicity was evaluated over a 48-hour period using impedance-based measurements on the same platform, with doxorubicin as a positive control. Both test compounds were evaluated at identical concentrations (0.1 μ M, 1 μ M, and 10 μ M). **Results:** Initial control experiments demonstrated that dofetilide produced a significant increase in field potential duration (FPD), confirming hiPSC-derived cardiomyocyte sensitivity to acute hERG block. Doxorubicin exposure resulted in a time-dependent loss of impedance, consistent with chronic cardiotoxicity and cell death. Glutamate demonstrated neurotoxicity at the highest concentrations by inhibiting cortical neurite outgrowth. The risk profile of the unknown compounds will be compared against these reference compounds. **Conclusions:** These findings support the application of hiPSC-derived neuronal and cardiac models as complementary NAMs for *in vitro* toxicity assessment. The ability to perform both toxicity assessments in a 96-well plate format allows for concurrent evaluation of neurotoxicity and cardiotoxicity. This approach provides a scalable, human-relevant platform for early safety de-risking of novel compounds and may facilitate more predictive, mechanism-based toxicological screening in drug discovery and development.

PS 3278 Potential Impact of Anion Channels on the Interpretation of NIS Assay Results in the Fisher Rat Thyroid Cells (FRTL-5)

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Background and Purpose: The Sodium/Iodide Symporter (NIS) assay is used as an *in vitro* screen to investigate a potential role for the inhibition of iodide uptake into thyroid cells on thyroid hormone synthesis. It is known that some compounds can

impact the assay by interfering with ATP synthesis or the electrochemical sodium gradient needed to drive the symporter. Other mechanisms can potentially confound the interpretation of the assay. Here we present data implicating changes in intracellular calcium with iodide excretion. Fischer Rat Thyroid Low Serum 5% (FRTL-5) cells have been shown to express a calcium activated anion channel called anoctamin 1 (ANO1), also known as transmembrane protein TMEM16A. The present work investigated a possible role for ANO1 to influence intracellular anion levels in FRTL-5 cells. **Methods:** FRTL-5 cells were treated with ionomycin to mobilize intracellular calcium, and internal iodide levels were monitored using the Sandell-Kolthoff method. An anoctamin-1 inhibitor (T16Ainh-A01) was used to inhibit the efflux of iodide from the cells treated with ionomycin. Perchlorate was used as a classical NIS inhibitor to demonstrate the sensitivity of the assay. Cell viability was monitored by measuring ATP levels in the cells. In separate experiments, endogenous calcium activated anion currents were measured in FRTL-5 cells using whole cell patch-clamp electrophysiology. Cells were held at -80 mV and stimulated with a voltage step pulse protocol from -100 to +120 mV at 0.2 Hz frequency. Data were recorded using a Molecular Devices pClamp 11 bundle. Ionomycin was perfused as positive control in the presence and absence of calcium into the cells. The impact of T16Ainh-A01 on anion currents through ANO1 channels in cells treated with ionomycin was also investigated. **Results:** In the NIS assay, the release of calcium from intracellular stores in FRTL-5 cells treated with ionomycin caused an apparent increase in iodide efflux. Treatment of the FRTL-5 cells with ionomycin plus T16Ainh-A01 weakly decreased the level of efflux of iodide from the cells compared to cells treated with ionomycin alone. In the patch-clamp assay, ionomycin was perfused into cells in the presence and absence of calcium in the internal solution, showing an enhancement of anion efflux for both chloride and iodide. Perfusion of cells with T16Ainh-A01 decreased the ionomycin-induced anion currents. **Conclusions:** The experiments described herein suggest that increases in internal calcium ion through ionomycin treatment decreased the level of iodide in FRTL-5 cells. Patch-clamp data shows that ionomycin-induced calcium release increases the efflux of anion (both iodide and chloride). T16Ainh-A01 decreased the ionomycin mediated anion efflux, however, it was not a strong inhibitor. This could suggest that T16Ainh-A01 is a weak anoctamin 1 inhibitor in FRTL-5 cells or multiple channels mediate calcium induced iodide efflux. Follow-up on these observations is important, as these findings suggest that a decrease in activity in the NIS assay can in some cases be due to other mechanisms, and be misinterpreted as NIS inhibition.

PS 3279 Monitoring Thyroid Hormone Action in Human Hepatocytes and Evaluating Chemical Exposure Effects to Support Thyroid Disruption Safety Assessment

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Background and Purpose: The EPA has emphasized the development of human-relevant new approach methodologies (NAMs) to identify chemicals that may disrupt thyroid hormone regulation and to reduce dependence on animal testing. This project aims to develop a human thyrocyte/hepatocyte screening model to elucidate the mechanism by which chemical exposures disrupt human thyroid kinetics and action with the goal of supporting population-level chemical risk assessment. Thyroid hormones play a key role in regulating energy balance by controlling cellular energy expenditure. TH levels are tightly regulated through multiple mechanisms; systemic levels are maintained by the hypothalamic-pituitary-thyroid (HPT) axis, the liver influences TH kinetics, and local intracellular deiodination modulates TH action at the tissue level. Intracellular deiodination of T4 to T3 separates liver TH levels from serum level. In the liver and other tissues, thyroid action controls cellular energy balance through T3 binding to thyroid receptors, which regulates the transcription of key regulatory proteins. Therefore, systemic TH levels may not accurately reflect tissue-specific TH activity. Chemicals including polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) and per- and polyfluoroalkyl substances (PFAS) can disrupt TH action in humans and animals. **Methods:** We tested hepatic metabolic endpoints using high-content confocal imaging to evaluate thyroid hormone action in response to chemical disruption. HepG2 cells were treated with chemicals, and imaging was used to assess fatty acid uptake (FAU), mitochondrial content, and mitochondrial membrane potential (MMP) using specific fluorescent stains. CYP1A1 luminescent assay was performed to evaluate the effect of chemical exposure on hepatic enzyme activity. **Results:** Treatment of HepG2 cells with 1,2,7,8-TCDD showed fatty acid uptake (FAU), mitochondria, and mitochondrial membrane potential (MMP) exhibited dose-response decreases with IC50s at 0.71, 0.46, and 0.09 nanomolar. PCDD/F congeners, 1,2,3,7,8-PCDF, and 1,2,3,4,6,7,8-HCDF, showed approximately 10-fold and 100fold lower IC50s compared to 1,2,7,8-TCDD. CYP1A1 luminescent assay also demonstrated dose-response relationships with 1,2,7,8-TCDD, 1,2,3,7,8-PCDF, and 1,2,3,4,6,7,8-HCDF with IC50s at 0.44, 3.00 and 8.4 nanomolar. PFOS significantly induced both MMP and FAU. However, as to the TH responsiveness, HepG2 monolayers did not respond to TH treatment, whereas HepG2 spheroids showed responsiveness to both TCDD and T4. We further investigated chemical disruption of hepatic metabolic endpoints using 3D hepatocyte spheroids. TCDD was found to reduce MMP, with an IC50 of 0.12 nanomolar, comparable to the effect observed in 2D cultures. In contrast, PFOS no longer induced MMP, and no combined effects were observed in PFOS and TCDD mixture experiments. **Conclusions:** Based on our current findings, MMP emerged as the most sensitive metabolic endpoint

for reflecting TH action in the liver. It is capable of detecting disruptions induced by low nanomolar concentrations of chemicals, including those naturally present in the environment.

PS 3280 Multimodal preclinical screening of glucocorticoid-induced disruption of bone remodeling in rat

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Background and Purpose: Bone toxicity is a critical consideration during drug development and may result from both intended pharmacologic mechanisms and off-target effects. While standard histopathological evaluation is routinely used in nonclinical toxicity studies, it often lacks the sensitivity to detect early or subtle skeletal alterations, particularly in short-term or exploratory dosing regimens. Early detection of bone changes is essential for mitigating downstream risk, especially in compounds with the potential to interfere with skeletal remodeling, growth, or maintenance. **Methods:** All animal procedures complied with institutional and national guidelines and were IACUC-approved. Sprague-Dawley rat serum (9-10/sex) was collected monthly for 8 months (starting ~1 month old) to assess age sensitivity of bone turnover biomarkers. In a separate study, male rats received subcutaneous DMSO/CMC (n=10) or prednisolone 7.5 mg/kg (n=10) for 14 or 28 days. Serum was collected at baseline, and days 2, 7, 15, 21, and 29 for bone biomarker analysis. Animals were euthanized at study endpoints; femur and tibia were collected, cleaned, fixed in 10% formalin, decalcified, sectioned, stained (H&E), and evaluated by a veterinary pathologist. Tibial growth plate thickness (2- or 8-month-old rats) was analyzed with Qu Path software; seven 1-mm measurements were averaged per sample. Group differences were tested with Tukey-Kramer. In the prednisolone study, proximal tibial physal thickness was analyzed with Visiopharm; Student's t-test compared groups. JMP® 14 was used for statistics. Hindlimbs were also collected for bone imaging. Rat mesenchymal stem cells were differentiated into osteoblasts ± prednisolone (12.5, 25, 50 µM), followed by Alizarin Red staining for matrix mineralization and quantification at 405 nm using ClarioStar. Cell viability (ATP) was measured with CellTiter-Glo and SpectraMax Plus 384. *Ex vivo* femur bone mineral density was assessed with InAlyzer2 DXA scanner. Micro-CT (Rigaku CT Lab GX130; 300 µA, 130 kV, 20 µm voxel, 14 min) evaluated distal femur trabeculae, analyzed in Dragonfly 2024. Serum P1NP, osteocalcin (formation), and CTX-1 (resorption) were quantified using IDS EIA kits and SpectraMax Plus384 according to the manufacturer. **Results:** To better characterize bone-related effects, we first compiled data from naïve Sprague-Dawley rats of ages typically used in early exploratory and later chronic toxicity studies. Then we applied these endpoints to assess changes in a rat model of glucocorticoid-induced bone loss. Two-month-old naïve male Sprague-Dawley rats were treated with prednisolone (7.5 mg/kg/day) for 14 or 28 days, with vehicle-treated animals serving as controls. Bone mineral density (BMD) was assessed *ex vivo* using dual-energy X-ray absorptiometry (DXA), which revealed a significant reduction in femoral BMD in the 28-day treatment group and a decreasing trend in the 14-day group. Micro-computed tomography (microCT) analysis of the distal femur demonstrated compromised trabecular microarchitecture in prednisolone-treated animals, with decreased trabecular thickness and increased separation consistent with decreased bone mass. Histological evaluation also revealed thinning of the physis, indicative of impaired longitudinal bone growth. Systemic effects of prednisolone were further evidenced by changes in serum bone turnover biomarkers, including N-terminal propeptide of type I procollagen (P1NP), osteocalcin, and C-terminal telopeptide of type I collagen (CTX-1), suggesting suppression of both bone formation and resorption. **Conclusions:** A multimodal approach improved the ability to detect and characterize early skeletal toxicity, providing a more comprehensive assessment than using standard histopathology alone. DXA imaging enabled the quantification of changes in bone density and Micro-CT enabled quantification of subtle bone architectural findings in a rat toxicity study with a bone toxicant. Incorporation of bone-specific endpoints (serum biomarkers and imaging assays) will aid in identifying compounds with potential impact on skeletal health guiding drug development.

PS 3281 Impact of climate change on the estrogenic activities of paraquat

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Background and Purpose: Rising global temperatures and altered aquatic physico-chemical regimes are likely to modify the environmental fate and biological activity of agrochemicals, yet few studies have quantified how climate-change influences the endocrine activity of commonly used herbicides. **Methods:** Here, we investigated how simulated climate-change stressors (elevated temperature, reduced pH and increased UV exposure) modulate the estrogenicity of paraquat using a yeast estrogen bioreporter assay (YES) with *in vivo* endocrine end-point assays in the African catfish *Clarias gariepinus*. Paraquat was preconditioned under control and climate-simulated treatments for 14 days to produce environmentally relevant transformation mixtures. Estrogenic activity of parent and transformed mixtures was quan-

tified using a recombinant *S. cerevisiae* YES (ER α and Luc) reporter, while sub-chronic exposure (28 day) of juvenile *C. gariepinus* to paraquat was used to assess biological responses such as plasma vitellogenin induction, 17 β -estradiol/testosterone ratios, gonadosomatic index (GSI), and gonadal histopathology. **Results:** When compared to control conditions, climate-simulated treatments produced transformation mixtures that elicited significantly higher reporter activation in the YES assay (over 1.8-4.0-fold; $p < 0.05$) and induced measurable vitellogenin synthesis and feminizing shifts in sex-steroid profiles in exposed fish ($p < 0.05$). Histopathological analyses revealed dose-dependent intersex features and reduced GSI consistent with endocrine disruption. **Conclusions:** These results suggest that climate-driven alteration can increase the estrogenic potential of paraquat and the integration of climate-change parameters and its transformation products into ecological risk assessments is crucial for more protective regulatory decision-making.

PS 3283 Quercetin enhances steroidogenesis in mouse Leydig cells MLTC-1 and I-10 exposed to atrazine

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Background and Purpose: Testosterone is the principal male sex hormone necessary for male fertility and overall well-being. Approximately 95% of total testosterone in the male body is produced by Leydig cells within the testes in a process called testicular steroidogenesis. Exposure to pesticides, diseases, medication, and the aging process can result in reduced testosterone levels in men. With a functional hypothalamic-pituitary-gonadal axis, testosterone deficiency can be attributed to: (1) decreased Leydig cell numbers and/or (2) their reduced steroidogenic capacity. The present study focuses on the Leydig cell level and investigates the effect of the herbal medicine quercetin (Quer) on the viability and steroidogenic capacity of mouse Leydig cells exposed to the pesticide atrazine (ATZ). **Methods:** Two mouse Leydig cell lines MLTC-1 and I-10 were treated with either DMSO (control), ATZ (3, 10, 30, and 100 μ M), Quer (1, 3.3, 10, and 30 μ M), or co-treatment of Quer + ATZ (Quer 3.3 μ M + ATZ 33 μ M or Quer 3.3 μ M + ATZ 100 μ M) for 2, 4, and 6 days, with medium/chemical change every two days. At each time point, cell viability was assessed, culture medium was collected for progesterone (P4) and testosterone (T) quantification with ELISA, and total RNA was extracted for RT-qPCR analysis of gene expression of key steroidogenic genes, StAR, Cyp11a1, Hsd3B6, and Hsd17B3. **Results:** When treated with ATZ alone, the viability of both MLTC-1 and I-10 cells decreased throughout the 6-day treatment concentration-dependently. For I-10 cells, this decrease was seen as early as Day 2 with the lowest concentration of ATZ (3 μ M). Interestingly, this decrease in cell viability was also accompanied by a concentration-dependent stimulation of T production in both cell lines. However, such stimulation was not evident for P4 production. On the other hand, Quer alone treatment at lower concentrations did not cause significant changes in cell viability at earlier time points, but rather decreased viability at the last time point, Day 6, with the highest concentration (30 μ M). In MLTC-1 cells, T production increased with higher Quer concentrations, most notably at 30 μ M across the treatment, with a 4.7-fold increase compared to control on Day 4. Similarly, P4 production also increased with higher Quer concentrations at later time points. For I-10, T production was stimulated on Day 2 and on Day 6 at 3.3 and 10 μ M. Like MLTC-1 cells, the increase in T production was most notable at 30 μ M on Day 2 (2.4-fold compared to control). P4 production was also increased on Days 2 and 6 at all concentrations. The two co-treatments (Quer 3.3 μ M + ATZ 33 and Quer 3.3 μ M + 100 μ M) had different effects on each cell line. For MLTC-1, Quer + ATZ 33 μ M caused a significant increase in cell viability compared to control and the individual treatments (Quer alone and ATZ 33 μ M) on Day 4. However, this protective effect did not sustain on Day 6. For I-10 cells, the co-treatment with the higher ATZ concentration (i.e., Quer + ATZ 100 μ M) on Day 4 significantly increased cell viability compared to ATZ 100 μ M. The protective effect of Quer persisted on Day 6, resulting in significantly increased cell viability compared to both control and ATZ 100 μ M. For both MLTC-1 and I-10 cell lines, addition of Quer augmented T production. For MLTC-1, this stimulation was observed in both co-treatments on Day 2 (Quer + ATZ 33 μ M: 2.4-fold; Quer + ATZ 100 μ M: 3.6-fold, compared to control) and for Quer + ATZ 100 μ M on Day 4 (2.9-fold compared to control). A similar stimulation was also observed for P4 production in MLTC-1 cells in Quer + ATZ 100 μ M co-treatment at all time points. For I-10, T production in both co-treatment groups was significantly increased on Days 2 and 4 compared to control and Quer alone. Similar to MLTC-1 cells, T production was stimulated the most in Quer + ATZ 100 μ M co-treatment on Day 2. Additionally, P4 production was also elevated in both co-treatments compared to ATZ alone (33 μ M and 100 μ M) treatments. **Conclusions:** In both MLTC-1 and I-10 mouse Leydig cells, Quer enhanced steroid production in the presence of atrazine. These results indicate that there may be crosstalk between Quer and ATZ, which influences testicular steroidogenesis, leading to enhanced steroid production in mouse Leydig cells.

PS 3284 Long-term exposure to a phthalate mixture reprograms white and brown adipose tissue phenotypes through estrogen signaling pathways in female mice

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Background and Purpose: Phthalates are synthetic chemical compounds commonly used in consumer products that act as endocrine disrupting chemicals. Phthalates are frequently prevalent as mixtures, and women are more likely to be exposed to phthalates than men. Although phthalates are obesogens and affect metabolic function, limited information was available on whether chronic exposure for 12 months to an environmentally relevant phthalate mixture alters the adipose tissue phenotype in female mice. For this reason, we tested the hypothesis that chronic exposure to a mixture of phthalates changes white and brown adipose tissue (WAT and BAT) morphology and adipose gene expression in female mice. **Methods:** Female CD-1 mice (6 weeks of age) were fed vehicle control (corn oil) or a mixture of phthalates (0.15 ppm, 1.5 ppm, and 1500 ppm) through the chow for 12 months. The mixture was composed of DEP (35.2%), DEHP (21.0%), DBP (14.9%), DIBP (8.6%), DiNP (15.1%), and BzBP (5.1%). White adipose tissue and brown adipose tissue (WAT and BAT) were analyzed for morphological characteristics and expression of adipogenesis, proliferation, angiogenesis, inflammation, collagen deposition, and steroidogenic factors, and estrogen receptor (ER) markers. **Results:** The results indicate that the phthalate mixture increased estradiol levels compared to control. In WAT, the mixture decreased adipocyte size and increased adipose number compared to controls. The mixture significantly increased inflammatory infiltrates and mast cell number compared to controls. In addition, the mixture-exposed groups contained adipocytes surrounded by macrophages. The mixture exposure reduced the expression of the adipogenic marker leptin (*Lep*) and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*Ppargc1a*) compared to controls. Further, the mixture increased an angiogenesis and inflammatory marker vascular endothelial growth factor a (*Vegfa*) and chemokine ligand 5 (*Ccl5*) compared to controls. Interestingly, mixture exposure changed several markers of steroidogenesis. Specifically, the mixture increased cytochrome P450 side-chain cleavage (*Cyp11a1*) and androgen receptor (*Ar*), but decreased cytochrome P450 aromatase (*Cyp19a1*) exposure compared to controls. In BAT, the mixture increased adipocyte and cell droplet number and decreased cell size compared to controls. Further, mixture exposure increased whitening area, blood vessel number, inflammatory infiltrates, BAT collagen deposition, ER α and ER β protein expression, and expression of the inflammatory marker chemokine (C-C motif) ligand 2 (*Ccl2*) and decrease the brown adipogenesis markers uncoupling protein 1 (*Ucp1*) and *Ppargc1a* and beta-3 adrenergic receptor (*Adrb3*) compared to controls. Further, the mixture decreased lipid droplet marker perilipin 1 (*Plin1*) and growth factor marker fibroblast growth factor 2 (*Fgf2*). **Conclusions:** These data suggest that exposure to a mixture of phthalates promotes abnormal WAT and BAT morphology and adipose gene expression in female mice, which may be related to a change in steroidogenesis and estrogen signaling on female adipose tissue. Supported by Brasillinois and NIH R01ES03112.

PS 3285 In life low-volume sampling and bioanalysis of thyroid hormones on a short-term mode of action study in Han Wistar rats: 6-PTU case study.

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Background and Purpose: Vital to homeostasis, altered thyroid function can impact thermoregulation, metabolism, behaviour and neurodevelopment. Consequently, thyroid hormones are now routinely measured on a number of OECD test guidelines that evaluate systemic and reproductive toxicity (e.g. OECD 408, 414 and 443). However, there are currently no internationally validated short-term screening studies to evaluate mechanisms of thyroid disruption *in vivo*. Historically, thyroid hormone analysis has been conducted on terminal fasted or unfasted blood samples (~2 mL) collected from the orbital sinus, sublingual vein, cardiac puncture or via decapitation under isoflurane anaesthesia. Considered a potential 3R's benefit, the purpose of the current study was to elucidate temporal effects on thyroid hormones in-life, via low volume blood sampling (0.3 mL) on Days 4, 8 and 14 without overnight fasting. **Methods:** As part of a multi-test item mode of action study, a known thyroid peroxidase inhibitor, 6-propyl-2-thiouracil (6-PTU) was administered to male and female Han Wistar (RccHan®/WIST) rats via gavage for 14 days (n=10/sex/group). A comparable control group received the 0.5% w/v carboxymethyl cellulose vehicle only. In-life blood samples were collected from the main study animals for thyroid hormone (T4, T3 and TSH) analysis on Days 4, 8 and 14 and for toxicokinetic assessment on Days 1 and 14. Blood samples for the thyroid hormone analysis were allowed to clot and centrifuged at 2000 g for 10 minutes, in order to obtain two aliquots of serum for LC-MS/MS quantification of Thyroxine (T4) and triiodothyronine (T3) or Luminex Milliplex assay analysis of Thyroid Stimulating Hormone (TSH). All bioanalysis was conducted using GLP validated analytical methods, which are presented in a separate publication (Brewin S, 2025). Additional endpoints assessed in the study included body weights, food consumption, terminal organ

weights and histopathology of liver and thyroid. **Results:** Repeat dose administration of 6-PTU in male and female Han Wistar (RccHan®:WIST) rats elicited the expected dose-dependent effects in thyroid parameters, which was supported by toxicokinetic analysis. Dose dependent increases in thyroid weights were observed in both sexes, which correlated with thyroid follicular cell hypertrophy and decreased follicular cell vacuolation. Dose and time-dependent effects on thyroid hormones were successfully identified in life, using lower serum volumes which increased data quality and reduced the number of animals required per group. Time-course effects on thyroid hormone levels in response to 6-PTU administration are presented. **Conclusions:** The study demonstrated advancements in sampling practices and analytical capabilities that refine and reduce animal use in thyroid mode of action studies. The validation of an analytical method to measure T3 and T4 from 35 µL of serum (Brewin S, 2025) facilitated the in-life blood sampling on Days 4, 8 and 14 from rats using lower blood volumes, which offered clear advantages over terminal sampling. The study design significantly reduced the number of animals required to meet the study objectives, representing a refinement and reduction in animal use consistent with the Principles of the 3R's. Furthermore, the in-life sampling enabled time-course evaluation of thyroid hormones within individual rats, allowing a direct correlation to other study endpoints.

PS 3286 Thyroxine (T4) and Triiodothyronine (T3): Validation of Methodology for the Determination of Concentrations in Surrogate Matrix and Serum Using Low Sample Volumes

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Background and Purpose: The monitoring of T3 and T4 levels in blood is used as a measure of potential disruption of the endocrine system, following exposure to certain chemical types. Concentrations of these thyroid hormones are often assessed as endpoints in toxicology studies to identify potential effects on thyroid function. The analysis of T3 and T4 is routinely required to support toxicological studies in the relevant animal species. One of the main challenges for rodent studies is the limited blood volumes, making it difficult to conduct multiple tests requiring separate analytical methods from the same sample or animal. Using a micro-sampling technique and low blood volume method enables a reduction in animal numbers whilst obtaining in-life real time thyroid data. An LC-MS/MS method was developed and validated for quantitation of T3 and T4 levels in rat serum, based on a 10 µL serum sample volume, in accordance with the SANTE/2020/12830 guidance. The method was developed to cover a range of concentrations, based on the endogenous levels and the ranges observed in previous studies. **Methods:** The developed method of analysis for T3 and T4 in low sample volume involved extraction/precipitation with acetonitrile/water, followed by dilution with water (T3) or water/acetonitrile (T4). Sample extraction was performed using both surrogate matrix (containing PBS, BSA and tween20) and rat serum. Surrogate matrix confirmed method specificity at the LOQ, which serum cannot demonstrate due to endogenous analyte levels. Quantitation was performed by reverse phase LC-MS/MS with the use of isotope labelled internal standards, using solvent-based calibration solutions. Other tests included final extract and fortification solution stability. Instrument matrix effects were addressed by the use of the internal standards. **Results:** The method was successfully validated within a GLP study for T3 and T4 in surrogate matrix and rat serum, covering the required concentration ranges and achieving the required LOQ of 0.2 ng/mL and 10 ng/mL in surrogate matrix for T3 and T4, respectively. For serum, the lowest validated concentrations were 0.5 ng/mL and 50 ng/mL for T3 and T4, approximately equivalent to the endogenous levels. The upper validated concentrations were 2 ng/mL and 200 ng/mL for T3 and T4 respectively. **Conclusions:** The analytical method was shown to be reliable and robust for the quantitation of T3 and T4 over the range of concentrations tested, meeting the SANTE validation acceptance criteria. Repeatability in T3/T4 concentrations was demonstrated within a number of replicate samples (n=15) using 10 µL of rat serum, confirming acceptable method performance when using the low sample volume. The use of solvent-based calibrations allows for accuracy in quantitation of the endogenous concentrations in serum. The use of surrogate matrix confirmed acceptable method performance for detecting potential treatment-related changes in both directions in serum T3/T4 levels of a study sample. This GLP-validated method has been used for routine study sample analysis supporting a range of toxicology studies.

PS 3287 Nonylphenol and Cetyl Alcohol Polyethoxylates Disrupt Thyroid Hormone Receptor Signaling to Promote Adipogenic and Obesogenic Effects

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Background and Purpose: Chronic health conditions, including obesity, are rising globally, with over 42% of U.S. adults affected and over \$300 million spent annually on related illnesses. Traditional explanations—diet, activity, and genetics—fail to fully account for this trend. Emerging evidence points to endocrine disrupting chemicals (EDCs), specifically obesogens, as contributing factors. This study investigates

two classes of widely used nonionic surfactants—nonylphenol ethoxylates (NPEOs) and cetyl alcohol ethoxylates (CetAEOs)—found in common household products and environmental samples. **Methods:** For adipogenesis assays, exposed cells included: murine pre-adipocytes, zebrafish mesenchymal stem cells (MSCs), and human MSCs. These cells were exposed to various concentrations and NPEO chain lengths. For *in vivo* work, Zebrafish were exposed to varying concentrations and chain lengths of NPEOs and CetAEOs. Nuclear receptor assays were conducted in zebrafish and human MSCs, along with co-exposure experiments. Osteogenesis assays were conducted solely on human MSCs. Most adipogenic data was obtained through Nile Red Staining. **Results:** hMSCs exposed to NPEOs and AEOs showed a heightened increase in triglycerides per cell. Zebrafish exposed to medium-chain NPEOs showed increased body mass and visceral fat, while CetAEOs promoted subcutaneous fat with slightly reduced weight. Both chemicals cause hyperactivity and decreased energy expenditure in larvae. Mechanistic studies revealed these ethoxylates interact with nuclear receptors. NPEOs acted as estrogen receptor agonists, while CetAEOs showed antagonistic activity. Both chemical groups indirectly antagonized thyroid receptor beta (TRβ). Co-treatment with thyroid hormone (T3) reversed ethoxylate-induced fat accumulation in stem cells, implicating thyroid disruption as a key pathway. **Conclusions:** Overall, our findings suggest that common consumer surfactants can disrupt metabolism and promote adiposity by interfering with hormone signaling, particularly thyroid pathways, across multiple biological models.

PS 3288 Multi-model predictions of potential endocrine disruptors in cosmetic ingredients: Comparing *in vivo* and *in vitro* data to *in silico* predictions

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Background and Purpose: Endocrine-disrupting chemicals (EDCs) are considered a serious health threat, contributing to major diseases. Classical targets of EDCs include nuclear receptors such as estrogen (ER), androgen (AR), and thyroid (TR), among others. These receptors are part of the so-called EATS, which includes several bioassays to detect activation/inhibition of estrogen, androgen, and thyroid receptor signaling as well as steroidogenesis ("E-A-T-S"). Considering the current regulatory scenario for cosmetics, safety assessment is constrained, requiring reliance only on available data and New Approach Methodologies (NAMs), specifically *in vitro* and *in silico* testing. This study aimed to assess the performance of *in silico* models in predicting endocrine activity of cosmetics ingredients, comparing them with *in vitro* and *in vivo* data available in the literature for the substances evaluated. **Methods:** Sixty substances with a warning for endocrine disruption were selected as potentially positive (PP), according to the definition of OECD GD 150, for substances that might be expected to lead to endocrine disruption, and twenty without the warning were selected as potentially negative (PN). These eighty substances were assessed by computational predictions for three hormonal receptors (ER, AR, and TR) using three software platforms (Danish QSAR, Vega QSAR, and Endocrine Disruptome), which included quantitative structure-activity relationship (QSAR) and molecular docking models. Model performance was evaluated based on coverage and concordance with *in vitro* and *in vivo* data. Finally, a majority consensus approach was applied to the *in silico* predictions to classify substances as having endocrine activity potential (>0.5) or non-endocrine activity potential (<0.5), by assigning scores of 0 and 1 for negative and positive results, respectively. **Results:** The Endocrine Disruptome was the most comprehensive tool, providing predictions for 95% of all substances. Danish QSAR exhibited good coverage for the ER and AR receptors, reaching 96.7% for the PP substances and 95% for the PN substances, but poor coverage for TR (59.3%). Vega QSAR covered 90% of the PN substances. For the PP substances, performance was better for ER (85%) and TR (98.3%), while coverage for AR was 68.3%. The concordance between predictions and *in vivo/in vitro* data was low for PP substances across all platforms. Endocrine Disruptome predicted the most positive results among the PP substances, with rates of 18.3% for ER, 23.3% for AR, and 45% for TR. Danish QSAR predicted positive results for 30% of the substances for ER, 15% for AR, and 6.7% for TR. Vega QSAR presented the lowest rates of positive prediction, being 23.3% for ER, 1.7% for AR, and no positive prediction for TR. In contrast, for the PN substances, the concordance with the *in vivo/in vitro* data was high, as only the Endocrine Disruptome predicted two positive results for AR. Finally, upon applying the majority consensus approach (considering only the *in silico* results), the analysis indicated that, within the PP substance group, approximately 35% are considered potentially active for ER, 23.3% for AR, and 30% for TR. For the PN substance group, the consensus demonstrated high agreement with the absence of activity, no substance showed potential activity for the ER and TR, and only one substance (5%) was classified as potentially active for the AR receptor. **Conclusions:** Given the increasing demand for NAMs in hazard identification, *in silico* methods serve as valuable tools, particularly for addressing data gaps. While the models showed high reliability in identifying PN substances, the low concordance observed between the positive predictions for the PP group and *in vivo/in vitro* data highlights the need for caution when interpreting isolated data. This suggests that the tools used in this study can be useful for hazard identification purposes, with a good performance especially in predicting the negatives. Conversely, any positive outcome should be considered with precaution. In those cases complementary predictions or *in vitro* results might be required.

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Background and Purpose: Prioritization of chemicals that may disrupt androgen signaling is central to the United States Environmental Protection Agency (US EPA) Endocrine Disruption Screening Program (EDSP). The program recently leveraged scores from the ToxCast androgen receptor (AR) Pathway Model, which integrates data from 11 *in vitro* assays, to prioritize chemicals for Tier 1 ED testing (i.e. Group 1 Chemicals). A high percentage of Group 1 chemicals exceeded the AR Pathway Score cutoff of 0.1, with most predicted to have AR antagonism - specifically, 28 out of 30 Group 1 chemicals were predicted to be AR antagonists. This retrospective analysis uses previously reviewed List 1 chemical data to assess whether AR antagonism scores correlate with *in vivo* anti-androgenic effects and proposes a tiered follow-up strategy for currently prioritized Group 1 chemistries. **Methods:** AR Pathway Model data (scores, flags, and cytotoxicity) for EDSP List 1 chemicals, previously reviewed by the Agency, were obtained from Kleinstreuer *et al.* (2017). US EPA risk assessment documents for List 1 chemicals were then reviewed for Tier 1 and 2-generation study results, where available. Additionally, assay-level data were examined to identify those most influencing antagonism predictions. **Results:** Our analysis, consistent with Kleinstreuer *et al.* (2017), found that integration of cytotoxicity and antagonism confirmation flags improved prediction accuracy. Across all compounds tested in ToxCast, the Odyssey Thera AR dimerization assays (OT_AR_ARSRC1 at 48 and 96 hour time points), showed the highest hit rate (15-21%), followed by Tox21 AR antagonist assays (TOX21_AR_BLA_Antagonist_ratio and TOX21_AR_LUC_MDAKB2_Antagonist) (~18%). Of the 49 List 1 chemicals, 8 had AR Pathway Model scores >0.1; 5 of these had antagonism confirmation flags or low confidence scores (<3), and 3 were negative in Tier 1 testing. In contrast, chemicals with high-confidence antagonism confirmation flags having AR Model Pathway scores suggesting predicted antagonism, showed concordant effects in the Hershberger assay supporting the suitability of this assay for targeted follow-up. **Conclusions:** Evaluation of EDSP List 1 chemicals supports refining interpretation of AR Pathway Model results by integrating antagonism confirmation flags, confidence scores, and cytotoxicity evaluation. For chemicals with high-confidence AR Pathway Model antagonism predictions, a tiered strategy prioritizing *in vitro* assays and the Hershberger assay is recommended over full Tier 1 testing.

PS 3290 **L-type voltage gated calcium channel blocker nifedipine blocks MIN6 cell depolarization with or without diphenhydramine; a live-cell fura-2 fluorescence imaging study.**

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Background and Purpose: Several antipsychotic medications have reported diabetogenic effects with patients taking them having a 2 fold higher risk of becoming type II diabetic (T2DM). Furthermore, specific antipsychotic drugs most often associated with T2DM also inhibit histamine 1 receptors (H1R). Diphenhydramine (DPH), is one of the most commonly used H1R inhibitors used over the counter for treatment of seasonal allergies (brand Benadryl®) and as a sleep aid (brand ZzzQuil®). It is unknown if DPH alters pancreatic beta cell function which could contribute to the onset of T2DM. This study was performed to examine if there are DPH effects on the excitability of MIN6 cells, an *in vitro* model of the pancreatic β -cell. **Methods:** Live fura-2 fluorescence of MIN6 cells was performed in the presence and absence of L-type calcium channel blocker (nifedipine 5 μ M) and agonist (BayK 8644 5 μ M) to examine the role of these channels in MIN6 cell response to DPH. MIN6 cells were incubated in 5 μ M fura-2 AM then all cells were depolarized by perfusing 40 mM KCl HEPES buffered saline for 2 min. After returning to baseline fluorescence, treated cells were perfused in buffer containing DPH (10 μ M) with and without 5 μ M nifedipine and 5 μ M BayK 8644 for 2 min then depolarized a second time in a 40 mM KCl solution that contained the same concentration of DPH 5 μ M with and without nifedipine and 5 μ M BayK 8644. Saline-control cells were perfused a second time with 40 mM KCl buffer only. **Results:** There were statistically significant ($p \leq 0.05$) decreases in both the slope and peak fluorescence of fura-2 in nifedipine-treated cells with or without 10 μ M DPH. No changes in time to onset and time to peak of fura-2 fluorescence were detected. These results indicate that the L-type calcium channel blocker, nifedipine at 5 μ M, inhibited 40 mM KCl-induced depolarization and importantly, that 10 μ M DPH did not alter this response. No significant differences were detected in BayK 8644-treated MIN6 cells for the quantitative endpoints examined. **Conclusions:** In contrast to our previous studies showing that 10 and 50 μ M DPH caused changes in time to onset and peak fura-2 fluorescence; 10 μ M DPH, in this study, did not alter these endpoints. Furthermore that DPH did not change MIN6 cell response to nifedipine or BayK 8644 would suggest that any actions that DPH has on MIN6 cells is not mediated via L-type calcium channels. Further research is needed to determine the dose-dependent effects of DPH on MIN6 cell fura-2 fluorescence, insulin release and *in vivo* effects on blood glucose.

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Background and Purpose: Apical toxicological effects observed in rats at high dietary levels of fenbuconazole have been consistently associated with hepatic toxicity. To further elucidate the mechanistic basis for these findings, integrated *in vivo* and *in vitro* studies were conducted to evaluate hepatic enzyme induction, aromatase inhibition, and steroid hormone synthesis. **Methods:** In two *in vivo* mechanistic studies, nine-week-old female rats received diets containing 0, 8, 80, or 800 ppm fenbuconazole (~0.6, 6, or 60 mg/kg bw/day). In the proestrus study, animals were treated for ~7 weeks and euthanized on proestrus; in the pregnancy study, treatment began 3 weeks prior to mating and continued through gestation (GD19-21). Serum hormone levels and hepatic CYP enzyme activities were assessed. Complementary *in vitro* assays were conducted including a human recombinant microsome assay to assess aromatase inhibition, and an H295R Steroidogenesis Assay to evaluate effects on testosterone (T) and 17 β -estradiol (E2) production. **Results:** At ≤ 80 ppm *in vivo*, no treatment-related effects were observed. At 800 ppm, systemic and hepatic toxicity was evident, with CYP2B1 and CYP3A2 induction up to 31-fold and CYP1A1 suppression. In pregnant rats, estradiol and corticosterone were significantly reduced, while progesterone was slightly elevated. *In vitro*, fenbuconazole inhibited human aromatase activity with an average LogIC₅₀ of -5.89 M (IC₅₀ = 1.29×10^{-6} M), achieving 1.4% residual activity. In the H295R assay, fenbuconazole produced a statistically significant 1.5-fold decrease in E2 at 1.78×10^{-5} M without affecting T modulation. **Conclusions:** Collectively, these data demonstrate that apical toxicological effects of fenbuconazole occur only at doses associated with overt hepatic toxicity, identifying the liver as the most sensitive target organ. Other systemic effects are considered secondary to hepatic perturbations. Mechanistically, induction of hepatic enzymes and weak inhibition of aromatase and steroidogenic pathways contribute to altered steroid hormone profiles, further supporting the liver as the central driver of systemic toxicity.

PS 3292 **LC-MS/MS-Based Deiodinase (DIO1, DIO2, DIO3) Inhibition Assays: Validation and Application to Human DIO1 Proficiency Testing for Endocrine Disruption Assessment**

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Background and Purpose: Deiodinase (DIO) enzymes are key regulators of thyroid hormone (TH) activation and inactivation, and inhibition of these enzymes is recognised as a molecular initiating event (MIE) contributing to TH disruption. Reliable *in vitro* assays are therefore essential to support the mechanistic evaluation of potential endocrine-disrupting (ED) chemicals. Building on our previously developed LC-MS/MS-based deiodinase inhibition assays for rat and human DIO1, DIO2 and DIO3, which have been validated in compliance with Good Laboratory Practice (GLP) principles, this study aimed to demonstrate assay robustness and to extend its application to the testing of proficiency items for human DIO1. The objective was to evaluate the method's suitability for proficiency testing and for future use in regulatory and research contexts focused on thyroid hormone disruption. **Methods:** Recombinant rat and human DIO enzymes are ectopically expressed in HEK293 cells. Cell lysates prepared from HEK293 cells expressing recombinant rat and human DIO1, DIO2, and DIO3 were used as enzyme sources. The LC-MS/MS-based bioanalytical (BioA) method quantitatively measures reaction products specific to each isoform: 3,3'-diiodothyronine (T2) for DIO1, 3,5,3'-triiodothyronine (T3) for DIO2, and 3,3',5-triiodothyronine (rT3) for DIO3 to determine enzyme activity and inhibition. For the current study, approximately 20 proficiency items were tested for their inhibitory potential toward recombinant human DIO1. Enzymatic conversion of rT3 to T2 was monitored by LC-MS/MS, and inhibition data were compared to published literature values. 6-Propyl-2-thiouracil (PTU) served as the positive control inhibitor. **Results:** The deiodinase inhibition assays demonstrated high sensitivity and precision, consistent with previous GLP validation data. The use of LC-MS/MS to monitor the production of the reaction products T2, T3 and rT3- for DIO1, DIO2 and DIO3 respectively, enables a higher degree of selectivity towards each analyte, consistent across all three isoforms, coupled with increased sensitivity over alternative detection techniques. The human DIO1 proficiency testing demonstrated excellent reproducibility and strong concordance with literature trends confirming the assay's analytical robustness and biological relevance. The LC-MS/MS readout provided high specificity for each analyte and enabled accurate quantification even at low enzyme activity levels thus ensuring reliable determination of inhibition potency. Application to approximately 20 human DIO1 proficiency items showed reproducible enzymatic activity inhibition measurements, which were in strong concordance with published literature values thus confirming both the analytical robustness and biological relevance of the assay. Compared with traditional Sandell-Kolthoff (SK) reaction-based methods, the LC-MS/MS BioA approach provided superior sensitivity and selectivity allowing reliable quantification of low-level enzyme activity- efficient discrimination of high, medium and low-level enzyme activity inhibitors, and accurate determination of inhibition potency for each analyte. **Conclusions:** GLP-validated LC-MS/MS-based deiodinase inhibition assays for rat and human DIO1,2 & 3 demonstrate robust, sensitive and selective measurement of enzymatic activity analytical plat-

form for investigating TH pathway interference. Application of the human DIO1 assay to proficiency testing demonstrates its suitability for use in inter-laboratory comparisons and regulatory screening frameworks and highlights their reproducibility and alignment with literature data. The LC-MS/MS BioA methodology offers improved sensitivity and specificity over traditional SK-based methods thus making it a reliable tool for assessing the ED potential of chemical compounds. Collectively, these assays strengthen the available toolkit for identifying and characterising chemicals with potential TH-disrupting activity and contribute to the broader implementation of mechanistic *in vitro* approaches in endocrine disruption testing strategies.

PS 3293 Robustness and Predictive Utility of the TruVivo® T4 Glucuronidation Assay in Primary Hepatocytes for Regulatory Safety Evaluation

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Background and Purpose: No *in vitro* or *in vivo* methodologies exist with international validated guidelines that can quantitatively assess liver-mediated thyroid hormone disruption across species to inform the human relevance of chemically induced thyroid toxicity. This highlights a critical need for NAMs that incorporate both relevant test species and human models for chemical safety evaluation. Previous study from our laboratory established 1. assay positive control (PC; PCB153) 2. historical control database (HCD) for vehicle and PC to define T4G assay variability and set acceptance criteria. The present study evaluated 26 compounds for their potential to alter thyroxine (T4) glucuronidation using primary rat and human hepatocytes within the TruVivo® system (co-culture model of feeder cells and primary hepatocytes). Expanding the compound set is essential to refine interpretation criteria for regulatory application. **Methods:** 26 compounds were selected based on literature evidence of nuclear receptor activation (CAR, PXR, AhR and PPAR) and preclinical or clinical data indicating alterations in circulating T4 levels. An initial screen of 26 compounds was conducted to determine solubility limits (macroscopic) and cytotoxicity (MTT assay). Subsequently, the compounds were pre-screened for T4 glucuronidation using a TruVivo® test system. Hepatocytes from one human male donors and one pooled male rat lot were exposed daily for 7 days to test compounds (3 dilution each), a positive control (PCB153; 30 µM) and reference compounds (PCN; 10 µM, PB; 500 µM, RIF; 10 µM) at non-cytotoxic concentrations (<20%). Physiological level of ¹³C₆T4 (0.05, 0.1µM rat and human) was added with the 7th exposure. T4 glucuronide (T4G) was quantified in the medium 24 hours post-T4 addition using a validated LC-MS/MS. The ΔT4G (T4G relative to DMSO) was calculated to assess compounds response. Post-exposure cytotoxicity was measured using ATP Glo® assay. From this, 12 compounds (PCB126, pentachlorophenol, carbamazepine, phenytoin, lansoprazole, and oxiconazole nitrate, levetiracetam, lamotrigine, flumazenil, ketoconazole, diclofenac sodium, and loratadine) were selected for further evaluation in TruVivo® test system (4 donor/lot). These evaluations were conducted under GLP conditions and established assay acceptance criteria across two laboratories. **Results:** The mean±SD T4G rates for vehicle control samples were 2.3±0.4 and 19±1.2 pmol/10⁶ cells/24 hours for human and rat hepatocyte respectively and fall within the HCD range. The PC showed mean±SD ΔT4G induction of 4.5±1.9 and 79.4±12.2 pmol/10⁶ cells/24 hours for human (in one instance didn't not fall within the HCD range) and rat (within the HCD range) hepatocyte respectively. Screening of test compound revealed that, in rat hepatocytes, 10 compounds induced a mean ΔT4G response ranging from 5 to 52.4 pmol/10⁶ cells/24 hours, while the remaining 16 compounds showed responses of <0 pmol/10⁶ cells/24 hours. In human hepatocytes, 6 compounds demonstrated ΔT4G responses of > 5 to 36.4 pmol/10⁶ cells/24 hours, 3 compounds showed responses of <0 pmol/10⁶ cells/24 hours, and remaining fell within the mid-range of 0 - 4.9 pmol/10⁶ cells/24 hours. These findings illustrate a quantitative species-specific modulation between human and rat hepatocytes. The post-exposure toxicity criteria were met, as neither the PC nor the two reference compounds (RIF & PCN) exhibited any cytotoxicity. Based on these results, 12 compounds were selected to represent a broad spectrum of chemical classes with varied ΔT4G in both species. These compounds are currently being evaluated to assess the dose-dependent responses, definition of positive and negative thresholds and further characterize quantitative species-specific modulation. **Conclusions:** TruVivo® T4G assay showed reproducibility with repeated data falling within HCD ranges, confirming robustness of the assay acceptability criteria. The ongoing evaluation is a step toward refinement of interpretation criteria, contributing to the establishment of a NAM for regulatory safety assessment of thyroid-disrupting chemicals.

PS 3294 Assessing different *in vitro* hepatocyte models using *in vitro* to *in vivo* extrapolation to predict liver-mediated thyroid hormone clearance in rats

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Background and Purpose: The development of plant protection products involves extensive *in vivo* testing, ranging from subchronic to chronic cancer bioassays using thousands of animals with thyroid being a common target organ. A key mode of action for chemically induced thyroid toxicity in rodents involves liver-mediated enhancement of thyroxine (T4) metabolism and clearance. Although rats remain the preferred test species, there are known quantitative differences in liver-mediated thyroid toxicity where rats are established to be more sensitive than humans. To accurately interpret biological responses, it is essential to quantitatively map the key event relationships underlying the liver-thyroid adverse outcome pathway (AOP) in rats. With the advancement of new *in vitro* models and growing regulatory support for reducing animal use in toxicity testing, new approach methodologies (NAMs) offer the possibility to assess key events associated with rodent thyroid toxicity and address human relevance without additional animal use. This study explores two *in vitro* hepatocyte models for characterizing liver-induced thyroid disruption in rats, using quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) to assess hepatic UGT induction and T4 metabolism. **Methods:** Two separate rat hepatocyte coculture models, a Wistar sandwich culture and a Sprague Dawley triculture (TruVivo), were exposed for seven consecutive days to known reference inducers of hepatic cytochrome P450 and UDPGT enzymes in rats. T4 was added to the final dose and supernatants analyzed by HPLC-MS/MS for T4 glucuronidation. CYP and UGT gene expressions in the liver were investigated using RNAseq in the TruVivo model. In the Wistar model, on Day 7 mRNA was extracted for RT-qPCR (Phase I and II genes) and P450 enzyme and UGT-T4 activity were measured. Supernatants were also analyzed for T4 clearance, glucuronidation and T3/rT3 formation. The *in vitro* CYP/UGT expression and activity and T4 clearance were utilized to extrapolate the 5% benchmark concentration (BMC) and its lower confidence bound (BMCL) to derive the *in vivo* administered equivalent dose (AED) using reverse dosimetry. These were then compared to the *in vivo* benchmark dose (BMD) and its lower confidence bound (BMDL). **Results:** Both *in vitro* hepatocyte models showed significant increases in the rates of T4 glucuronidation when exposed to known positive controls. In the TruVivo model, PB and PCN increased glucuronidation compared to vehicle controls, with PCB153 inducing the highest rate of glucuronidation and UGT-T4 activity, making it the reference positive control. All three compounds also upregulated liver phase I and II enzyme biomarkers. In the Wistar culture, T4 glucuronidation was markedly increased in hepatocytes treated with PCN and PB, with PCN showing a marked increase in UDPGT-T4 activity whereas only marginal increases were observed with PB. QIVIVE using *in vitro* data produced the most accurate oral AED estimates when based on plasma AUC (0-24 h). Extrapolation of BMCL from mRNA and hepatic enzyme activity showed better accuracy than extrapolation from T4 clearance *in vitro*, when compared to the *in vivo* data obtained from male adult rats. Furthermore, the predicted AED was significantly lower than the BMD(L) values for T4 levels obtained from *in vivo* studies. **Conclusions:** Regulatory assessment of thyroid toxicity is shifting from animal studies towards alternative methods, emphasizing the need for robust *in vitro* approaches to characterize liver-mediated thyroid AOPs. We examined the effects of thyroid-disrupting compounds on T4 clearance and metabolism using two rat hepatocyte models and compared findings with short-term *in vivo* studies in Wistar and Sprague Dawley rats. Points of departure (PODs) were derived from *in vivo* studies and evaluated against predicted *in vitro* PODs using QIVIVE. Our study confirmed species differences supporting rat liver-mediated thyroid clearance is not biologically relevant for human risk assessment due to higher hepatic activity in rats. These results support the use of optimized *in vitro* systems as predictive tools for thyroid disruption, reducing animal use and enabling early de-risking of agrochemicals to improve next-generation risk assessment (NGRA).

PS 3295 Predictive *in silico* assessment of endocrine-disrupting activity in bioactive peptides

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Background and Purpose: Bioactive peptides (BPs) are short chains of amino acids with a wide range of biological activities, making them valuable in medicine and the food industry. However, their resemblance to natural hormones allows certain BPs to bind to nuclear receptors and alter cell signaling, raising concerns about possible endocrine interference. Thus, understanding these molecular interactions is essential to anticipate and prevent possible toxic effects related to peptide-based products. Therefore, this study aimed to examine the endocrine disruption potential of the most representative BPs using a combined *in silico* and machine learning framework, aimed at mapping receptor binding patterns and identifying structural features related to hormonal modulation. **Methods:** The work began with the extraction of the main BPs reported in the scientific literature between 2019 and 2024 through a systematic approach; Antihypertensive, antioxidant, anticancer and multifunctional effects with experimentally validated bioactivity were taken into account. The molecular structures of the selected BPs were drawn in ACD/ChemSketch and converted

to SMILES format using the NIH Translator tool. The potential for endocrine disruption was then assessed using the Endocrine Disruptome web platform, which simulates molecular docking in 14 human nuclear receptors, including AR, ER α / β , GR, LXR α / β , PPAR α / β / γ , RXR α , and TR α / β . Peptide-receptor binding energies ≤ -7.0 kcal/mol were considered physiologically significant. Interaction matrices were standardized and analyzed by principal component analysis (PCA) and K-means clustering to reveal receptor binding trends. Finally, a supervised machine learning model was integrated to validate and refine the predictive clustering, estimating variable importance scores and confirming the discriminative power of peptides previously classified as highly promiscuous. **Results:** Initially, 132 BPs were extracted from the articles consulted; among these, 97 showed significant affinity for at least one nuclear receptor, yielding 277 peptide-receptor interactions. Most peptides interacted primarily with the glucocorticoid receptor (27.1%), followed by the thyroid receptors TR β (11.6%) and TR α (9.8%) and the androgen receptor (9.8%). While 76.3% of BPs showed low promiscuity (binding to ≤ 3 receptors), only 2.1% were classified as highly promiscuous, interacting with seven or more receptors. The PCA explained 43.6% of the variance, revealing distinct groups consistent with receptor selectivity profiles. Dipeptide H3-01 (WF) and anticancer peptide C5 (RWQWRWQWR) showed the strongest multireceptor affinities (-9.8 to -11.9 kcal/mol) toward LXR β , LXR α , GR, and PPAR γ , suggesting potential for pan-receptor modulation. Machine learning validation strengthened the PCA-based classification, confirming that these peptides are predictive outliers with high endocrine binding probabilities and unique physicochemical signatures (aromatic density, charge distribution, and hydrophobicity index). **Conclusions:** This integrative analysis suggests that certain bioactive peptides behave as endocrine-active compounds able to influence several nuclear receptors. Combining computational modeling with a machine learning validation step increased confidence in the receptor-binding patterns observed and strengthened the identification of peptides showing high binding promiscuity. Altogether, the results point to a small subset of molecules with potential endocrine activity that deserves closer toxicological scrutiny. Early application of predictive toxicology frameworks could help refine safety assessments and ensure that peptide-based nutraceuticals and therapeutics advance with stronger evidence of biological selectivity and reduced endocrine risk.

PS 3296 Luteolin promotes steroidogenesis in I-10 mouse Leydig cells exposed to atrazine

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Background and Purpose: Testosterone, the primary male sex hormone, is essential for male fertility and overall health. About 95% of total testosterone is synthesized by testicular Leydig cells via steroidogenesis. Disruption of this process by factors like pesticide exposure can lead to testosterone deficiency. Atrazine (ATZ), a widely used herbicide, can impair steroidogenic function, while luteolin (Lut), a flavonoid, exhibits antioxidant properties. This study examines luteolin's effects on ATZ-induced dysfunction in I-10 Leydig cells by assessing viability, steroidogenesis, and gene expression. **Methods:** Mouse Leydig I-10 cells were treated with DMSO (control), ATZ (3.3, 10, 33, and 100 μ M), Lut (1, 3.3, 10, and 33 μ M), and a co-treatment of ATZ + Lut (ATZ 33 μ M + Lut 3.3 μ M or ATZ 100 μ M + Lut 3.3 μ M) for 2, 4, and 6 days, with medium/chemical change every two days. Cell viability was measured at each time point, with testosterone (T) and progesterone (P4) levels quantified by ELISA, and the expression of key steroidogenic genes (StAR, Cyp11a1, Hsd3 β 6, and Hsd17 β 3) analyzed by RT-qPCR. **Results:** ATZ alone reduced cell viability in a concentration-dependent manner but temporarily increased T levels. Lut alone enhanced both P4 and T production at higher concentrations (10 and 30 μ M). In the co-treatment, Lut protected I-10 cells from ATZ-induced cytotoxicity and transiently stimulated both P4 and T production. Specifically, the ATZ 100 μ M + Lut 3.3 μ M co-treatment showed the strongest effects, with P4 and T rising 2.6-fold and 1.9-fold, respectively, on Day 2, before returning to baseline by Day 6. These findings suggest co-exposure of Lut and ATZ may confer modest stimulation on steroidogenesis in I-10 cells. **Conclusions:** In I-10 mouse Leydig cells, luteolin promoted survival and steroidogenesis in the presence of atrazine, suggesting a modulatory interaction that benefits I-10 cell vitality and enhances its steroid production.

PS 3297 Endocrine disrupting effects of jet fuel: Changes in rodent estrous cycle and ovary size after 6-month oral exposure

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Background and Purpose: Recent incidents involving contaminated water, such as the Red Hill water crisis, have shown that we need a better understanding of the effects of oral exposure (i.e., ingestion) of jet fuel. Ingestion has not historically been widely studied as a standard route of jet fuel exposure for health assessments given that ingestion does not present as a typical occupational exposure risk. Endocrine-disrupting chemicals (EDCs) are garnering greater attention and wider concern for their previously unknown effects mimicking, blocking, or interfering with the body's hormones. We have previously demonstrated that the military aviation fuel Jet Propellant (JP)-5 is capable of modulating nuclear human estrogen receptor

(ER) activity in an *in vitro* HEK293 cell line stably expressing an ER-luciferase gene reporter, suggesting that JP-5 can act as an EDC. Here, we assess the endocrine effects of *in vivo* 6-month occupational exposures to JP-5 in female rats. **Methods:** Animal activities were IACUC protocol approved. To evaluate JP-5's effects on the endocrine system, female Sprague Dawley rats were exposed to 1 (LOW), 10 (MID), or 100 (HIGH) mg JP-5 per kg body weight (BW) diluted in corn oil via gavage five days/week for six months. Control rats were exposed to the vehicle only (corn oil). These rats underwent ~ 14 continuous days of vaginal lavage pre-exposure, ~ 16 wk into exposure, and the last two weeks of exposure. Samples were fixed and stained using 0.1% methyl blue to determine estrous cycle. Plasma samples were collected during humane euthanasia ~ 6 -7 wk post-last dose of JP-5 to assess delayed hormonal effects via enzyme linked immunosorbent assay. A separate subset of rats also underwent 6-month oral JP-5 exposure, with plasma samples collected 24 h post-last dose to assess immediate hormonal effects. Ovaries were collected from both cohorts to measure changes in Ovary:BW ratio. Group differences were analyzed using a one-way analysis of variance and Dunnett's post-hoc test. **Results:** There were no group differences in estrous cycle at baseline pre-exposure. After ~ 16 wk of oral JP-5 exposure the LOW, MID, and HIGH groups experienced increased number of days in diestrus ($p < 0.05$). However, by the end of the exposure period only the MID and HIGH group had increased number of days in diestrus ($p < 0.05$). Immediately after 6-month JP-5 exposure there was a dose-dependent decrease in plasma estradiol (E2, $p < 0.01$), and a reduction in progesterone (PROG) in the HIGH group ($p < 0.01$). Although ~ 6 -7 wk post exposure found no group differences in hormone levels, a lesser but significant relationship between JP-5 exposure level and E2 ($r = -0.35$, $p < 0.001$) existed. Ovary:BW ratio did not differ between groups 24 h post-last dose of JP-5, but ~ 6 -7 wk post exposure found LOW JP-5 to have greater Ovary:BW ratio. This larger Ovary:BW ratio negatively correlated to plasma corticosterone levels ($r = -0.786$, $p < 0.01$), while E2 correlated to parathyroid hormone (PTH) levels ($r = 0.915$, $p < 0.001$). Multiple linear regression analyses found PTH to be a significant predictor of Ovary:BW ratio immediately following JP-5 exposure and ~ 6 -7 wk following exposure. **Conclusions:** Our study suggests long-term ingestion of JP-5 leads to changes in estrous cycle, hormone levels, and ovary size. Reduction in E2 and PROG levels coincide with increased time spent in diestrus. Although hormonal group differences were not present, correlational analyses indicate that there are sustained effects over a month after JP-5 exposure ends. Sustained low levels of JP-5 may contribute to increases in ovary size, albeit PTH may mediate these effects. Further research is needed to investigate the potential EDC effects of JP-5 on ovaries, including hormone related disorders such as polycystic ovarian syndrome and endometriosis. No DoD endorsement implied. This work was funded by Defense Health Program, Military Operational Medicine Research Program 330, Joint Program Committee 5.

PS 3298 Associations Between Co-Exposure to Environmental Toxins and Type 2 Diabetes in U.S. Adults: Insights from NHANES on Perchlorate, Nitrate, and Thiocyanate

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Background and Purpose: Perchlorate, nitrate, and thiocyanate are environmental anions known to disrupt thyroid function by competitively inhibiting iodide uptake via the sodium-iodide symporter (NIS), potentially reducing thyroid hormone synthesis. Although thyroid dysfunction is linked to metabolic disorders, the relationship between these anions and type 2 diabetes mellitus (T2DM) has not been fully elucidated in human populations. **Methods:** A total of 5,196 adults aged ≥ 20 years from the National Health and Nutrition Examination Survey (NHANES 2011-2020) were included. Nine Survey-weighted multivariable logistic regression models were used to evaluate the associations between urinary perchlorate, nitrate, and thiocyanate and T2DM. Weighted quantile sum (WQS) regression was applied to assess the combined effects of co-exposure to these anions. **Results:** Higher urinary perchlorate concentrations were significantly associated with increased odds of T2DM (OR = 1.92; 95% CI: 1.10-3.37; $p < 0.05$) in fully adjusted models. WQS regression showed that co-exposure to perchlorate, nitrate, and thiocyanate was positively associated with T2DM; each unit increase in the WQS index corresponded to a 33% higher risk of T2DM (OR = 1.33; 95% CI: 1.07-4.65; $p < 0.001$). After adjusting for age, gender, race/ethnicity, and urinary creatinine (Model 2), the association remained significant, indicating a 45% higher risk of T2DM per unit increase in the WQS index (OR = 1.45; 95% CI: 1.045-7.00; $p = 0.02$). Perchlorate and thiocyanate contributed the greatest weights within the mixture. **Conclusions:** Higher urinary perchlorate levels and co-exposure to perchlorate, nitrate, and thiocyanate were significantly associated with T2DM. Perchlorate and thiocyanate were the primary contributors to the observed mixture effect. These findings highlight the metabolic relevance of environmental anion exposure and underscore the need for further mechanistic and mixture-based investigations.

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Background and Purpose: Rodents are the standard model for regulatory toxicity testing; however, they are particularly sensitive to thyroid disruption. Thyroid-related findings in rodent studies are common and generally arise from hepatic mechanisms that lack human relevance. A well-characterized pathway involves liver enzyme induction—particularly UDP-glucuronosyltransferases (UGTs)—which accelerates the clearance of thyroid hormones like thyroxine (T4). This can lead to compensatory thyroid follicular cell hypertrophy and, in some cases, tumor formation. However, these liver-mediated thyroid effects are not considered human-relevant, as the underlying mechanisms and thresholds differ significantly between species. Historically, *in vivo* animal studies have been the foundation of thyroid safety evaluations for agrochemicals. However, there is growing momentum within the scientific and regulatory communities to adopt New Approach Methodologies (NAMs) that reduce reliance on animal testing and improve human relevance. This study aims to validate an *in vitro* liver-thyroid organ-on-chip model capable of investigating mechanisms of thyroid toxicity. **Methods:** Cryopreserved human primary hepatocytes from mixed donors were co-cultured with hepatic stellate cells at a 24:1 ratio directly in the small compartment of the HUMIMIC Chip2 system (TissUse, Berlin, Germany) utilizing a polycarbonate Dynarray microcavity membrane. Thyroid microtissues underwent a five-day pre-incubation in Matrigel-coated 96-well plates, following protocols established by Dr. Deisenroth, prior to hydrogel overlay and subsequent transfer into the large compartment of HUMIMIC Chip2 chips. The liver/thyroid co-cultures were maintained on-chip with thyroid stimulating hormone (TSH) stimulation preceding exposure to two reference chemicals: liver-mediated thyroid disruptor (PCB153) and direct thyroid disruptor (methimazole). Quantification of albumin, T4, and T4 metabolites (T4-glucuronide and/or T4-sulfate) was performed using liquid chromatography-mass spectrometry. Additionally, immunostaining was employed to confirm the functional integrity of both liver and thyroid tissues. **Results:** Primary thyrocytes cultured on Matrigel-coated plates self-assembled into rounded, hemispherical 3D microtissues and exhibited pronounced responsiveness to TSH stimulation, with an average production of T4 at 7.5ng/mL. As anticipated, methimazole treatment produced a concentration-dependent decrease in thyroid hormone secretion, whereas PCB153 exposure did not induce any measurable changes in the thyroid microtissue model. Liver spheroids remained viable and functional for over 14 days, forming bile canaliculi with MRP2 expression and producing albumin as well as thyroid hormone metabolites (T4-glucuronide and/or T4-sulfate) following T4 supplementation. Methimazole treatment did not affect T4 metabolite concentrations, whereas PCB153 exposure significantly increased T4G levels, indicating a pronounced liver-mediated effect. The integrated liver/thyroid co-culture system secreted measurable thyroid hormones, which declined following methimazole administration, while albumin production remained stable throughout the two-week study. **Conclusions:** These findings confirmed that both the liver and thyroid models were fully characterized and functioned as intended. It underscores the value of an integrated liver/thyroid co-culture system for the early investigation of mechanisms of thyroid disruption. Furthermore, the model offers a mechanistic framework to support demonstrations of non-human relevance for liver-mediated thyroid disruption pathways. A follow-up study is currently underway to optimize the co-culture system and enhance its predictive performance.

PS 3300 *In Vitro* assessment of AhR/CAR/PXR activators within the thyroid endocrine disruption (T-) modality framework: Implications for human relevance

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Background and Purpose: Thyroid hormones (THs) regulate metabolism, growth, and neurodevelopment. Xenobiotics can disrupt TH homeostasis by interfering with the thyroid axis through distinct molecular initiating events (MIEs), which may lead to altered, often decreased, circulating TH levels. EU regulation 2018/605 and ECHA-EFSA guidance require evaluation of endocrine-disrupting potential, including thyroid (T-) modality. Key MIEs include inhibition of the sodium/iodide symporter (NIS), thyroid peroxidase (TPO), and deiodinases (DIO1, DIO2, DIO3), which respectively impair iodide uptake, TH synthesis, and peripheral conversion of thyroxine (T4) to triiodothyronine (T3) or reverse T3 (rT3). In parallel, activation of hepatic nuclear receptors such as the Aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR) can induce phase I (CYP) and phase II (UGT) enzymes, enhancing hepatic clearance of conjugated THs, mainly via glucuronidation. However, the human relevance of liver enzyme-mediated thyroid effects remains a regulatory challenge in the EU. This study integrates multiple new approach methodologies (NAMs) covering both thyroid MIEs and hepatic metabolism pathways to investigate the mechanisms underlying TH disruption. A set of test substances, including AhR, CAR and/or PXR activators, with or without reported effects on serum TH levels in humans, was tested for inhibition of NIS, TPO and DIO1-3 and for indirect liver enzyme-mediated thyroid effects. **Methods:** NIS-mediated iodide uptake was assessed in HEK293 cells stably expressing human

NIS using the Sandell-Kolthoff reaction. TPO activity was measured in human thyroid microsomes using the Amplex UltraRed method. DIO (1, 2 and 3) activities were assessed in HEK293 cell pellets expressing each human DIO isoform, with T3 and rT3 quantified by LC-MS following T4 incubation. Test substances were assessed across different assays, with respective positive and negative controls included to ensure assay specificity and reproducibility. Liver-mediated effects were investigated in cryopreserved rat and human primary hepatocytes cultured in a 2D-sandwich configuration (2 male and 2 female batches per species). Cells were exposed for 7 days to test substances or to reference CYP inducer compounds β -naphthoflavone (BNF), Phenobarbital (PB), Rifampicin (RIF) or 5-pregnen-3 β -ol-20-one 16a-carbonitrile (PCN). CYP1A, CYP2B and CYP3A mRNA expression and related enzymatic activities were measured as markers of AhR, CAR and PXR activation, respectively, along with UGT mRNA expression and T4-glucuronidation (T4-UGT) activity. T4 disappearance and T4 metabolite formation were quantified in both species to assess interspecies differences in TH metabolism and clearance. **Results:** In line with previous results (Asselin et al., 2024; Friedman et al., 2016) with CAR/PXR activators RIF and PB showing mild (PB) or strong TPO inhibition and mild DIO1 inhibition (PB), our present data show that 5/9 AhR, CAR and/or PXR test substances for which effects on serum THs have been reported in humans exhibit multiple mode of actions (MoAs). These findings suggest that liver enzyme induction alone is unlikely to explain the observed alterations in serum TH levels. Ongoing work using the same set of test substance aims to address liver-mediated mechanisms, focusing on CYP and UGT increase and T4 metabolism in human and rat hepatocytes to clarify the contribution of hepatic pathways to TH homeostasis disruption. **Conclusions:** These findings illustrate that AhR/CAR/PXR activators can influence TH homeostasis through multiple mechanisms, including direct effects on TH synthesis. By combining these complementary assays, the work contributes to the refinement of thyroid (T-) modality testing strategies and to improving human relevance assessments within the EU endocrine disruptor regulatory framework.

PS 3301 Differentiating Thyroid Modes of Action in Short-Term Rodent Toxicity Studies Using Toxicogenomics

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Background and Purpose: Alterations in thyroid parameters in rat toxicity studies can arise from direct thyroid disruption or via indirect mechanisms such as liver enzyme induction and systemic toxicity. Distinguishing between these thyroid modes of action (MOAs) is key to understanding human relevance but is rarely possible through traditional toxicology endpoints. Through rapidly evolving omics technologies such as transcriptomics and metabolomics, it is now possible to generate vast amounts of molecular information from tissue samples collected in toxicity studies, potentially refining and reducing the overall use of animals. This study investigated whether toxicogenomic analysis of liver, thyroid, and plasma in short-term rat studies would allow mechanistic differentiation between established direct thyroid MOAs, including inhibition of Thyroid Peroxidase (TPO), Sodium Iodide Symporter (NIS), or Deiodinase (DIO), and thyroid effects secondary to liver enzyme induction or systemic toxicity. **Methods:** Male and female Han Wistar rats (n=10/group) were treated for 14 days with reference chemicals representing direct thyroid MOAs (methimazole, 6-propyl-2-thiouracil, sodium perchlorate) or liver enzyme induction (phenobarbital) at 3 dose levels per chemical, alongside respective concurrent vehicle control groups. Two levels of food restriction were also included in the study to emulate the physiological stress typically observed with systemic toxicity (reduced food consumption and lower bodyweight gain). Study endpoints included toxicokinetic analysis of each reference chemical at 2 timepoints, in-life thyroid hormone analysis (T4, T3, TSH) at 3 timepoints, body weights, and terminal organ weights and histopathology on all animals. Transcriptomics data (liver, thyroid) and metabolomics data (liver, plasma) were generated on all groups to investigate the molecular profiles associated with each MOA. **Results:** Toxicokinetic analysis for each reference chemical confirmed appropriate separation of systemic exposure levels across groups, corresponding with observed dose-related changes in apical thyroid endpoints. Toxicogenomic analyses revealed distinct, dose-related profiles for each reference MOA, achieving mechanistic differentiation between direct and indirect thyroid MOAs at the molecular level. **Conclusions:** Toxicogenomic analysis enabled detection of molecular effects following short-term dosing that allowed successful differentiation between direct and indirect thyroid MOAs in rats. This work demonstrates the potential for omics-based MOA categorization of test chemicals with thyroid effects, based on similarity with reference profiles of known thyroid MOAs. Ultimately, this approach could be used to inform regulatory decision-making and reduce uncertainty in human health risk assessments for test chemicals with rodent thyroid effects.

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Background and Purpose: Adverse Outcome Pathways (AOPs) are a conceptual framework that describe the mechanistic links between biological processes and events at the molecular level to adverse effects at the individual and population levels. The thyroid hormone signaling pathway, which involves the thyroid hormones T3 and T4, is one such molecular-level process critical for the development and viability of vertebrates, including fish. **Methods:** In this study, CRISPR/CAS technology was used to better understand the role of key thyroid hormone signaling pathway enzymes, deiodinase 3a and 3b (Dio3a, Dio3b), which regulate thyroid hormone concentration in tissues through inactivating T3 and T4, to assist in AOP development. Zebrafish Dio3a^{-/-} and Dio3b^{-/-} mutant lines with a 31-base pair (bp) deletion and 11-bp insertion, respectively, were produced using CRISPR/CAS-12a and three generations of genotyping and selective breeding. Equal numbers of either mutant Dio3a or Dio3b embryos and wildtype (WT) embryos (n=120-144) were incubated in 24-well plates for six days (6-144 hours post-fertilization, hpf) and observed daily, taking note of phenotypic abnormalities including edema, reduced pigmentation, altered swim bladder inflation, and deformed body shape. At 144 hpf, larval fish were pooled into 20-23 groups per genotype (3-6 fish per group), and RNA was sequenced. **Results:** Phenotypic data observed between Dio3a^{-/-} and Dio3b^{-/-} versus WT groups displayed similar trends. Rates of survival were lower for Dio3a^{-/-} compared to WT (77% vs. 85% for WT), while abnormalities remained similar amongst groups (5% vs. 6%). Swim bladder inflation at 144 hpf was higher amongst the WT group (73% vs. 65% for Dio3a). Meanwhile, amongst the Dio3b mutant group, there was a significant difference in survival (70% vs. 93% for WT; p<0.0001), with the frequency of abnormalities in the surviving groups averaging 10% (Dio3b^{-/-}) and 16% (WT). Swim bladder inflation at 144 hpf exhibited no difference between WT and Dio3b^{-/-} groups. RNA sequencing revealed large differences in global gene-expression patterns between WT in both mutant groups, with 1638 differentially expressed genes (DEGs) in the Dio3a^{-/-} fish and 1506 DEGs in Dio3b^{-/-} fish. **Conclusions:** Overall, stable lines of zebrafish with Dio3a/b mutations were generated with CRISPR/CAS-12a that will serve as a valuable system to identify causal relationships between key events for future studies focused on thyroid-related AOPs. The contents of this abstract neither constitute nor necessarily reflect US EPA policy.

PS 3303 Developing A Curated Resource of Rodent Pubertal Studies in Support of the US EPA Endocrine Disruptor Screening Program (EDSP)

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Background and Purpose: Endocrine disrupting chemicals may interfere with the endocrine system functions such as normal reproduction and developmental processes in humans and wildlife. The United States Environmental Protection Agency (US EPA) Endocrine Disruptor Screening Program (EDSP) seeks to screen for and protect against possible endocrine effects associated with chemicals using a tiered testing approach. The male and female rodent pubertal studies (890 Series Health Effects Test Guidelines OCSPP 890.1450 and 890.1500) are EDSP Tier 1 assays that assess a chemical's potential to disrupt endocrine-mediated development by examining changes during sexual maturation. These studies help determine if chemicals can interfere with the estrogen, androgen, and thyroid hormone pathways in the developing endocrine system by assessing endpoints specific to pubertal development in both male and female rats. **Methods:** An effort was initiated to comprehensively identify documents from 1990 to present that describe studies with data similar to the pubertal test guidelines. Using study evaluation criteria, studies were designated as "guideline"-adherent, "guideline-like" or "useful" and prioritized for curation. Statistically significant and treatment-related findings were extracted via an application-based curation workflow using EPA's Health Assessment Workspace Collaborative (HAWC) program. Study metadata included rat strain, sex (male or female), animal source and husbandry, exposure lifestage, diet, route of exposure, and exposure duration. Curation is ongoing. **Results:** Preliminary assessment of the data includes 136 pubertal curations (59 male, 57 female) covering 86 chemicals, the majority of which are biocides. Out of the 147 unique endpoints curated, 94 endocrine endpoints relevant for both male and female rats were identified in relation to the reproductive and endocrine systems, affecting organs such as the testis, uterus, thyroid, and pituitary gland. Potential estrogenic effects evaluated include age and body weight at vaginal opening, organ weights and histology of uterus and ovary, and estrus cyclicity endpoints. Potential androgenic effects evaluated include age and body weight at preputial separation, and organ weights and histology of the epididymis and testis. Potential thyroidogenic effects assessed may include hormone measurements (e.g. T4), organ weights and histology of the thyroid. **Conclusions:** The rodent pubertal assays are part of the larger EDSP Tier 1 battery designed to provide a comprehensive screen of a chemical's potential endocrine activity. Developing a curated resource facilitates the discovery of reference compounds and produces a database of results from an established guideline study that could be used to build scientific confidence in New Approach Methods. This abstract does not necessarily reflect U.S. EPA policy.

PS 3304 Application of a NOAEL and LOAEL Boundary Approach to Identify a Point of Departure for Derivation of Minimal Risk Levels for Hazardous Substances

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Background and Purpose: For ATSDR to develop minimal risk levels (MRLs) for hazardous substances, selection of the most appropriate point of departure (POD) is paramount. When available and adequate, ATSDR preferentially selects PODs from epidemiological data; otherwise, appropriate experimental animal laboratory data are used. When adequate dose-response data are available, benchmark dose modeling (BMD) is used. In the absence of such data, the NOAEL/LOAEL (N/L) approach is used, in which ATSDR sets the POD at either the highest NOAEL below the lowest LOAEL or the lowest free-standing LOAEL (if there is no accompanying NOAEL). Often, when MRLs are based on human data, POD selection defaults to the N/L approach since human data often lack multiple exposure levels required for robust dose-response analysis within a single study. However, reliance on the N/L approach often leads to uncertainties in POD selection from epidemiological studies due to substantial overlap in reported NOAELs and LOAELs for the critical effect. Here, we present an alternative approach that utilizes data from multiple studies to identify an exposure that is most likely to be the N/L boundary, a threshold exposure level at which adverse effects may or may not occur. **Methods:** For this presentation, illustrative cases utilizing the N/L boundary methodology to identify a POD are reported for the chronic-duration inhalation MRLs from the Toxicological Profiles for Mercury (Hg) and Carbon Disulfide (C2S). For this approach, we made the following assumptions: 1) A N/L boundary exists and is located somewhere within the range of overlapping NOAELs and LOAELs; 2) Each NOAEL and LOAEL in this range represents an independent estimate of the N/L boundary; 3) The best estimate of the N/L boundary is the weighted median of the set of overlapping NOAELs and LOAELs (weighted for study size); and 4) The best value for the POD is the lower 95% confidence limit on the median (to account for uncertainty in the estimate). To calculate the N/L boundary, overlapping NOAELs and LOAELs included all LOAELs ≤ the highest NOAEL for the critical effect, plus all NOAELs ≥ the lowest LOAEL for the critical effect. **Results:** For Hg vapor, the most sensitive effect following chronic inhalation exposure was tremors. The highest identified NOAEL was an estimated air concentration of 8.74 µg Hg/m³ by Boogaard et al. (1996) and the lowest identified LOAEL was an estimated air concentration of 4.90 µg Hg/m³ by Wastensson et al. (2006, 2008); air concentrations were estimated from urinary Hg levels using a pharmacokinetics model. A total of seven epidemiological studies identifying a NOAEL or a LOAEL in the approximated "overlap range" of estimated air concentrations of 4-9 µg Hg/m³ were included in the N/L boundary calculation. Based on these studies, a weighted median N/L boundary (95% Confidence Interval [CI]) of 4.92 (2.84, 7.00) µg Hg/m³ was calculated, resulting in a selected POD of 2.84 µg Hg/m³. For C2S, the most sensitive effect following chronic inhalation exposure was impaired nerve conduction velocity. The highest identified NOAEL was 5.64 ppm by Yoshioka et al. (2017) and the lowest identified LOAEL was 2.9 ppm by Godderis et al. (2006). A total of seven epidemiological studies identifying a NOAEL or a LOAEL in the identified "overlap range" of measured air concentrations of 2.9-5.64 ppm were included in the N/L boundary calculation. Based on these studies, a weighted median N/L boundary (95% CI) of 4.76 (4.02, 5.64) ppm was calculated, resulting in a selected POD of 4.02 ppm. **Conclusions:** Use of a weighted median N/L approach allows for multiple human studies to contribute to the POD determination for MRL calculations. This approach can result in reduced uncertainty associated with basing a POD on a single human study, especially when there is an overlap in reported NOAELs and LOAELs for the critical effect. Where appropriate, this approach should be considered and applied for future POD determinations based on epidemiological data. **Disclaimer:** The findings and conclusions in this presentation have not been formally disseminated by the Centers for Disease Control and Prevention/ATSDR and should not be construed to represent any agency determination or policy.

PS 3305 Framework for Intracochlear Accepted Daily Intake Determination for Human Health Risk Assessment

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Background and Purpose: The cochlea is separated from systemic circulation by the blood-labyrinth barrier (BLB) limiting the passage of molecules in or out. Direct delivery of therapeutics into the cochlea chambers (i.e. intracochlear administration) allows for efficient drug delivery, bypasses the barriers of the middle ear, and allows for more targeted and controlled dosing. Intracochlear administration involves directly injecting into the peri-lymphatic chambers through the round window membrane. Due to the unique nature of intracochlear administration, an accepted daily intake (ADI) specific to this route of administration is necessary. The goal of this study is to establish a framework for determining intracochlear ADIs from parental ADIs and provide examples to illustrate the utility of the framework. **Methods:** Both systemic and local effects must be considered when establishing an intracochlear ADI from a parental ADI. A framework for human health risk assessment

was developed considering: *Toxicokinetic considerations*: 1) bioavailability assumed to be 100% since substances are delivered directly into the cochlea; 2) substance assumed to be equally distributed throughout the cochlea; 3) metabolic pathways assumed to be similar to systemic pathways; elimination is assumed to be *de minimis* due to BLB. *Toxicodynamic considerations*: 1) the inner ear expected to be sensitive to neurotoxic substances; 2) receptor density assumed to be similar to systemic receptor density. *Irritation and Sensitization Effects*: 1) cochlear cells demonstrate immune responses after exposure to ototoxic substances; 2) if a substance is a known irritant or sensitizer, the local concentration following intracochlear administration should be evaluated to determine irritation or sensitization potential; 3) acute ICM administration of a known eye irritant at ppm levels did not result in any treatment-related local irritation. *Clinical Experience*: 1) substances administered into the cochlear rapidly dilute and diffuse within the intracochlear space; 2) diffusion along the entire cochlea is achieved with a volume of 90 μ L; 3) the cisterna magna may be connected to the intracochlear space by CSF flow. **Results**: A framework for determining intracochlear ADIs from parental ADIs was developed using information considered from the intracochlear route of administration. Firstly, the parenteral ADI is adjusted by applying a 10x factor for intracisternal magna (ICM) administration to account for additional uncertainty. Secondly, based on the volume difference between the cisterna magna and intracochlear space (approximately 3 vs 0.2 mL), an additional 10x factor is applied. Therefore, a total factor of 100 is applied to the parenteral ADI to determine an intracochlear ADI. If a substance is neurotoxic, an additional 10x factor is recommended unless substance-specific neurotoxicity information is available. Lots The first case study is for Triton X-100 with a parenteral ADI of 750 μ g/day. Triton X-100 is not neurotoxic but was reported to be irritating to the eye at 0.3% (3 μ g/ μ L). Therefore, a factor of 100 was applied to the parenteral ADI yielding an intracochlear ADI of 7.5 μ g/day. Assuming that 7.5 μ g is injected into the intracochlear space with a volume of 190 μ L, the worst-case local concentration is estimated to be 0.04 μ g/ μ L, which is 75 times lower than the concentration reported to be irritating to the eye. The second case study is for Poloxamer-188 with a parenteral ADI of 460 μ g/kg/day. Poloxamer-188 is not neurotoxic or irritating. Therefore, a factor of 100 was applied to the parenteral ADI yielding an intracochlear ADI of 4.6 μ g/kg/day. The intracochlear ADIs illustrated in these case studies are not expected to cause adverse systemic effects. Further, the local concentrations inside the intracochlear space are not expected to cause adverse local effects. Consultation with a clinical neurologist indicated that the safety risk is considered low at these concentrations. **Conclusions**: The utility of this proposed framework is illustrated by the two case studies in this study. While this framework provides a qualitative approach to determining intracochlear ADIs, it is a preliminary approach. Further refinement with quantitative data would further refine this approach and increase its applicability.

PS 3306 Using a Framework Approach to Determine Parenteral Accepted Daily Intake for Benzonase

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Background and Purpose: Enzymes are a diverse group of proteins that have a variety of functions. The use of enzymes in pharmaceutical manufacturing and therapeutics has increased over the years. Due to the diverse nature of enzymes, their safety profiles may vary considerably. Unfortunately, there is no consensus on how to determine health-based exposure limits (HBELs) for enzymes. Many industries default to the exposure limit of a highly sensitizing enzyme, subtilisin, and few enzyme-specific HBELs are available. A preliminary framework for HBEL determination for enzymes was previously presented by Fung et al. The goal of this study was to apply the preliminary framework and determine an accepted daily intake (ADI) for benzonase. **Methods**: In the preliminary framework presented by Fung et al., the recommendation is to first determine whether enzyme-specific data is available when determining a HBEL. If enzyme-specific data is not available, a default approach is taken based on enzyme characteristics including whether the enzyme is biocatalytic, endogenous, and/or sensitizing. If enzyme-specific data is available, a data driven approach should be taken. **Results**: Benzonase is a genetically engineered endonuclease used to remove residual nucleic acids in the purification process of pharmaceutical manufacturing. Benzonase-specific toxicological data is limited but data is available for similar endonucleases including DNase I (endogenous human enzyme that catalyzes the hydrolysis of extracellular DNA); therefore, a data driven approach was taken to determine the parenteral ADI for benzonase. The point of departure (POD) was determined from DNase I data. The amount of DNase I that can be added to the serum while remaining below the threshold of activity (concentration where DNA hydrolysis is observed) was calculated by subtracting the 95th percentile concentration of serum DNase I of 6 ng/mL from the threshold of activity required for catalysis of 50 ng/mL, yielding a value of 44 ng/mL. Benzonase was reported to have 34-fold greater activity compared to DNase I; therefore, the amount of benzonase that can be added to serum while staying below the threshold of activity is 1.3 ng/mL. Assuming that benzonase is distributed throughout the blood and a total blood volume of 4,700 mL, the amount of benzonase that can be added while staying below the threshold of activity is 6,000 ng/day. The value of 6,000 ng/day or 6 μ g/day was selected as the POD. The POD is derived from a minimum active serum concentration in humans and accounts for difference in activity of DNase I compared to benzonase; therefore, further adjustment factors are not warranted. Together, a parenteral ADI of 6 μ g/day

is recommended for benzonase. **Conclusions**: This study demonstrates the utility of preliminary framework presented by Fung et al. where enzyme-specific data is available. Additional case studies using this framework may help refine the framework to determine HBELs for enzymes without data.

PS 3307 Risk Assessment and Pharmacokinetic Modeling of Incidental Ingestion of Ethanol from Mouthwash

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Background and Purpose: Mouthwashes containing ethanol for antiseptic properties have a long history of use and well-established oral hygiene benefits by significantly enhancing the control of dental biofilm and reducing gingival inflammation. Despite the substantial health benefits from ethanol-containing mouthwashes, there has been ongoing scrutiny by regulatory and health authorities of products containing ethanol resulting from the classification of consumption of alcohol-containing beverages as a potential carcinogen. Unlike alcohol-containing beverages, mouthwash products are not intended to be ingested, and the potential ethanol exposure is substantially lower for mouthwash products. Therefore, we conducted a risk assessment that benchmarked estimated and modeled incidental exposure to ethanol in mouthwash products against endogenous blood levels, dietary exposure, and health-based guidance values (HBGVs) for ethanol. **Methods**: Several approaches were used to estimate and benchmark exposure to ethanol from mouthwash use for risk characterization purposes. First, an internal dose, or systemic exposure dose (SED), was calculated from three use scenarios, including average use, 90th-percentile use, and maximum-label use. Data for average use and 90th-percentile use were based on previously reported exposure data in representative European countries. The estimated exposure to ethanol from various mouthwash-use scenarios was compared to typical dietary intake levels to contextualize the relative magnitude of exposure. Second, a physiologically based pharmacokinetic (PBPK) model was used to estimate ethanol blood levels associated with the three use scenarios, which were then compared to typical endogenous ethanol blood levels to further understand the relative magnitude of exposure. Finally, the SEDs from the three use scenarios were benchmarked against different HBGVs to determine whether these exposures are consistent with levels considered to be without safety concern. **Results**: The estimated potential exposure to ethanol from average use, 90th-percentile use, and maximum-label use of a 26.9% ethanol-containing mouthwash in a 60-kg person was 4.46 mg/kg/day, 7.65 mg/kg/day, and 14.15 mg/kg/day, respectively. All use scenarios, including maximum-label use which is nearly two-fold above 90th-percentile use, were within the range of dietary intake levels (excluding alcoholic beverages). Using PBPK modeling for twice daily 20-mL (maximum-label) mouthwash use, the predicted C_{max} of 1.16 mg/L and a 24-h constant blood alcohol concentration of 0.16 mg/L derived from the predicted AUC of 3.84 (mg/L)·hr are within range of average endogenous blood ethanol concentrations. All mouthwash-use scenarios—including average, 90th-percentile, and maximum-label use—resulted in relatively low estimated ethanol exposures that were below various reference and HBGVs, including the HBGV of 1 g/day for ethanol (16.7 mg/kg/day in a 60-kg human), a conservative value that is considered to be protective against chronic health endpoints. The findings suggest that ethanol from mouthwash use does not significantly contribute to systemic levels of ethanol, falls within dietary intake levels, and remains below HBGVs. **Conclusions**: Taken together, this assessment concludes that incidental oral exposure of ethanol-containing mouthwashes represents a *de minimis* health risk.

PS 3308 Quantitative Risk Assessment for t-Butyl Alcohol in Drinking Water

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Background and Purpose: Tert-butyl alcohol (TBA, CAS #75-65-0) is a branched-chain tertiary alcohol used in fuel blending, industrial solvents, and chemical synthesis. It is also a primary metabolite of methyl tert-butyl ether (MTBE) and ethyl tert-butyl ether (ETBE), both of which have been widely used as gasoline oxygenates. TBA has been detected in groundwater and drinking water systems, yet it remains unregulated by the U.S. EPA and Health Canada. This assessment was conducted under NSF/ANSI/CAN 600 standard to establish an oral reference dose (RfD) and corresponding drinking water criteria for TBA, supporting product certification and public health protection. **Methods**: A comprehensive toxicological review was conducted, including chronic and subchronic toxicity, carcinogenicity, genotoxicity, developmental and reproductive toxicity, and ADME data. The key study selected was an NTP chronic drinking water bioassay in F344/N rats and B6C3F1 mice. The critical effect was increased absolute kidney weight in female rats. Benchmark dose modeling yielded a BMDL₁₀ of 91 mg/kg-day, which was allometrically scaled to a human equivalent dose (HED) of 22 mg/kg-day. A composite uncertainty factor of 30 was applied to account for inter- and intra-species differences, resulting in an oral RfD of 0.7 mg/kg-day. Drinking water criteria were calculated using U.S. EPA 90th percentile intake rates. **Results**: The derived RfD supports a total allowable concentration (TAC) of 4 mg/L and a single product allowable concentration (SPAC) of 0.4 mg/L. Cancer

endpoints were evaluated based on thyroid follicular cell hyperplasia and adenomas observed in mice. Although U.S. EPA previously applied linear low-dose extrapolation, recent mechanistic studies (Borghoff et al., 2025) support a threshold-based mode of action involving CAR/PXR activation, hepatic enzyme induction, and disruption of thyroid hormone homeostasis. This anti-thyroid MOA is consistent with a non-mutagenic, receptor-mediated mechanism. Benchmark dose modeling of thyroid hyperplasia yielded a BMDL10_{HED} consistent with the kidney endpoint. Recent TBA studies including bacterial mutation, *in vivo* micronucleus and comet assays, and transgenic rodent mutation studies with MTBE and ETBE showed no DNA damage or increased mutation rates and strongly support that TBA does not act through a genotoxic mechanism. Developmental toxicity studies showed fetal effects only at high doses (≥ 1000 mg/kg-day), typically accompanied by maternal toxicity. In a key oral study, decreased fetal body weight and increased skeletal variations were observed at 1000 mg/kg-day. The NOAEL for developmental effects was 500 mg/kg-day, corresponding to a NOAEL_{HED} of 126 mg/kg-day. A short-term exposure level (STEL) of 100 mg/L was derived using maternal and fetal endpoints, adjusted for a 30x uncertainty factor and a 0.033 L/kg intake rate for pregnant women. **Conclusions:** This assessment provides a comprehensive, threshold-based risk characterization of TBA in drinking water. The derived RfD, TAC, SPAC, and STEL are protective of both cancer and non-cancer endpoints. The updated genotoxicity data and mechanistic evidence support a non-genotoxic, receptor-mediated MOA for thyroid effects. The recent IARC classification of TBA as Group 3 further supports the conclusion that TBA is not classifiable as to its carcinogenicity to humans. The proposed drinking water criteria ensures public health protection under NSF/ANSI/CAN 600.

PS 3309 Derivation of an oral reference dose and drinking water criteria for lithium

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Background and Purpose: NSF is an independent non-governmental organization that provides health-based risk assessments for drinking water contact chemicals in accordance with the published Health Effects Standard, NSF/ANSI/CAN 600. Although lithium is not intentionally added to drinking water, low levels have been observed to extract from cement and concrete drinking water system components undergoing certification. Accordingly, the purpose of this research was to derive an oral reference dose (RfD) and corresponding drinking water criteria for lithium. **Methods:** A comprehensive literature search for toxicological data on lithium compounds was conducted. *In vivo* and *in vitro* studies were critically evaluated against guidelines and for data quality. Clinical and cohort studies involving human participants that included clear information on dosing and adverse event reporting were also reviewed. Since lithium doses in the clinical studies were typically reported as blood plasma levels, conversion to oral doses was facilitated by a dosimetric adjustment that considered pharmacokinetic data identifying typical human clearance values for lithium compounds. The RfD was subsequently derived by dividing the selected oral POD by appropriate uncertainty factors. To calculate drinking water criteria, 90th percentile drinking water intake rates identified in the U.S. EPA Exposure Factors Handbook were incorporated. **Results:** The therapeutic use of lithium salts in the treatment of psychiatric disorders dates back to at least the mid-20th century; thus, the dataset relevant to human toxicity consists primarily of clinical trials and hospital-based cohort studies, with relatively few guideline-compliant studies in laboratory animals. However, taken collectively, the human data revealed several consistent lithium-induced effects to the kidney (polyurea), thyroid (increased TSH and hypothyroidism), and nervous system (reduced nerve conduction velocity and reduced performance in tests of memory). As the human studies were largely designed to evaluate effects after long-term therapeutic doses, it was not possible to determine a NOAEL or critical effect. Therefore, the above “constellation of effects” associated with serum concentrations of approximately 0.6 mmol/L (that increased in severity at higher doses) were considered co-critical effects. The selected POD of 0.6 mmol/L was extrapolated to an oral dose of approximately 2.0 mg/kg-day based on the median of the clearance values identified from a range of pharmacokinetic studies in humans (0.02 L/kg-hr) and assuming an oral bioavailability of 100%. To derive the oral RfD for lithium, a total uncertainty factor of 300 was applied to the POD of 2 mg/kg-day, which included a factor of 10 to account for the lack of a clear NOAEL, a factor of 10 to account for intraspecies differences in sensitivity, and a factor of 3 to address the lack of a chronic study. Therefore, the oral RfD was 2 mg/kg-day divided by 300, or 6.67 mg/kg-day. From the oral RfD, a total allowable concentration (TAC) of 40 µg/L (rounded) was derived based on the 90th percentile intake rate of 0.034 L/kg-day for adults and application of a 20% relative source contribution to account for potential exposure to lithium from sources other than drinking water. To address the possibility of multiple sources of lithium in drinking water, a single product allowable concentration (SPAC) of 4 µg/L was identified, assuming 10 sources of contamination. Lastly, a short-term exposure level (STEL) of 100 µg/L was derived from the same POD that was used to derive the TAC, using the drinking water intake for a child 0-12 months of age (0.15 L/kg-day) and an uncertainty factor of 100. **Conclusions:** Based on the selected POD and the application of appropriate uncertainty factors that consider interspecies and intraspecies differences in sensitivity, the TAC, SPAC, and STEL drinking water action levels derived in this assessment are considered protective of public health. Despite the lack of suitable guideline-compliant studies in animals, confidence in the overall assessment is high due to the extensive human clinical data available for lithium compounds.

PS 3310 Identification of Proposition 65 MADLs for Methyl Chloride

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Background and Purpose: Methyl chloride is a naturally-occurring component of environmental media, arising from chemical reactions occurring in the oceans and through combustion of grass, wood, and coal. Methyl chloride currently serves as an industrial intermediate in the production of silicone-based fluids, elastomers, and resins, which are widely used industrially and in consumer products. The State of California's Office of Environmental Health Hazard Assessment (OEHHA) included it as a developmental toxicant on the list of substances known to the State to cause cancer or reproductive toxicity (i.e., Proposition 65), based on the Authoritative Body mechanism: NIOSH determined that methyl chloride is a male reproductive toxicant and developmental toxicant. OEHHA has not established a safe harbor level for methyl chloride for either endpoint. Therefore, this work applied OEHHA's published methodologies to methyl chloride's health effects dataset to calculate a developmental toxicity maximum allowable dose level (MADL) and to update a previously-calculated MADL for male reproductive toxicity. **Methods:** Three key studies identified by NIOSH and included in a U.S. EPA submission were evaluated to identify the critical developmental effect and to establish a point of departure (POD) for the developmental toxicity MADL. The literature search was also updated for both endpoints. Inhalation exposure concentrations were converted from analytical exposure concentrations in units of ppm to continuous exposure concentrations and then to average daily doses in units of mg/kg/day using calculated inhalation rate and study-specific body weight values. From the available studies, the point of departure for each endpoint was selected and the MADLs were calculated using procedures outlined by OEHHA. **Results:** Developmental toxicity studies were performed in rats, mice, and rabbits and involved inhalation exposure during applicable periods of organogenesis. In rats, fetal effects included reduced crown-rump length, reduced body weight, and reduced ossification at a nominal concentration of 1,500 ppm, a concentration that also significantly reduced maternal body weight gain. In two studies with mice, significant maternal toxicity occurred at concentrations of 1,500 and 750 ppm, and fetal heart malformations occurred at concentrations of 500 and 750 ppm. Specifically, the bicuspid or tricuspid valves and their supporting structures were absent or malformed. In contrast, no maternal or developmental toxicities were observed in rabbits. The mouse is therefore the most sensitive species based on the concentrations at which developmental effects not attributable to maternal toxicity were observed. Using time-weighted average body weights and calculated inhalation rates, the mouse NOAECs of 100 ppm and 250 ppm were converted to NOAELs of 1,161 mg/kg/day and 2,876 mg/kg/day, respectively. The corresponding LOAECs were converted to LOAELs of 5,451 mg/kg/day and 5,752 mg/kg/day, respectively. The highest NOAEL lower than the lowest LOAEL was 2,876 mg/kg/day and was selected as the point of departure for the developmental toxicity MADL calculation. This value was multiplied by the default body weight of 58 kg for pregnant women specified in 27 CCR and then divided by a factor of 1,000 to yield a developmental toxicity MADL of 166.8 mg/day for methyl chloride. The literature search for male reproductive effects did not identify any additional studies. The point of departure for the male reproductive toxicity MADL therefore remains the NOAEC of 150 ppm (309.6 mg/m³) based upon testicular effects and reduced fertility observed in a 2-generation rat study. Using the study's reported body weight data and a calculated inhalation rate, the NOAEC of 309.6 mg/m³ was converted to a NOAEL of 50.9 mg/kg/day. When multiplied by the default body weight of 70 kg for adults specified in 27 CCR and then divided by a factor of 1,000, the male reproductive toxicity MADL for methyl chloride is 3.6 mg/day. **Conclusions:** In summary, exposures to methyl chloride less than 166.8 mg/day do not pose a risk of developmental toxicity and exempt responsible parties from Proposition 65 label warning requirements in the absence of agency-established toxicity criteria. Similarly, exposures to methyl chloride less than 3.6 mg/day do not pose a risk of male reproductive toxicity. The large difference between these two MADLs illustrates the importance of considering all relevant endpoints when performing Proposition 65 compliance assessments.

PS 3311 Establishment of Proposition 65 maximum allowable dose levels (MADLs) for ethylene dibromide

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Background and Purpose: Ethylene dibromide has historically been used as a lead gasoline additive and fumigant. Although no longer used as such in the U.S., it remains an industrial intermediate, and exposures to contaminated environmental media, water in particular, may also occur. Originally listed on California's Proposition 65 for carcinogenicity, ethylene dibromide's Proposition 65 listing now includes the male reproductive toxicity and developmental toxicity endpoints as a result of National Institute for Occupational Safety and Health (NIOSH) and United States Environmental Protection Agency (U.S. EPA) assessments. California's Office of Environmental Health Hazard Assessment (OEHHA) has not established safe harbor maximum allowable dose levels (MADLs) for ethylene dibromide. This work evaluates toxicological data available for ethylene dibromide to establish MADLs for use in Proposition 65 compliance evaluations. **Methods:** This work critically assessed data included in agency evaluations, including NIOSH, U.S. EPA, and the Texas Commission on Environmental Quality (TCEQ), and newer data identified from a search of the peer-reviewed literature. Dosimetric adjustments and dose-response analyses identified the critical developmental effect and the critical male reproductive effect associated with ethylene dibromide exposure. Using methodologies

established by OEHA, MADLs were calculated for each endpoint. **Results:** In classical developmental toxicity studies, pregnant female rats and mice were exposed to ethylene dibromide via inhalation during their periods of organogenesis. Statistically significant effects included reduced live litter size, reduced live fetal body weight, reduced ossification, and/or changes in pup neurobehavioral parameters. The incidence of hydrocephalus was increased (not significant) in rats and mice as well. These effects often occurred together with significant maternal toxicity, complicating identification of true no effect levels for development. Additional studies revealed male-mediated developmental effects: male rats were exposed via inhalation to ethylene dibromide and then mated with untreated females, resulting in reduced litter size, pup neurobehavioral effects, or altered brain neurotransmitter enzyme activities in progeny. Considering all available data, a LOAEC of 154 mg/m³, based on hydrocephalus in mice, was selected as the point of departure for the MADL calculation. After adjustment to a continuous dose, application of default inhalation rate and body weight factors, and application of a factor of 1,000 as required by OEHA, a developmental toxicity MADL of 928 µg/day was established. For male reproductive toxicity, testicular degeneration or atrophy was a common finding in chronic studies in rats and mice. Additionally, two epidemiology studies in exposed workers identified effects on sperm quality, such as reduced sperm count and reduced sperm motility. Most of these studies did not assess reproductive function, however. In one study that evaluated functional effects, male rats were exposed via inhalation and then mated with untreated females, and testicular atrophy occurred together with reduced male fertility. In male rabbits, ethylene dibromide inhalation exposure prior to mating resulted in reduced sperm motility but did not affect fertility. Since the MADL is based on the most sensitive health effect, lowest effect levels from studies identifying a potentially adverse effect were converted to mg/kg/day equivalent intake values to facilitate the dose-response assessment. An epidemiology study in papaya workers provided the lowest daily intake estimate of 0.046 mg/kg/day, equivalent by OEHA's definition to a no effect level of 0.0046 mg/kg/day, and served as the basis for the male reproductive toxicity MADL. When multiplied by a default adult body weight of 70 kg, the resulting MADL for male reproductive toxicity is 0.322 µg/day. **Conclusions:** This work establishes safe harbor MADLs that can be used in Proposition 65 regulatory compliance determinations. It also illustrates some of the challenges inherent in the evaluation of reproductive and developmental toxicity, such as distinguishing between secondary effects due to maternal toxicity and primary effects on the developing organism; assessing male-mediated developmental effects; interpreting effects of unclear toxicological significance; and making the best use of limited epidemiology data.

PS 3312 Consideration of Carcinogenicity and Mode of Action Information by an Independent Expert Panel to Support Derivation of No-Significant-Risk-Level Values for Vinyl Acetate Monomer

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Background and Purpose: The carcinogenicity and cancer mode of action (MOA) of vinyl acetate monomer (VAM) has been well-studied. VAM was recently placed on the Proposition 65 list as a carcinogen by the state of California, and prompting the need for the derivation of a No-Significant-Risk-Level (NSRL) value. **Methods:** A triple-blinded, independent panel of five experts was engaged to review information on the carcinogenicity and MOA of VAM to provide input on key decisions for the calculation of NSRL values using best available science. Combined, this panel possessed more than 200 years of relevant experience and more than 1,000 scientific publications. Based upon the weight of evidence (including considerations for study quality), the panel expressed higher confidence in using effects observed at the point of contact, rather than relying on those observed in systemic tissues, as the critical effects supporting NSRL derivation. **Results:** The panel considered a cancer MOA involving increased cell replication to be best supported by the weight of evidence (mean confidence score of +3.8±1.1, on a scale of -5 to +5). In contrast, a cancer MOA involving direct genotoxicity was not supported for VAM (mean confidence score of -3.6±2.0, on a scale of -5 to +5). Because VAM produces effects at the point of contact (i.e., upper respiratory tract following inhalation exposures; upper gastrointestinal tract following oral exposures), separate NSRL values were derived for inhalation and oral routes of exposure. **Conclusions:** Based upon the decisions recommended by the panel at each step of the derivation process (endpoint/dataset, dosimetry, point of departure, low-dose extrapolation), inhalation and oral NSRL values of 20,000 and 110,000 µg/day were calculated, respectively. Confidence in the inhalation NSRL value was considered high by the panel (mean confidence score of 9±1 on a scale of 0 to 10), whereas confidence in the oral NSRL was considered medium (mean confidence score= 6.4±3.8, on a scale of 0 to 10). Based upon best available science (data and methods), human exposures below these NSRL levels are not expected to pose a significant cancer risk.

PS 3313 Confidently determining reliable intake limits from robust carcinogenicity studies

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Background and Purpose: The assessment of carcinogenic potency is crucial for ensuring human safety, particularly within the pharmaceutical industry where the therapeutic benefit of a drug can justify the presence of low levels of carcinogenic impurities. In such cases, the acceptable intake (AI) is determined by the carcinogenic potency of the impurity. For carcinogens, the potency is expressed as the TD₅₀, which is the dose at which the probability of remaining tumour-free over a lifetime exposure is halved, and serves as the basis for deriving AI limits. This work aimed to improve the reliability and transparency of TD₅₀-based AI derivation. **Methods:** The original Carcinogenic Potency Database (CPDB) TD₅₀ model proved irreproducible due to its unpublished methodology, ambiguities in the workflow, and errors in data curation prior to TD₅₀ calculation. This has prevented the calculation of TD₅₀s from new studies and as a result a new implementation of the TD₅₀ model ("Lhasa TD₅₀") was developed [Thresher *et al* 2019] and made freely available via the Lhasa Carcinogenicity Database (LCDB). To further strengthen safety assessor confidence, a comprehensive reassessment has been conducted which has led to significant enhancements to the Lhasa TD₅₀ model, enabling more robust results to be produced. In addition, applicability metrics such as the Akaike Information Criterion and X² goodness-of-fit are available to assessors to evaluate the appropriateness of TD₅₀ calculations for specific dose-response datasets to support expert review. Additionally, the framework for assessing study reliability has been refined through the incorporation of an A+ grade, allowing confirmation of which studies are suitable for the assessment of potency. Building on this information, a two-stage approach combining top reliability grading and Pareto optimization has been developed to identify the 'most sensitive site in the most robust study', the preferred point of departure (PoD) for AI limits under ICH M7. **Results:** This method correctly identifies a single preferred study in 98% of the time (with no more than three being proposed in any case), dramatically reducing the time needed for selecting the PoD. For validation of this method, the studies from the ICH M7 addendum and the regulatory guidance on nitrosamines were correctly identified. **Conclusions:** These improvements provide a transparent workflow that supports more efficient decision making.

PS 3314 Systematic Evaluation of the Carcinogenic Potential of Di-isononyl Phthalate in Humans

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Background and Purpose: Di-isononyl phthalate (DINP) is a high molecular weight phthalate widely used in the manufacture of polyvinyl chloride and other commercial products. This assessment aims to systematically evaluate the evidence base for DINP, including human and animal cancer studies and mechanistic data, to inform an overall conclusion regarding the carcinogenic potential of DINP in humans. **Methods:** Peer-reviewed DINP literature were identified from systematic searches conducted in PubMed and Embase, supplemented by hand searching. Publicly available good laboratory practice and/or guideline laboratory reports were identified through review of regulatory documents. Mechanistic data were organized according to the key characteristics of carcinogens (KCCs). All identified data underwent critical appraisal. Data were integrated using principles of WHO IPCS for human and animal cancer data alongside concepts for activity assessment of mechanistic data. **Results:** Four epidemiological studies that evaluated the association between DINP exposure (via DINP-specific metabolites) and cancer were identified. Three studies reported no statistically significant (SS) positive increases in breast cancer incidence or mortality risk associated with DINP exposure. A case-control study found a SS increase in prostate cancer risk associated with DINP exposure, but this finding was determined to represent an imprecise association because it was based on a very small sample size and lack of consideration of confounding or covariates except matching on age. Current evidence from human studies does not support an association between DINP and cancer in humans. Four reliable dietary rodent cancer bioassays (3 in rats, 1 in mice) were identified. Inconsistent SS increases in liver and kidney tumors, and mononuclear cell leukemia (MNCL) were reported. A SS increase in hepatocellular adenomas, carcinomas, and/or their combined incidence was observed in 3 of the 4 studies. Mode of action (MoA) analysis supports that DINP elicits liver tumors via the peroxisome proliferator-activated receptor alpha (PPARα) MoA, which is widely considered to lack human relevance. A small but SS increase in renal tubular carcinomas was observed in 1 of 2 studies in male F344 rats; it is postulated that DINP acts through the non-human relevant α2u-globulin nephropathy MoA. In the F344 but not SD rat bioassays, a dose-dependent and SS increase in MNCL was observed. MNCL is a common lesion in aging F344 rats and is not considered relevant in predicting human cancer. Collectively, these animal data provide strong evidence that DINP is unlikely to be a human carcinogen. A robust mechanistic dataset of 127 studies was identified, with endpoints mapping to 9 of 10 KCCs. Reliable data in exposed

humans from 42 studies showed inconsistent findings for KCC2 (genotoxicity), KCC4 (epigenetic alterations), and KCC5 (oxidative stress). Reliable data from *in vivo* animal studies, *in vitro* assays (human and mammalian), and *in silico* models from 85 studies showed DINP was neither genotoxic (KCC2) nor immunosuppressive (KCC7). Inconsistent signals of activity were reported for KCC1 (electrophilic), KCC4 (epigenetic alterations), KCC5 (oxidative stress), and KCC8 (modulates receptor-mediated effects). Activity related to KCC10 (alters cell proliferation, cell death and nutrient supply) was evident in liver and kidney of *in vivo* rodent models, consistent with studies that demonstrated rodent-specific MoAs for kidney and liver tumors described above. **Conclusions:** Limited epidemiological data do not support an association between cancer outcomes in humans and exposure to DINP. While rodent bioassays have shown tumor formation following DINP exposure, the underlying MoAs are not considered relevant to humans. Mechanistic data support this interpretation, demonstrating a lack of genotoxicity and only inconsistent activity across the other 9 KCCs. Collectively, this systematic evaluation suggests that DINP is unlikely to be carcinogenic in humans.

PS 3315 Setting Occupational Exposure Limits (OELs) for Carcinogens: A Focus on Benzene

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Background and Purpose: Since the 1950s, regulation of occupational carcinogen exposure has progressed from hazard identification to quantitative evaluation, driven by advances in cancer biology. This narrative review compares two approaches for setting Occupational Exposure Limits (OELs) for carcinogens, focusing on benzene. **Methods:** A narrative review was conducted to qualitatively synthesize and summarize the occupational limits established by different regulatory agencies and researchers. **Results:** The first approach applies the carcinogenic mode of action (MOA), using linear extrapolation for primary genotoxicants and non-linear extrapolation for secondary genotoxicants. The second approach quantifies *in vivo* genotoxicity data, employing mathematical modeling to define the point of departure (POD) for non-linear extrapolation, regardless of the MOA. As benzene is an indirect genotoxicant, it is considered to have a threshold value. Based on the MOA approach, different European institutions and commissions have proposed OELs ranging from 0.05 to 0.25 ppm, and the ACGIH has recommended a TLV/TWA of 0.02 ppm (with skin notation). Based on quantitative genotoxicity approach, the proposed OELs range from 0.021 to 0.07 ppm for benzene. **Conclusions:** Current evidence indicates that workplace benzene concentrations above 0.3 ppm do not adequately protect the health of exposed workers. Extending follow-up of existing cohorts and conducting well-designed epidemiological studies are essential to reduce uncertainties and to confirm the most sensitive toxic effect, thereby supporting the establishment of a more realistic OEL for benzene and minimizing the likelihood of over- or underestimating the associated risks.

PS 3316 Establishing an Endogenous Threshold for AICAR to Support NSF Certified for Sport® Compliance

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Background and Purpose: The NSF Certified for Sport® program (NSF 306) verifies that certified products do not contain athletically banned substances (ABS), including, but not limited to, those prohibited by the World Anti-Doping Agency (WADA). WADA includes substances on its Prohibited List if they meet at least two of the following criteria: potential to enhance performance, potential to cause adverse effects, or violation of the spirit of sport. Once banned, the default acceptability criterion in finished products is absence of the substance at the analytically feasible detection threshold. Although adulteration is typically considered the source of ABS, in some cases, ABS may naturally occur in supplement ingredients as unintended constituents, or may be naturally produced by the body at low background concentrations. In either case, these endogenous levels may be above the analytical detection limit, but would be unlikely to meet WADA criteria for a prohibited substance. 5-Aminoimidazole-4-carboxamide riboside, also known as AICAR or acadesine, is banned in sport as a metabolic modulator and has been detected in dietary supplements (>20 ng/g). However, AICAR occurs endogenously in humans, plants and yeasts, and as a by-product of microbial fermentation due to its role in purine biosynthesis. This evaluation was prompted to determine whether an endogenous level of AICAR in dietary supplements can be scientifically justified to allow certification of products with a detectable level of AICAR that still complies with anti-doping policies. **Methods:** This evaluation integrated data from published human and animal studies, including population biomonitoring, pharmacokinetic assessments, performance enhancement models, and toxicity studies to characterize endogenous levels in humans and dietary supplement ingredient sources and to confirm absence of performance enhancement and adverse effects at the expected levels of exposure. **Results:** Although a definitive endogenous level for AICAR in humans could not be established due to limited exposure-controlled data, athlete population studies (with unreported supplement use) suggested that the fifth percentile of background

urinary concentrations was ~60 ng/mL and the 99th percentile was ~1,500 ng/mL. Oral bioavailability of AICAR was reported to be low (~5%) based on plasma AUC comparisons following oral and intravenous dosing. In healthy volunteers, a 167 mg/kg oral dose (~10 g) yielded peak urinary concentrations of 9,800 ng/mL at 8 hours, indicating therapeutic exposure and rapid elimination. Animal studies showed increased endurance after repeated intraperitoneal doses of 500 mg/kg in mice (~60 mg/kg or 3.6 g human equivalent) and no adverse effects after repeated intraperitoneal doses of 750 mg/kg in rats (~180 mg/kg or 10.8 g human equivalent). Intravenous doses of 50-210 mg/kg (3-12.6 g) were well-tolerated in patients with chronic lymphocytic leukemia. Concentrations of AICAR in yeast were reported to be ~2.4 mg/g, far exceeding supplement detections of 30-60 ng/g. Normalized to relevant supplement serving sizes, supplement detections equated to ~2×10⁻⁵ to 1×10⁻³ mg/day exposures. A qualitative threshold of 0.5 mg/day (~0.008 mg/kg-day) was proposed as an acceptable exposure limit to allow variability in endogenous ingredient concentrations while remaining well below doses linked to performance enhancement, toxicity and those that would contribute meaningfully to background urinary concentrations. A margin of exposure analysis indicated that consumer exposure at the endogenous threshold was > 7,500 times lower than human equivalent intraperitoneal doses with performance enhancing effects in mice, which were expected to be exaggerative of typical oral exposure considering low bioavailability. **Conclusions:** The proposed threshold of 0.5 mg/day AICAR is expected to be protective of toxicological effects, performance enhancement, and the risk of a positive doping test. Adopting this threshold within NSF 306 supports accurate interpretation of analytical findings, enables differentiation between AICAR presence consistent with levels naturally occurring in food and exogenous adulteration intended for performance enhancement, and ensures compliance with banned substance policies.

PS 3317 Database calibrated toxicity values for human health assessment based on existing toxicology data for one thousand chemicals

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Background and Purpose: Traditional human health assessments rely upon expert judgment of dose-effect linkages observed in animal toxicology or human studies and can take considerable time and resources to complete. In this presentation, we describe a new database-calibrated assessment process (DCAP) to generate oral, non-cancer human health toxicity values using a new computational method without the need for expert input. This computational approach to chemical risk assessment greatly decreases the time needed to complete human health assessments. **Methods:** The DCAP approach converts information in the US EPA Toxicity Values Database (ToxValDB) that contains dose-response summary values (DRSVs) from *in vivo* toxicity studies to oral, chronic human equivalent doses using a series of standard conversion factors. A point-of-departure (POD) is then calculated for a given chemical using a calibration percentile that is benchmarked to critical effect PODs from previously published human health assessments. Traditional and process-specific uncertainties are incorporated to derive a calibrated toxicity value (CTV) that is likely to be without appreciable risk of adverse non-cancer health effects over a lifetime. **Results:** Application of the DCAP approach is illustrated by through the development of 1,001 CTVs for chemicals that currently lack human health assessments. **Conclusions:** The DCAP computational approach has the potential to establish toxicity reference values for environmental chemicals currently lacking human health assessments in a fraction of the time required for traditional expert-based assessments.

PS 3318 Component-based toxicological screening of spearmint (*Mentha spicata*) aerial parts essential oil and safe-supported exposure limits for humans.

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Background and Purpose: Dose-response assessment is an important application of toxicology used in translational medicine and human health risk assessment. While sufficient evidence for dose-response assessment is available for many discrete compounds, the toxicology of many natural complex substances (NCS) is understudied. When there is insufficient NCS data, risk assessment protocols instruct evaluation of the NCS on a component basis. Component-based dose-response assessments can be performed when the composition of an NCS is characterized and adequate constituent toxicological data is available. Here, a component-based toxicological assessment of spearmint (*Mentha spicata*) aerial parts essential oil (EO) was performed to estimate exposure limits for use in human health risk assessments. Additionally, findings from this assessment are compared to research on spearmint EO. **Methods:** Spearmint EO was characterized using gas chromatography mass spectrometry analysis of 21 material lots. Constituents were organized into toxicological groups and descriptive statistics were derived for each compound group. Toxicological data was evaluated for all constituents ≥1% including repeated dose systemic toxicity, developmental toxicity, fertility toxicity, and genotoxicity.

Constituents with insufficient data to determine a point of departure (POD) were considered “not assessed” for that endpoint. The percentage of assessed constituents was calculated as a normalized value for each endpoint. Constituent POD values were obtained from toxicological risk assessments, safety dossiers, and expert opinions. Reference doses (RfD) were extrapolated for each constituent using body surface area scaling to convert POD to a human equivalent dose (HED). An uncertainty factor of 10 was used for interindividual variability. Risk-based exposure limits for the NCS were calculated for each constituent by dividing the constituent RfD by its normalized percentage in the NCS. From there, an adult safe-supported daily exposure value for the NCS was determined. For the purposes of this assessment, an adult safe-supported daily exposure value is defined as the greatest exposure to the material from a discrete route of administration that does not exceed the risk-based exposure limit of any constituent. **Results:** The main constituents evaluated in spearmint EO were carvone and limonene. Altogether, sufficient data was available to evaluate most constituents in spearmint EO for repeated dose systemic toxicity (91%), developmental toxicity (90%), fertility toxicity (89%), and genotoxicity (94%). No genotoxic compounds were identified. Carvone was identified as the primary exposure-limiting constituent for repeated dose systemic, developmental, and fertility toxicity. Of these endpoints, repeated dose systemic toxicity exposure limits for carvone were the most restrictive. Assuming a dermal absorption value of 100% for carvone, the safe-supported exposure limit for healthy adults and sensitive populations was determined to be 103 mg/person/day (RfD: 1.7 mg/kg bw/day) spearmint EO for all routes of exposure combined. **Conclusions:** The critical study for spearmint EO repeat dose toxicity is a 90-day oral subchronic study (OECD 408) in rats that concluded a NOAEL of 100 mg/kg bw/day. This equates to an RfD of 1.6 mg/kg bw/day for spearmint EO, which agrees with the RfD found in this compound-based assessment. No genotoxic constituents were identified in spearmint EO, which aligns with findings from bacterial reverse mutation and *in vivo* micronucleus assays showing that spearmint EO is not genotoxic. As limitations, read-across data was not included in this analysis unless read-across was used to derive POD values in reputable toxicological risk assessments. Also, thresholds of toxicological concern and Cramer classifications for constituents were not considered. Given the GRAS status of spearmint EO and its widespread use in cosmetic and food products, this research may be used to inform posology for clinical trials and human health risk assessments. Furthermore, this research serves as an example comparing the toxicological profile of a mixture to its components.

PS 3319 A physiologically-based pharmacokinetic (PBPK)/ pharmacodynamic (PD) model for rats and humans for the insecticide acephate

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Background and Purpose: Acephate is an organophosphate insecticide and its most sensitive toxicological endpoint is acetylcholinesterase (AChE) inhibition. The uncertainty of regulatory risk assessments can be reduced by better understanding the difference in pharmacokinetics and AChE inhibition in rats, the species for which there is the most toxicology data, and humans, which is the focus of risk assessment. **Methods:** A PBPK/PD model was developed to simulate acephate dosimetry and pharmacokinetics in rats and humans for oral and inhalation exposures. Acephate is metabolized *in vivo* to methamidophos, which is the primary cause of AChE inhibition, though acephate itself also contributes. The model simulates tissue dosimetry and clearance, including conversion of acephate to methamidophos, metabolism of acephate and methamidophos to other metabolites, and urinary excretion, which is the primary mode of clearance for both acephate and methamidophos. The model was fit using both rat and human pharmacokinetic data, and the model's ability to estimate AChE inhibition was tested with rat *in vivo* studies. Fitting was accomplished using Markov Chain Monte Carlo, and the posterior distribution of the fitted parameters was used for uncertainty analysis. **Results:** The model successfully simulated both rat and human plasma concentrations following oral doses. The model also provided good estimates of AChE inhibition in a series of rat *in vivo* studies for acephate and methamidophos and for oral and inhalation exposures. The fitted human model was applied to estimate points-of-departure (PODs) for risk assessment. **Conclusions:** A PBPK/PD model was built for acephate. The model adequately simulated dosimetry and AChE inhibition in rats and dosimetry in humans. It was applied to estimate PODs for human risk assessment.

PS 3320 Quantifying Variability in Chemical Sensitivity Attributable to Social Non-Chemical Stressors: an Approach Using a Systematic Review of Human Studies

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Background and Purpose: The extent to which social non-chemical stressors (SNCS) (e.g., poverty, discrimination, perceived stress, etc.) contribute to variability in the human response to chemical exposures is not well-characterized. Quantification of this variability in chemical risk assessment is needed to establish protective default

factors when creating health guidance values (HGVs). **Methods:** We propose a novel approach for quantifying the variability attributable to SNCS. We first developed and registered a protocol to implement the Navigation Guide systematic review methodology, a structured and transparent framework tailored to incorporate environmental health data into decision-making. Using this method, we conducted literature searches in PubMed and Web of Science to identify human studies that quantified the combined effects of one of several key chemicals (lead, particulate matter, or organophosphate chemicals) with SNCS on child cognition. We included studies that assessed interactive effects of chemicals and SNCS, or that stratified chemical effects by SNCS, so that susceptibility attributable to SNCS could be quantified. **Results:** From a previous systematic review, we *a priori* identified 64 studies as eligible according to our inclusion criteria. Our current literature search yielded 4,232 eligible studies, which will allow us to expand the number of relevant included studies. From our included studies, we will calculate the ratio of chemical effects in high- versus low-SNCS subpopulations (within a given study) for a standard chemical contrast (i.e., one standard deviation), to derive a “modification factor”. Considering a unitless ratio of effects allows us to synthesize across diverse epidemiologic study populations, chemicals and dose ranges, and SNCS indicators and constructs, to estimate a general magnitude and range of the modification factor. We will use meta-regression to estimate how the factor varies by chemical, type of SNCS, developmental stage of exposure and outcome assessment, geography, and other factors, allowing the factor to be extrapolated to specific health risk-related scenarios. **Conclusions:** Our quantitative results, based on a rigorous systematic review, may strengthen chemical risk assessment approaches to better account for SNCS and lead to more health protective HGVs. Furthermore, our results can inform the practice of cumulative risk assessment broadly, which currently does not adequately account for SNCS.

PS 3321 Human Health Risk Evaluation of Pesticide Residues on Fresh Fruits and Vegetables in California: An Integrated Statewide Monitoring and Enforcement Program

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Background and Purpose: To protect public health and enforce pesticide regulations, the California Department of Pesticide Regulation (DPR) operates the California Pesticide Residue Monitoring Program (CPRMP), a globally unique program that combines real-time health risk evaluation with regulatory enforcement. CPRMP samples both domestic and imported fresh produce to identify illegal pesticide residues and ensure compliance with US Environmental Protection Agency (US EPA) established tolerances. A pesticide tolerance is defined as the highest residue levels of a pesticide legally permitted on a specific commodity. Samples are collected statewide from wholesale and retail outlets, distribution centers, and farmers markets, with an emphasis on commodities frequently consumed by children and diverse demographic groups. Each sample is quantitatively analyzed for approximately 500 different pesticides to identify violations. Two types of violations are monitored by CPRMP: 1) “over tolerance” (OT), where residue levels exceed the established tolerance limit, and 2) “no tolerance established” (NTE), where a pesticide is detected on a commodity without an approved tolerance. DPR conducts rapid health risk evaluations for both types of violations to protect the public. **Methods:** When produce has been identified that exceeds a tolerance, a human health risk evaluation is initiated, focusing on assessing acute dietary exposure as chronic consumption of the same lot of fresh produce is considered unlikely. Exposure is estimated using commodity-specific consumption rates derived from National Health and Nutrition Examination Survey (2003-2018) data for the most sensitive subpopulation. These estimates are then compared to pesticide-specific acute oral reference doses (aRfDs), which are the maximum oral safe doses established by regulatory agencies including DPR, US EPA, European Food Safety Authority, World Health Organization and others. The aRfDs established by DPR are preferred when available, otherwise those established by US EPA, EFSA, WHO, or other agencies are used in order of preference. Estimated exposures that exceed the aRfD indicate potential acute health risks to consumers and trigger more stringent enforcement actions than those taken when no health risk is identified. When a sampled commodity contains residues from multiple pesticides known to share a common mechanism of toxicity, a hazard index approach is used to calculate cumulative risk. **Results:** In 2023, CPRMP collected 3,544 produce samples from over 500 locations across California. Analysis showed that 97% of samples were compliant with US EPA pesticide tolerances, while 3% of samples contained illegal pesticide residues. Notably, of those violations, 83% involved imported produce. Among California-grown samples, fewer than 1% had violations. Only 20 samples out of 3,544 (0.6%) had residues at levels posing a potential health risk; nineteen of these were imported. Ultimately, fewer than 1% of all samples, both domestic and imported combined, were determined to pose a potential acute health risk. **Conclusions:** This work highlights real-time evaluation of human health risk associated with pesticide residues on fresh produce. The case studies presented demonstrate the ongoing need for surveillance and health risk evaluation of pesticide residues on agricultural commodities. This integrated program serves as a model for other regulatory entities seeking to protect public health through science-based enforcement.

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Background and Purpose: Existing models for evaluating lung cancer risk associated with cigarette smoke typically depend on epidemiological data or a limited range of toxicity endpoints. These approaches may not adequately account for cumulative risks arising from extended exposure to multiple carcinogens, such as "Cohort of Concern" (CoC) substances, which are known to pose elevated cancer risks even at low concentrations. There is a need for a more comprehensive framework that incorporates mutagenicity, carcinogenic potency, and duration of exposure. This study was designed to develop and implement such a model for identifying CoCs among cigarette smoke constituents and quantifying the corresponding lung cancer risk. **Methods:** A risk assessment framework using data from the CPDB and LCDB was developed. Carcinogenic potency was quantified by calculating the Cancer Risk Specific Dose (CRSD) from the TD_{50} (tumorigenic dose rate 50%). Substances with a CRSD < 0.025 $\mu\text{g/kg/day}$ were classified into the CoC. Mutagenicity was assessed via Ames test data. The relationship between carcinogenic potency and acute toxicity was analyzed using the metric $\text{Log}(0.5/TD_{50})+7$. A theoretical mutational model was applied to estimate lung cancer risk (P), factoring in the mutation slope of key chemicals, the number of driver gene mutations required ($k=7$), and stem cell division frequency (d). This model, $P=1-(1-(1-(3E-06*(1+\text{mutation slope})))^d)^k)^8*1.5E+07$, was used to evaluate the risk contribution of major cigarette smoke constituents: Benzo[a]pyrene (B(a)P), NNK, NNN, acrolein, and nicotine. **Results:** B(a)P, NNK, and NNN were identified as potent CoC substances due to their low TD_{50} values (0.1-1.8 mg/kg/day), resulting in CRSDs below the 0.025 $\mu\text{g/kg/day}$ cutoff threshold, and their strong positive Ames test results. In contrast, acrolein and nicotine were excluded from the CoC; acrolein due to a lack of a definitive TD_{50} and weak mutagenicity, and nicotine due to the absence of a TD_{50} and a negative Ames test. Application of the mutational model demonstrated that exposure to the CoC substances (B(a)P, NNK, NNN) leads to a substantial increase in cumulative risk. The model estimated a **10- to 25-fold increase in lifetime lung cancer risk** for individuals smoking one cigarette per day compared to one pack per day, after one year of continuous exposure. **Conclusions:** This study establishes a multi-parameter framework that integrates carcinogenic potency, mutagenicity, and exposure duration to refine lung cancer risk assessment from cigarette smoke. By moving beyond traditional models, this approach provides a more mechanistic basis for risk quantification and highlights the significant risk posed by prolonged exposure to a potent Cohort of Concern, even at low daily intake levels.

PS 3323 Comprehensive Toxicological Evaluation of a Novel E-Vapor Product Using a Multi-Tiered Risk Assessment Framework

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Background and Purpose: Understanding the toxicological profile of electronic nicotine delivery systems (ENDS) is essential for evaluating their potential health risks. This study presents a structured toxicological risk assessment of a new close-pod ENDS that utilizes a novel induction-heating technology (VEEV inPRIME™, Philip Morris Products S.A., Neuchâtel). **Methods:** All flavor and base e-liquid ingredients met food or pharmaceutical grade quality. Their inclusion levels were evaluated based on regulatory requirements and a rigorous toxicological risk assessment, outlined in the four-step human health risk assessment process of the United States Environmental Protection Agency. Any potential leachable compounds from the device's components that might come into contact with the e-liquid or aerosol were evaluated under exaggerated temperature and pH conditions using extractable analysis. Analytical techniques included gas chromatography-mass spectrometry (GC-MS) for semi-volatiles, liquid chromatography-mass spectrometry for non-volatiles, and headspace GC-MS for volatile compound detection. In addition, a stability study has been conducted up to the e-liquid shelf life to assess whether or not any new hazards emerge over time. Aerosol chemical analysis focused on ENDS aerosols' performance analytes (e.g., propylene glycol, glycerol, and nicotine) and included the measurement of the 39 harmful and potentially harmful constituents (HPHCs) listed by the World Health Organization (WHO) in their *Report on the Scientific Basis of Tobacco Product Regulation*. Droplet size distribution (DSD) was measured using cascade impactor and laser diffraction methods that aimed to assess aerosol respirability. *In vitro* toxicological testing, comprising the Water-Soluble Tetrazolium 8 (WST-8) assay, the Ames test, and the micronucleus assay, were conducted in accordance with internationally accepted guidelines, comparing the e-vapor aerosol fractions to reference cigarette smoke fractions. Notably, the studies employed two distinct puff collection patterns to account for potential variability in aerosol generation over the full pod lifetime. This approach enables the puffs collected at the beginning, middle, or end of a pod to be assessed separately, as some ENDS may generate higher HPHC levels towards the pod's end, a factor that can influence the toxicological profile of the aerosol fractions. **Results:** The results demonstrate that all e-liquid ingredients were deemed toxicologically acceptable at their intended use levels, indicating low concern for consumer safety under the evaluated conditions. The device materials were found to be toxicologically acceptable, with no concerning chemical leaching observed under exaggerated usage condi-

tions or over the e-liquid shelf-life. Additionally, the aerosol produced by the product contained undetectable or below-quantifiable levels of all 39 HPHCs listed by the WHO, resulting in an average reduction of more than 99% compared to cigarette smoke for 9 representative HPHCs. DSD measurements highlighted that the aerosol emitted is within the respirable range, and no significant differences in DSD were identified compared to other benchmark ENDS products. *In vitro* studies showed that, under the conditions of the tests, the aerosol fractions exhibited no mutagenic, genotoxic, or cytotoxic effects under either puffing collection patterns. On the contrary, cigarette smoke fractions induced cytotoxicity and positive responses in the micronucleus and Ames tests. **Conclusions:** In summary, the applied risk assessment approach robustly substantiated the safety profile of the e-liquid formulations, device materials, and the collected aerosol of this novel ENDS, suggesting that the product is a better alternative for adult smokers who do not wish to quit nicotine use.

PS 3324 Mode of action and human relevance assessment for diisononyl phthalate (DINP)-induced liver tumors in rodents

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Background and Purpose: Diisononyl phthalate (DINP) is a high molecular weight phthalate widely used as a plasticizer in flexible PVC products. Chronic bioassays have indicated that DINP induces liver tumors in mice and rats. Rodent liver tumors are hypothesized to occur via the rodent-specific peroxisome proliferator-activated receptor alpha (PPARα)-activator mode of action (MOA). PPARα activation is the first and only key event shared across species; the downstream biological responses leading to tumor formation are widely considered to be unique to rodents and not relevant to humans. To further inform whether rodent liver tumors observed following DINP exposure are occurring through the rodent-specific PPARα MOA, a comprehensive assessment of the evidence base for DINP was conducted. **Methods:** The animal and human evidence base for DINP was evaluated in the context of the key events (KEs) and associative events outlined in a published MOA framework for PPARα activator-induced rodent hepatocarcinogenesis. The PPARα MOA for rodent liver tumors includes four KEs: (KE1) PPARα Activation, (KE2) Alteration of Cell Growth Pathways, (KE3) Perturbation of Cell Growth and Survival, and (KE4) Clonal Expansion. First, the weight of the evidence (WoE) for each KE and liver tumors (the adverse outcome [AO]) was evaluated for DINP and its metabolites. Next, the human relevance of this MOA and the potential for alternative MOAs for DINP-induced liver tumors were critically examined. The analysis incorporated aspects of the modified Bradford Hill Criteria and characterization of data gaps and uncertainties. Studies from the peer-reviewed literature and unpublished laboratory reports related to DINP and liver carcinogenesis were first identified from existing authoritative assessments of DINP, followed by literature searches in PubMed to identify literature pertaining to DINP, its metabolites and each KE and potential alternative MOAs. **Results:** WoE for KEs 1 and 3 and the AO strongly support the PPARα MOA for rodent liver tumors. Although limited data were available to inform KEs 2 and 4, these events can be reasonably inferred given the strong evidence for the other KEs and AO. Regarding alternative MOAs for liver tumors, evidence for genotoxicity and the aryl hydrocarbon receptor were not supported. DINP activation of PPARγ and constitutive androstane receptor/pregnane X receptor were weakly supported based on limited *in vitro* studies. Evidence possibly consistent with cytotoxicity (e.g., elevated serum liver enzymes and focal necrosis) occur at later timepoints and at higher DINP doses than those that elicit PPARα activation—indicating they are likely secondary to the PPARα MOA. Rodent liver tumors resulting from a PPARα MOA are generally regarded by the scientific community to lack human relevance. Comparative data from human and non-rodent mammalian models indicate decreased sensitivity and responsiveness to DINP and its metabolite MINP compared to rodents. Limited data for humans (*in vitro*) and monkeys (*in vivo*) suggest perturbation of cell growth and survival is not elicited by DINP or its metabolite. Further, no evidence was identified regarding DINP exposure and liver cancer in humans. Collectively, DINP-specific evidence supports a lack of human relevance for this MOA. **Conclusions:** Overall, the WoE for DINP strongly supports that the liver tumors observed in rodents following DINP exposure are occurring via the rodent-specific PPARα MOA. Moreover, based on quantitative and qualitative species differences in KEs of this MOA, the WoE indicates liver tumors observed in rodents following DINP exposure are likely not relevant to humans.

PS 3325 A Reassessment of Chronic Kidney Inflammation as the Critical Effect for Dimethyl Terephthalate

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Background and Purpose: Dimethyl terephthalate (DMT) is primarily used as a monomer in the production of polyethylene terephthalate which is widely used in medical devices. Kidney related effects after exposure to DMT have been identified as the critical effect through multiple reviews and reports. Metabolism of DMT to terephthalic acid (TPA) is a crucial step in the adverse outcome pathway of DMT.

Previous studies have reported kidney inflammation in female rats at doses of 125 mg/kg/d, while similar findings in mice lacked statistical significance. The U.S. EPA's 1989 IRIS assessment identified a LOAEL of 125 mg/kg/d for DMT, and Ball et al. (2011) derived a BMDL₁₀ of 148 mg/kg/d. Ball et al. suggested that the highest doses administered in the NCI (1979) study may have been insufficient to saturate urine with TPA, yet high enough to initiate an inflammatory response in renal tissue. Dai et al. (2005) further demonstrated sediment and precipitate formation associated with TPA, reinforcing the need to consider urinary chemistry in evaluating nephrotoxicity. Additional factors such as retrograde nephropathy, rodent-specific susceptibility to chronic progressive nephropathy (CPN), alpha-2u-globulin accumulation in male rats, and infectious disease influences complicate interpretation of kidney effects. CPN, a spontaneous and age-related disease of rodents, may be exacerbated by chemical administration and is a confounding factor in the interpretation of renal findings. Given the complexity of kidney effects observed across *in vivo* studies, further scrutiny of the critical effect is warranted. **Methods:** Considerations for risk assessment under ISO 10993-17:2023 framework are explored for these substances. We reviewed chronic kidney inflammation incidence data from 7 NTP studies on various chemicals cohoused with DMT to evaluate dose-response and trends. Chronic toxicity studies with known inducers of urolithiasis were reviewed for kidney related effects. Critical effects for different chemicals across the NTP cohoused studies were compared with those of DMT. Alternative PODs were proposed based on mechanistic plausibility and threshold considerations. **Results:** An increasing trend of chronic kidney inflammation was observed in female rats for 5 other substances studied concurrently with DMT by NTP. Male rats showed a high background incidence rate of chronic kidney inflammation across all studies likely attributable to alpha-2u-globulin. TPA and melamine showed increased incidence of chronic kidney inflammation compared to DMT as well as observed uroliths. Route specific metabolic differences after exposure to DMT are based on hydrolysis to TPA in the GI tract after oral exposure and a lack of first-pass metabolism and a slower esterase driven hydrolysis in the plasma/tissues after parenteral exposure contributes to differing urine chemistry dynamics which is critical to understanding potential kidney related effects. Further interspecies differences of rodent susceptibility to calculus formation, and sensitivity of human infants also warrant careful consideration within medical device risk assessment and application of uncertainty factors. If possible, re-examination of kidney histopathology from NTP would offer additional certainty over proper identification of chronic kidney inflammation in the cohoused studies. An alternative POD of decreased body weight observed across multiple studies may be relevant depending on exposure route and patient population. **Conclusions:** Selection of decreased body weight gains from a subchronic rat study can be justified as the critical POD. Preliminary analysis suggests that chronic kidney inflammation may represent a secondary effect linked to urinary chemistry rather than a primary toxicodynamic response. Rodents are more sensitive to urinary chemistry effects based on propensity to crystal formation and higher protein concentration. Reexamination of kidney histopathology from previous NTP studies could offer more confidence in interpretation of kidney related effects. Pathogenic infections cannot be ruled out as a factor in incidence of chronic kidney inflammation in the cohoused NTP studies. Threshold-based approaches incorporating urinary saturation data and considering crystal formation conditions could provide a more biologically relevant POD for DMT, reducing uncertainty in medical device safety evaluations.

PS 3326 Safety evaluation of hair straightening products containing glyoxylic acid with respect to formaldehyde release and oxalic acid formation

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Background and Purpose: Human health concerns due to heat-activated hair straightening product (HSP) use are debated as the active ingredient, glyoxylic acid (GA), is argued to cause acute kidney injury (AKI) via oxalic acid (OA) crystal formation. Furthermore, heat-decomposition to formaldehyde (FA) might expose users to airborne FA. This study evaluates FA and OA exposure from GA-based HSPs under realistic and worst-case use scenarios to assess consumer and stylist safety.

Methods: Airborne FA was measured using a realistic simulation test in a non-ventilated 15 m³ room. The HSP ($\pm$ GA) was applied to a mannequin head and the hair straightened using a flat iron (230°C). Air concentrations of FA were measured in the breathing zones of the stylist, mannequin head, and in the far field zone on the floor. FA concentrations were also measured following HSP treatment to mannequin heads or consumers in 2 salons. Stylist exposure to GA was measured in a realistic salon simulation test. Hand rinsing and sampling were performed prior to HSP treatment, after HSP application and rinse-off, and after flat ironing. Skin absorption of GA was assessed under realistic application conditions (20 mg/cm² HSP containing 16% GA, 20 min exposure), but also after unrealistic excessive HSP use (increased exposure duration or application amount) to investigate systemic exposure. The highest absorbed amount was used in a PBPK model to estimate the OA excretion after HSP application. Endogenous GA and OA levels were measured in skin from 4 donors. HSP safety was assessed by comparing (a) the OA systemic exposure of the consumer and stylist after HSP use with that from endogenous and dietary sources

and (b) OA urine levels after worst-case HSP use with those linked to normal endogenous and dietary sources, plus levels above which there is a risk of AKI. **Results:** FA air exposure ranged from 3.1 - 12.3 $\mu\text{g}/\text{m}^3$ for GA-containing HSP and 2.3 $\mu\text{g}/\text{m}^3$ for the placebo. In one salon, the background level reached 37 $\mu\text{g}/\text{m}^3$. Maximum levels measured in 2 additional salons following HSP treatment were 2-61 $\mu\text{g}/\text{m}^3$. All levels are below FA exposure limits: 120 $\mu\text{g}/\text{m}^3$ (ACGIH) and 100 $\mu\text{g}/\text{m}^3$ (WHO indoor air quality). The mean total (external) GA exposure of stylists was 7.72 mg. Most of the HSP was washed off post-exposure (20 min to 3 h) in skin absorption studies. The max bioavailable amount remaining was $17.2 \pm 22.9 \mu\text{g}/\text{cm}^2$ (10% GA with 3 h exposure) representing a worst-case extreme use condition since the HSP is applied to the hair, not the scalp. The total bioavailable amount of GA after application of the realistic HSP treatment (16% with 20 min exposure) was $5.22 \pm 2.39 \mu\text{g equiv.}/\text{cm}^2$ or max 4.41 mg for HSP applied to 580 cm² scalp. This is below endogenous OA levels in the skin ($18.7 \pm 5.1 \mu\text{g}/\text{cm}^2$ or max 381 mg in 16000 cm² total skin surface area, GA was not detected in skin). Assuming 25% of GA is metabolized to OA, the estimated systemic exposure is 0.0145 mg for stylist (3 applications/day) and 1.1 mg for consumers (single use). In comparison, dietary (10-25 mg/day) and endogenous sources (ascorbic acid, GA, glycine metabolism; 12-20 mg/day) contribute 22-45 mg/day which is 20-41 times higher than consumer exposure and >1500-times more than daily stylist exposure. Skin absorption of GA under worst-case conditions (10% GA with 3 h exposure) equates to a systemic exposure of 2326 mg GA. Using this input in a PBPK model (validated using *in vivo* data), the estimated OA renal excretion was 3.46 mg/day (bolus) and 3.42 mg/day (3 h infusion; equivalent to 3 h topical application). These amounts are much lower than the urinary excretion of 18 mg/day based on a high OA diet and the 40 mg/day threshold associated with AKI risk. **Conclusions:** This HSP safety assessment demonstrates that the release of FA under realistic use conditions is well below regulatory exposure limits. Systemic exposure to OA from HSP use is negligible compared to other sources, as well as endogenous levels in skin. Therefore, GA-based HSPs are concluded safe for consumers and stylists.

PS 3327 Incorporating Climatic Relevance Endpoints into GreenScreen for Safer Chemicals Assessments

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Background and Purpose: Clean Product Action's GreenScreen® for Safer Chemicals hazard assessment methodology specifies the evaluation of 18 human health, environmental, and physical hazard endpoints, and assigns an overall benchmark (BM) score of 1-4, with 1 being most hazardous and 4 being least. This project expanded the hazard framework to incorporate two climatic relevant endpoints: ozone depletion potential (ODP) and global warming potential (GWP). The United Nations' Globally Harmonized System of Classification and Labelling of Chemicals (GHS) defines ODP as "the ratio of integrated perturbations to total ozone, for a differential mass emission of a particular compound relative to an equal emission of CFC-11." According to the U.S. Environmental Protection Agency, GWP "is a measure of how much energy the emissions of 1 ton of a gas will absorb over a given period of time, relative to the emissions of 1 ton of carbon dioxide (CO₂). The larger the GWP, the more that a given gas warms the Earth compared to CO₂ over that time period. The time period usually used for GWPs is 100 years." There is precedence for including climatic potential endpoints in chemical hazard assessment tools, such as Cradle to Cradle Certified®'s Material Health Assessment Methodology and ChemFORWARD. These two endpoints would broaden and strengthen GreenScreen® assessments by identifying chemicals that are less hazardous and more sustainable. **Methods:** Chemicals screened for their ODP and 100-yr GWP would use measured or predicted values to assess its climatic potential endpoints. Definitions for Low (L), Moderate (M), High (H), and Data Gap (DG) are proposed. Like other endpoints in a GreenScreen® assessment, the confidence level is designated as high or low. The proposed GreenScreen® ODP criteria are defined as Low: ODP equal to zero or chemical is non-volatile (i.e., BP >250°C or Vapor Pressure <0.1 mm Hg at 20°C) or chemical is solid; High: ODP greater than 0 and/or GHS Category 1 for ODP; or Data Gap: Insufficient data for ODP. The proposed GreenScreen® GWP criteria are defined as Low: 100-yr GWP equal to zero or chemical is non-volatile or chemical is solid; Moderate: 100-yr GWP greater than zero and less than or equal to 10; High: 100-yr GWP greater than 10; or Data Gap: Insufficient data for GWP. For a chemical with a high hazard for ODP or GWP, the overall benchmark score is proposed to be BM-1. The presence of a data gap for ODP and/or GWP would not count toward the data gap rules in a GreenScreen® assessment. **Results:** Ten chemicals (6 BM-2 and 4 BM-1) with existing GreenScreen® assessments were assessed for ODP and GWP hazards, using publicly available ODP and GWP values and were scored following the proposed criteria. Following the proposed criteria, BM scores for five out of the 10 chemicals would change from BM-2 to BM-1, indicating a high hazard. One of these chemicals, 1,1,2,2-tetrafluoroethyl-2,2,2-trifluoroethyl ether (CAS #406-78-0), is currently listed on the TCO Certified Accepted Substances List with a BM-2 score. This illustrates a shortfall in the current GreenScreen® methodology for not considering this chemical's high 100-yr GWP value of over 900 and the chemical's potential impact on climate change. One of the BM-2 chemicals has an unchanged BM score due to data gaps. Of the four BM-1 chemicals, two would receive an additional subscore of BM 1f proposed for denoting the high climatic relevance endpoints, highlighting their potentially high impacts on climate change. **Conclusions:** This pilot project demonstrates that the addition of the ODP and GWP endpoints can have a differentiating impact on a chemical's GreenScreen® benchmark score. Additionally, basing climatic relevant

criteria on GWP or ODP values ensures that the latest science and measurements are utilized compared to checking a list that may represent outdated information. Methyl bromide (CAS #74-83-9) is the only chemical of the 10 chemicals assessed that has a GHS category 1 ozone depletion classification (H-420: Harms public health and the environment by destroying ozone in the upper atmosphere) and the only chemical listed in the Montreal Protocol annexes of ozone depleting substances. The addition of climate potential expands GreenScreen® beyond the traditional human health and environmental toxicology realm and extends the assessment paradigm into planetary health and sustainability endpoints.

PS 3328 Demonstrating the difference between hazard and risk: The relevance of high-dose experimental animal study results to real-world fragrance exposure in consumers

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Background and Purpose: Fragrance safety is very important to consumers and the industries that produce or use fragrances in their products. Since 1966, risk assessment of fragrances has been carried out by a dependable scientific infrastructure that works in full transparency at Research Institute for Fragrance Materials (RIFM). For a risk assessment, exposure and toxicity data are necessary. Risk assessment of fragrances and their constituents for systemic toxicity has been partly based on studies in rodents which were conducted historically or were required for regulatory purposes. Rodents were exposed to discrete fragrances to determine the highest dose level not associated with an adverse effect (no observed adverse effect level or NOAEL). To achieve this, fragrances are experimentally applied up to dose levels largely exceeding the minute levels of real-life exposure in people. The huge spread between the high-dose experimental exposure and real-life consumer exposure is a concept familiar to toxicologists but may be unfamiliar to the typical fragrance consumer. Here, a common fragrance will serve to demonstrate this concept. p-Cymene is a fragrance and flavoring found naturally in raspberries. To demonstrate that the real-life risk of inducing an adverse effect due to fragrance exposure is extremely low, two exposure scenarios were selected. First, exposure through food consumption and second, exposure through use of fragrance/fragranced products. In more detail we ask how many raspberries a person would need to consume to get an adverse effect from p-cymene ingestion, disregarding any potential effect of nutritional imbalances. Second, aggregate exposure to a perfume (or fine fragrances) by humans is calculated to match the dose level associated with an adverse effect seen in animal studies. **Methods:** The most conservative NOAEL of p-cymene from animal studies was identified after evaluating all OECD- and/or GLP-compliant repeated dose/ reproductive toxicity studies, to select the dose associated with an adverse effect. A robust OECD 422 study, which is a combined repeated dose and reproduction/developmental toxicity screening test in rats was selected, showing adverse effects at a dose of 100 mg/kg/day. A human equivalent dose (HED) calculation using an allometric exponent for body surface area (0.67, to account for the difference in metabolic rate between rats and humans), was applied to convert the adverse effect dose into a human equivalent dose. The amount of p-cymene in a raspberry was taken from VCF (Volatile compounds in food) database and reliable research articles. For exposure through use of fragrances, aggregate fragrance exposure the Creme-RIFM Aggregate Exposure model was utilized. The model explores usage data from tens of thousands of consumers in North America, Europe, and Asia and provides realistic exposure estimates that consider all fragrances the population is exposed to across all consumer products that are used. **Results:** Adverse effects at 100 mg/kg/day of p-cymene translated into a HED of 830 mg/day. Further, based on reliable literature and databases, it was determined that raspberries contain a mean of 0.0027 mg of p-cymene. Therefore, following human equivalent dose (HED) calculations and amount of p-cymene in raspberries, a person would need to ingest approximately 307,407 raspberries/day in order to match the adverse effect level seen in animals. For fragrance use, a content of 0.0022% of p-cymene in perfumes based on the 95th percentile (which is exposures from the highest users) values were estimated from the Creme-RIFM Aggregate Exposure model. It turned out that it would take 3722 sprays/day (equivalent to 3 L or 0.8 gal) to match the dose level associated with adverse effects in animal studies. **Conclusions:** In conclusion, the selected exposure scenarios highlight the essential importance of taking real life exposure into account to arrive at a realistic human risk assessment. Exposure science has evolved considerably, and results of high dose animal studies need to be put into perspective with real life consumer exposure. The selected exposure scenarios exemplify and underline the minute health risk associated with fragrance use.

PS 3329 Probabilistic cancer risk assessment associated with implantable medical devices: A case study

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Background and Purpose: This case study aims to evaluate the lifetime cancer risk associated with residual formaldehyde in medical devices using a probabilistic risk assessment method. Under the ISO 10993 guidance framework, by integrating chemical release data, toxicological thresholds, and probabilistic modeling on cancer risk, the case study demonstrates a more comprehensive risk assessment framework that accounts for implantation duration in relationship to cancer risk. Formaldehyde may be used to preserve biological tissues used in medical devices. Some formaldehyde may remain on or inside the devices after the tissues have been processed. When these devices are implanted in the body, the remaining formaldehyde can leach into nearby tissues, which may result in long-term exposure. This hypothetical case study demonstrates how route and duration of exposure can be integrated to conduct a probabilistic cancer risk assessment. **Methods:** To assess chronic exposure risk, calculations will follow ISO 10993-17: 2023, which provides a framework for determining the cancer risk specific dose (CRSD). CRSD is derived from the TD₅₀ value and normalized with 50,000, which is based on a hypothetical lifetime cancer risk of 1 in 100,000. The Maximum Estimated Exposure Dose (EEDmax) will then be compared to CRSD to determine the Margin of Safety (MOS), where MOS > 1 indicates a negligible carcinogenic concern. For a more refined risk characterization, a probabilistic cancer risk model was applied based on the publication by Abbott (Weng et al., *Regulatory Toxicology and Pharmacology*, 2026, Vol 164, 105976, ISSN: 0273-2300). This model allows estimation cancer risk during the duration of implantation over time. **Results:** McClurg et al. (1996) published a case study of bioprosthetic heart valves treated with formaldehyde initially absorbed about 770-800 nmole/mg dry weight, decreasing to roughly 370 nmole/mg after 16 weeks. Assuming proper washing before clinical use, the residual formaldehyde on the heart valve would likely be around 8 mg remaining within the implanted valves. When only worst-case exposure assumptions are made, the MOS overestimates the risk. To further evaluate cancer risk under clinical use conditions, a probabilistic cancer risk model was applied. The formaldehyde mutation slope of 0.49, determined by Ames assay, was used to evaluate implantation duration versus cancer potency over time. As a result, during the first 20 years of implantation, the probabilistic cancer risk was found to be 2.8 per 10,000,000, indicating that the potential cancer risk is 36-fold lower than the default hypothetical threshold of cancer risk of 1 in 100,000 recommended via ISO 10993-17: 2023. Therefore, the cancer risk for the duration this medical device is likely to be used poses a negligible carcinogenic risk. **Conclusions:** This case study shows that formaldehyde leaching from preserved implanted biological tissue poses a minimal carcinogenic risk when the totality of evidence is considered. It demonstrates how a comprehensive, multi-parameter framework that evaluates cancer potential throughout the lifetime of the device to draw realistic conclusions by combining scientific evidence with regulatory principles to improve the risk assessment in medical devices.

PS 3330 Is it actually dirty? A Probabilistic Risk Assessment of the EWG's Dirty Dozen

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Background and Purpose: Consumers often face mixed messages about the safety of the foods they eat. Each year, the Environmental Working Group (EWG) releases a widely circulated report ranking 47 commonly consumed fruits and vegetables based on their levels of pesticide residues. The 12 with the greatest pesticide contamination are designated the "Dirty Dozen." This list is intended to help consumers reduce potential exposure to harmful pesticides. However, guides such as the EWG's only incorporate hazard-based assessments and do not consider exposure assessments - and ultimately risk- during their publication. As such, this study aims to apply human health risk assessment methodologies to the EWG's Dirty Dozen list, utilizing a probabilistic screening-level framework to estimate pesticide exposure among U.S. consumers and assess potential chronic health risks. **Methods:** For each item on the 2025 Dirty Dozen list, the most frequently detected pesticides and its corresponding residue concentrations were obtained from the USDA Pesticide Data Program (PDP). Using the EPA Exposure Factor Handbook (EFH) and National Health and Nutrition Examination Survey (NHANES) consumption distributions were developed for adults and children separately. Next, dietary exposures were calculated for each pesticide-produce combination. These distributions were then compared to the population adjusted dose (PAD) distribution from EPA Office of Pesticide Programs. The distribution for the PAD was defined as a distribution centered on the PAD value with a one order of magnitude SD, per EPA's definition of a reference dose. **Results:** Our results indicated that the estimated chronic exposures were well below levels of concern for the population. We additionally calculated cumulative measures of exposures to multiple contaminants and found these to also be below the level of concern. Lastly, we calculated the quantities of produce required to reach potentially harmful exposure levels and demonstrate that these values far exceed normal consumption based on the distributions provided by EPA, even for children. **Conclusions:** In conclusion, we present a dietary risk assessment for the EWG's "Dirty Dozen" that demonstrates exposure does not exceed levels of concern based upon consumption patterns in the US.

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Background and Purpose: During the past decade, engineered stone fabrication has become the focus of renewed concern regarding respirable crystalline silica (RCS) exposure and silicosis risk. Several reports have suggested that engineered stone dust exhibits enhanced toxicity due to its finer particle size distribution, higher surface area, and/or resin and metal content, and that conventional exposure limits may be inadequate. Claims have also been made that standard engineering controls are ineffective. This study sought to evaluate the scientific foundation of these claims and determine whether engineered stone warrants a distinct toxicological or regulatory category. **Methods:** A non-systematic review of peer-reviewed publications, governmental assessments, and field exposure investigations during engineered stone fabrication was conducted. Emphasis was placed on toxicological studies comparing particle morphology, surface chemistry, and dose-response relationships across materials, as well as industrial hygiene data evaluating the effectiveness of wet methods, local exhaust ventilation, and respiratory protection. Regulatory evaluations by OSHA, NIOSH, and international agencies were reviewed to contextualize findings within current occupational standards. **Results:** Both quartz, the predominant silica polymorph in natural stone, and cristobalite, present at higher concentrations in some engineered stones, exhibit comparable dose-response relationships for silicosis. A review of current literature, supported by particle characterization studies, suggests that RCS particle size distributions from engineered stone do not materially differ from those generated during natural stone processing when fabrication techniques are equivalent. Dry cutting of both engineered and natural stones yields finer particles than wet methods. No convincing evidence shows that differences in surface area, zeta potential, or resin content translate into increased potency (relative to airborne RCS concentration), specifically for silicosis. Air monitoring data show that RCS concentrations during dry cutting and polishing of high-silica engineered stone (80% by volume or 90% by weight) can exceed OSHA's permissible exposure limit by several orders of magnitude, but similar exposures have been documented in granite (20-60% silica by volume or weight). Exposure assessment data indicate that low-silica content stone like marble (<5%) can generate RCS above the PEL if manipulated under improper controls, particularly during dry cutting without local exhaust ventilation (LEV) or respirators. Conversely, when wet methods, LEV, and proper housekeeping are employed, airborne RCS concentrations are reduced by 80-99%, often achieving levels below 0.05 mg/m³. Respirators with Assigned Protection Factors of 10 or greater provide an additional margin of safety for residual exposures. Clusters of silicosis among engineered stone workers consistently correspond to dry fabrication and inadequate control, not intrinsic material properties. Legal and administrative reviews confirm that existing federal and state standards already require multilingual training, exposure monitoring, and effective use of controls. Field data from California and Washington indicate noncompliance, particularly lack of fit testing and inconsistent use of wet cutting, is the primary cause of overexposures. Evidence from community-based training programs shows immigrant and limited-English-proficiency workers can be successfully trained when materials are accessible and culturally appropriate. **Conclusions:** Toxicological and epidemiological evidence does not support classification of engineered stone as a unique or unusually hazardous source of RCS. The presence of disease is best determined by cumulative RCS exposure, not material composition. Rather, observed silicosis cases reflect lapses in exposure control, training, and enforcement, not limitations of the current regulatory framework. The weight of evidence affirms that existing occupational standards, when implemented through established industrial hygiene practices, are sufficient to protect workers. Continued emphasis on compliance verification, multilingual worker education, and sustained engineering control performance remains the most effective means of preventing silica-related disease across all industries handling RCS.

PS 3332 Conditional Knockdown of USP22 Reveals Target Liability in Adult Mice

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Background and Purpose: USP22 is a histone deubiquitinating enzyme within the SAGA complex, implicated in oncogenesis and physiological functions. Complete USP22 knockout in mice results in preweaning lethality, while heterozygotes display dysregulations of immune and hematopoietic systems, abnormal gait. Reduced USP22 expression has been associated with sporadic cerebral cavernous malformation. To better characterize potential toxicological liabilities associated with USP22 loss-of-function, we employed an inducible USP22 knockout mouse model. **Methods:** 12-16-week-old, male and female mice underwent tamoxifen-induced USP22 knockdown. The mice were monitored over 6 months via clinical observation, body weight, clinical pathology, flow cytometry, serum cytokine release profile, antinuclear antibody detection, histopathology, and Magnetic Resonance Imaging (MRI) assessment of central nervous system (CNS). **Results:** USP22 protein levels were reduced by 90% in peripheral tissues. The knockout mice presented no obvious adverse clinical observations, but the male mice exhibited reduced body weight gain during the study. Serum cytokine profile revealed a progressive increase of pro-inflammatory mole-

cules (IFN γ , IL-2, IL-4, IL-5), with marked elevations at six months. By six months, the mice presented elevations in globulin (male 30%, female 50%), alkaline phosphatase (male 50%, female 34%), and triglycerides (male 26%, female 62%), and anti-dsDNA autoantibody levels were also significantly elevated, particularly in females. Flow cytometry confirmed a mild but insignificant increase in T cell population in both sexes and a significant rise in B cell population in females. Additionally, peripheral blood hematology suggested potential impact to the erythropoietic compartment of the bone marrow, including altered red blood cell parameters and reduced hemoglobin indicative of anemia without regenerative response (decreased reticulocytes) and thrombopenia (lower platelet counts with larger sizes). Histopathological findings included an increase of lymphocytes in mesenteric lymph node and increase of hypertrophy/hyperplasia of goblet cells in gastrointestinal tissues indicating immune-mediated pathological changes in the intestinal tract. Due to limited tamoxifen exposure in brain, only ~50% knockdown was achieved. There were not histological abnormalities identified in the brain and spinal cord, but MRI analysis detected modest but significant (5%; p0.05) total brain volume reduction in male knockdown mice. **Conclusions:** Inducible USP22 knockdown mice presented toxicities indicative of inflammation, immune dysregulation with potential autoimmunity, goblet cell hyperplasia and proliferation in the intestinal track and alteration of the erythroid compartment leading to anemia and thrombopenia. Additional studies will be needed to better understand the role of USP22 in the central nervous system and the implications of reduced brain volume in male mice.

PS 3333 The delay in the completion of vaginal opening induced by Epyrifenacil is a secondary effect caused by prepubertal anemia.

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Background and Purpose: Epyrifenacil is a novel uracil protoporphyrinogen oxidase (PPO) inhibiting herbicide developed by Sumitomo Chemical Co., Ltd. In a two-generation reproductive toxicity study of Epyrifenacil in rats, a delay in the completion of vaginal opening (VO), an indicator of sexual differentiation in female rats, was observed. While VO can be influenced by chemicals with endocrine-disrupting (ED) properties, it is also known to vary due to secondary factors such as growth retardation or stress. In this study, no changes were observed in other ED-related parameters, including anogenital distance and estrous cycle, or in body weight during prepubertal period. Furthermore, results from a series of *in vitro* assays (luciferase reporter gene, steroidogenesis, and aromatase assays) did not indicate any ED-related activity. To elucidate the mechanism underlying the delayed VO, we investigated the relationship between anemia, the only apparent systemic toxicity observed, and the completion of VO. **Methods:** Anemia was induced in juvenile female Crl:CD(SD) rats by drawing a certain amount of blood from the jugular vein (Experiment I) or by intraperitoneal administration of phenylhydrazine (PHZ), a well-known inducer of hemolytic anemia (Experiment II). Experiment I: Postnatal day (PND) 15 pups were randomly assigned to each group (control, two blood removal (BR) and satellite groups) based on body weight. In the BR groups, 1.0% or 1.5% of body weight of blood was drawn every 2 days from PND 16 and 28. Experiment II: PND 17 pups were divided into 3 groups (PHZ 10 mg/kg/day, PHZ 20 mg/kg/day, and vehicle control) in the same manner as Experiment I. PHZ was intraperitoneally administered to pups on PNDs 18 and 19. To monitor hematological parameters, blood was also drawn on PND 35 and PND 57 or 58 in the BR and satellite groups (Experiment I) and on PNDs 20, 24, 28, 35, 42, and 56 (Experiment II). Parameters assessed included clinical signs, body weight, food consumption, hematology, observation of VO, necropsy, organ weights (ovaries and uterus), and histopathological examinations (ovaries, uterus, vagina). **Results:** Both BR and PHZ treatment groups showed transient anemia characterized by a significant decrease in hemoglobin levels (approximately 20-35%) compared to the control group. Despite the presence of anemia, there were no noticeable changes in the body weight of these female rats on each postnatal day, indicating that their growth was comparable to that of the control group. Additionally, the anemia did not impact the weights or tissue structure (histopathology) of the reproductive organs after sexual differentiation. However, the age at VO was delayed in the anemic rats compared to the control group. Furthermore, these anemic rats had a higher body weight at the time of VO compared to the control group. These results imply that anemia in prepubertal females could be a contributing factor in delaying puberty. **Conclusions:** Epyrifenacil consistently induces anemia through PPO inhibition in mammalian toxicity studies; yet neither *in vivo* nor *in vitro* data indicate ED-related activity. In the two-generation reproductive toxicity study of Epyrifenacil in rats, and among the ED-related endpoints, only the delay in VO completion (accompanied by increased body weight at VO) was noted, which was associated with more severe anemia than those observed in BR and PHZ treatment groups. Taken together with these results and the weight of evidence, we concluded that the delay in the completion of VO following Epyrifenacil exposure was a secondary effect due to prepubertal anemia and not related to endocrine-disrupting effects.

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Background and Purpose: In drug discovery, proactive toxicological risk assessment of candidate compounds is crucial for avoiding clinical-stage failure and post-market withdrawal. However, traditional preclinical safety evaluation strategies have significant limitations: *in vivo* animal studies exhibit low human relevance (translatability) due to significant species differences and face dual pressures of ethics and cost; while conventional *in vitro* binding assays are inadequate for predicting functional biological effects. Notably, approximately 75% of clinical adverse drug reactions (ADRs) originate from dose-dependent off-target effects, highlighting an urgent need for more accurate early-risk identification tools. To address these challenges, the IQ DruSafe consortium—a preeminent alliance of global pharmaceutical companies—has championed the enhancement of preclinical predictive power through the expansion and refinement of secondary pharmacology screening strategies. This study is aligned with this initiative and aims to investigate the implementation of an advanced *in vitro* secondary pharmacology screening system utilizing functional assay formats. By generating richer pharmacological information beyond mere binding affinity, this strategy seeks to enable a more accurate and earlier identification of potential safety liabilities, thereby providing highly translatable safety insights for the optimization of lead compounds. **Methods:** This study adopted a high-throughput screening strategy integrating multiple functional assay technologies (including FLIPR calcium flux, HTRF, ADP-Glo, FP, etc.). The strategy included two modes: single-point primary screening and multi-concentration dose-response curve screening. To achieve high throughput and high precision, the platform integrated automated instruments (such as acoustic liquid handling systems and multi-mode plate readers) to perform curve-based screening tests. We applied this strategy to test the dose-response effects of various antidepressants, pergolide, and osimertinib on a panel covering 77 important safety-related targets. **Results:** The results indicated that this screening strategy can reveal differentiated off-target risk profiles. Testing the antidepressants amitriptyline, citalopram, and bupropion from different generations using the ICSTP SAFETYPANEL™ 77 successfully quantified their off-target interaction risks. The results showed that amitriptyline exhibited off-target activity and strong inhibitory effects on 22 safety-related targets (15 GPCRs, 4 ion channels, 3 transporters); citalopram had 6 off-target interactions (3 GPCRs, 1 ion channel, 1 transporter, 1 nuclear receptor target); while bupropion had the cleanest off-target profile, with activity only at the DAT target, which is its primary target. These findings are highly consistent with their known clinical safety profiles. Furthermore, the dose-response screening mode based on functional assays provided significant advantages, such as effectively differentiating full agonists from partial agonists and identifying complex non-linear biological effects. Multi-concentration testing helped avoid false positive or false negative misjudgments caused by compound solubility limitations or aggregation tendencies. The use of 1 mM ATP concentration in all kinase target assays, a condition closer to the physiological state, improved the predictive accuracy for *in vivo* inhibitory potency. The integration of automated processes significantly increased sample throughput and detection efficiency. **Conclusions:** This study confirms that a secondary pharmacology screening strategy utilizing functional assay formats, multi-concentration dose-response curves, and integrated automation can provide more comprehensive and predictive data for drug safety assessment. This strategy helps identify and mitigate potential safety risks mediated by off-target effects early in compound optimization, thereby improving the success rate and cost-effectiveness of new drug development and promoting the development of safer and more effective drugs.

PS 3335 Pragmatic Modification of Systematic Evidence Map Scope in Support of the ATSDR Toxicological Profiles Program

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Background and Purpose: The Agency of Toxic Substances and Disease Registry (ATSDR) has recently adopted systematic review methodology into the review of substances under consideration for toxicological profile update or development via incorporation of interactive systematic evidence maps (SEM). SEMs are preferable to previously conducted internal chemical evaluations due to their transparency, repeatability, and resulting publicly accessible interactive databases of health effects studies. However, conducting SEMs proved to be challenging while maintaining schedules and adhering to budgetary constraints. This became especially apparent when the Populations, Exposures, Comparators, and Outcomes (PECO) eligibility criteria developed for the pilot SEM (methylene chloride) was applied to data-rich compounds, resulting in a cumbersome number of studies selected for retrieval and/or data extraction. Therefore, additional compound-specific exclusion criteria were developed to manage scope, narrowing the focus to the intended goal: identifying studies that could potentially impact minimal risk levels (MRLs; i.e., low-exposure studies) and/or filled data gaps identified in profiles. Here, we present two case studies illustrating how ATSDR developed fit-for-purpose modifications to ATSDR's SEM protocol to manage scope for two data rich compounds: carbon tetrachloride (CCl₄) and toluene. **Methods:** Due to the high volume of selected studies, a re-review of

included studies at the title/abstract and full-text stage was conducted to determine if there was a specific study type (or types) that was being included by the pilot PECO criteria that would be uninformative for the intended purpose of the SEM. For CCl₄, it was determined that studies that administered a known toxic dose with the intent to amend (ameliorate or exacerbate) hepatotoxicity and studies that used CCl₄ as a model of toxicity or positive control should be excluded. The rationale for exclusion was 1) studies are uninformative for hazard identification (evaluating a known target of CCl₄ toxicity), and 2) studies are uninformative for low dose extrapolation (evaluating known toxic doses). For toluene, due to a robust epidemiological database, it was determined that only human studies containing quantitative reporting and analysis of measured toluene exposure levels were adequate for data extraction. Therefore, studies that lacked quantitative exposure data, estimated exposure using a job-exposure matrix, or only presented a combined analysis of benzene-toluene-ethylbenzene-xylene (BTEX) exposure were excluded. Case study reports were also excluded. **Results:** Of the initial 11,805 references identified in the literature search for CCl₄, 7,387 studies were excluded as studies designed to amend (ameliorate or exacerbate) the known hepatotoxic effects and 989 studies were excluded as model of toxicity/positive control studies. Together, those study types represented 71% of the studies returned in the literature search. Excluding these studies dramatically narrowed the scope of the SEM to the highly relevant 24 studies identified for data extraction. For toluene, of the initial 5,448 references, 57 case reports, 35 human studies lacking adequate exposure data, and 57 human studies reporting only BTEX analyses were excluded. Exclusion of these studies reduced human data extraction by 62%, from 241 to 92 studies. **Conclusions:** For both CCl₄ and toluene, narrowing the scope of the SEM to the most relevant study types by adding compound-specific exclusion criteria to the PECO criteria saved an extreme amount of effort and resources. By focusing on the intended goal of the project, the systematic review approach can be designed to be fit-for-purpose in order to manage the timeline and budget while still developing a transparent, repeatable, and useful SEM product. **Disclaimer:** The findings and conclusions in this presentation have not been formally disseminated by the Centers for Disease Control and Prevention/ATSDR and should not be construed to represent any agency determination or policy.

PS 3336 Systematic Evidence Map for CCl₄ and Interactive Visualization

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Background and Purpose: The Agency of Toxic Substances and Disease Registry (ATSDR) has recently adopted systematic review methodology into the review of substances under consideration for toxicological profile updates or development via incorporation of interactive systematic evidence maps (SEM). SEMs are preferable to previously conducted internal chemical evaluations due to their transparency, repeatability, and resulting publicly accessible interactive databases of health effects studies. The carbon tetrachloride (CCl₄) profile, published in 2005, was selected for application of the SEM. **Methods:** Two independent screeners conducted title and abstract screening of the search results in DistillerSR for references that met the PECO eligibility criteria. References included at title and abstract screening were retrieved and full-text review was conducted by two independent screeners in DistillerSR to confirm PECO relevance. At both title/abstract and full-text review, screening conflicts were resolved by discussion between the primary screeners, with consultation by a third screener (if needed) to resolve any remaining disagreements. It was determined that studies that administered a known toxic dose with the intent to amend (ameliorate or exacerbate) hepatotoxicity and studies that used CCl₄ as a model of toxicity or positive control should be excluded. The rationale for exclusion was 1) studies are uninformative for hazard identification (evaluating a known target of CCl₄ toxicity), and 2) studies are uninformative for low dose extrapolation (evaluating known toxic doses). References that were categorized as PECO-relevant health effects studies in humans and animals advanced to high-level data extraction in DistillerSR. Results of the Literature Screen and Data Extraction were exported into Tableau Public to generate interactive data visualizations. **Results:** Of the initial 11,805 references identified in the literature search for CCl₄, 7,387 studies were excluded as studies designed to amend (ameliorate or exacerbate) the known hepatotoxic effects, and 989 studies were excluded as model of toxicity/positive control studies. Together, those study types represented 71% of the studies returned in the literature search. The literature screening identified 9 human and 15 animal health effects studies. A majority of human studies were chronic-duration, and most animal studies were acute-duration. 100% of the human studies evaluated exposure via biomarkers, so exposure was attributed to multiple/unknown exposure routes. 49% (33) of animal studies focused on the inhalation route and 51% (34) focused on the oral route. The most-studied endpoints in inhalation studies included death, body weight, respiratory, hepatic, and renal. Findings from human and animal studies are consistent with the existing Toxicological Profile for CCl₄ (2005): 1) The hepatic and renal systems are toxicity targets of CCl₄; 2) There is some evidence for respiratory effects in humans and animals; 3) Animal studies generally report effects in cancer studies, but findings from human studies are mixed; and 4) The evidence for effects in other organ systems is mixed. **Conclusions:** The 2025 CCl₄ SEM identified toxic endpoints consistent with the most recent toxicological profile from 2005. The interactive data visualization of these data created in Tableau can be accessed via links in the published SEM available on the ATSDR web page (SYSTEMATIC EVIDENCE MAP (SEM) FOR CARBON TETRACHLORIDE). **Disclaimer:** The findings and conclusions

PS 3337 Dosimetry Refinement for Tiered Screening Human Health Risk Assessment: A Case Study

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Background and Purpose: Military operations frequently involve exposure to chemicals for which suitable health-based exposure limits are often lacking. Human health risk consults to support operational decision-making require quick identification of any serious toxicological concerns. A case study was conducted to demonstrate a modernized, tiered approach to prospective derivation of health-based screening values (HSVs), with a focus on *in vitro* New Approach Methods (NAMs) of varying complexity. Derivation of HSVs from *in vitro* toxicity data, a process known as *in vitro* to *in vivo* extrapolation (IVIVE), is typically performed with highly simplified toxicokinetic models in early stages of a tiered approach. Comparing toxicokinetic models of varying conservatism and complexity provides additional opportunities to refine the prospective risk assessments as envisioned for the individual levels of a tiered approach. **Methods:** This case study used seven chemicals with existing *in vitro* and *in vivo* toxicity information (malathion, malaoxon, diazinon, diazoxon, aldicarb, chlorobenzene, and tris (2-chloroethyl) phosphate). These substances were amenable to high throughput toxicokinetic modeling using the "high throughput toxicokinetics (httk)" R package (based on a conservative, generic model) and had existing "bespoke" (custom or semi-generic) human physiologically based pharmacokinetic models. **Results:** We found that differences in model structure (notably, presence or absence of extrahepatic metabolism), central tendency parameter values, and parameter value distribution assumptions contributed to differences in IVIVE results between httk and the bespoke models. Typically, when httk-based IVIVE was used for this limited suite of substances, HSVs were substantially lower than when bespoke models were used (as much as 64,000-fold in these case studies). **Conclusions:** Substances for which metabolism was the primary route of clearance (rather than elimination unchanged in urine) were especially prone to have highly conservative HSVs when the httk models were used, as would typically be expected in early stages of a tiered assessment of a novel substance. In particular, features such as the potential for extrahepatic metabolism should be considered when interpreting the predictions from generic pharmacokinetic models. No Department of Defense endorsement implied.

PS 3338 Evaluating New Chemicals Under the Toxic Substances Control Act (TSCA): Application of US EPA's ChemInformatics Modules for Identification of Information for Human Health Risk Assessment

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Background and Purpose: US Environmental Protection Agency's Office of Pollution Prevention and Toxics, New Chemicals Division (NCD) is responsible for conducting human health and environmental risk assessments for new chemical substances submitted under the Toxic Substances Control Act (TSCA) prior to their entry into US commerce. Typically, limited to no toxicological information is available for submitted new chemical substances. To address data gaps, a read-across approach using similar chemicals with acceptable test data is frequently employed for hazard identification and selection of toxicity values for quantitative risk assessment. The ChemInformatics Modules (CIM), developed by US EPA, is a publicly available web-based platform that provides a centralized location for chemical information (e.g., physicochemical properties, hazard and safety data, structural alerts) typically used in a new chemical human health risk assessment, which includes information from federal, state, and international sources. CIM integrates search functions for structure, substructure, and similarity. This poster presents the framework used by NCD to incorporate systematic use of CIM into read-across assessments for new chemical substances. **Methods:** NCD has developed a framework leveraging CIM to facilitate identification of analogues for which publicly available toxicological test data are available, as well as chemical category information for read-across that provides coverage of the hazard endpoints and toxicity value needs of a NCD human health risk assessment for a new chemical substance. The search strategy is iterative and includes both hierarchical analogue searches and analogue/chemical category exploration approaches with visualization of available toxicological information for hazard coverage. Standardized reports are generated that present the curated information in a user-friendly format for hazard assessment and toxicity value selection. **Results:** Systematic use of CIM by NCD human health assessors, in accordance with the newly developed framework, has led to more standardized identification of relevant toxicological information from publicly available sources to address data gaps in hazard identification and toxicity value selection. This has improved the

efficiency, consistency, and transparency of human health risk assessments for new chemical substances. The framework will also provide a resource for stakeholders preparing new chemical submissions to US EPA under TSCA. **Conclusions:** Application of a newly developed hierarchical framework incorporating systematic use of CIM in read-across assessment has improved the efficiency, consistency, and transparency of NCD human health risk assessments for new chemical substances submitted under TSCA. *These views are those of the authors and do not reflect views or policies of US EPA.*

PS 3339 Skip to Recipe: the importance of food disaggregation in performing lifestage-specific dietary exposure assessments

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Background and Purpose: Characterizing dietary exposure is a critical part of the risk assessment for chemicals that are intentionally added to or are unintentionally present in a nation's food system (e.g., food additives, contaminants, pesticide residues, etc.). In the USA, nationally representative food consumption data is available from the What We Eat in America (WWEIA) survey, which is the dietary intake portion of the National Health and Nutrition Examination Survey. WWEIA contains data from two non-consecutive days of 24-hour dietary recall, where many of the consumed foods being reported are mixed dishes (e.g., cracker, cake). However, in many cases, the concentration information that would inform chemical exposure (e.g., pesticide residue data) is collected on the constituent parts of these foods (i.e., wheat flour, sugarcane sugar). Thus, a reliable means to disaggregate mixed foods into food commodity ingredients provides a critical link between dietary recall information and accurate exposure assessments. The objective of this study is to characterize how these recipes used to disaggregate mixed foods can impact consumption estimates and to demonstrate the importance of understanding the implications of recipe assumptions when characterizing lifestage-specific sensitivities. **Methods:** To examine the impact of recipes on dietary exposure assessment, consumption estimates were generated for the approximately 1000 unique ingredients using two unique databases to break down the mixed foods reported in the WWEIA surveys: the US Environmental Protection Agency's Food Commodity Intake Database (FCID) and the US Food and Drug Administration's (FDA) Food Disaggregation Database (FDD). This required methodically translating ingredients utilized in FDD to an identical or analogous item in FCID to ensure the same nomenclature was used across datasets. The translation was performed from FDD to FCID because the latter consistently provided more granularity, and further disaggregation of FDD could be more reliably and consistently performed than reaggregation of FCID ingredients. Thus, the result of this translation was frequently an increase in the granularity of FDA's disaggregation by using existing FCID recipes to extend those in FDD. Once translation was complete, the recipe databases were used to generate bodyweight-normalized, per capita commodity consumption estimates for dietary intake data from the 2005-2010 WWEIA surveys. These consumption estimates were then evaluated to identify areas of divergence. **Results:** The consumption estimates were largely similar for the 2005-2010 WWEIA surveys, regardless of whether the recipes from the translated version of FDD or FCID was used for mixed food disaggregation. This indicated that corresponding exposure assessments would likely be robust across the whole of the surveyed population. However, the FCID recipes include numerous ingredients in very small amounts to account for various plausible preparations of a given food. These commodities introduce low volume consumers to FCID-generated consumption estimates that are almost entirely absent from those generated with FDD. Likewise, FDD does not include specificity for ingredients in baby foods, which may reduce lifestage-specificity if data are available for ingredients within that specific production chain. Given that infants and young children may be a sensitive subpopulation, this represents a critical divergence and one that would warrant consideration in cases where exposure is being evaluated on commodities that are heavily utilized in baby food or infant formula. **Conclusions:** A database of recipes for disaggregating mixed foods is a critical component of dietary exposure assessments, particularly for chemicals that are measured in or added to the constituent ingredients. Because of their translational use, the assumptions made at the various levels of recipe development will have downstream impacts on consumption estimates and exposure. It is therefore critical that the recipes themselves are readily available for review and evaluation, but it is similarly important that the underlying assumptions are transparently characterized and that the impact of recipes on exposure is assessed during development and testing. Likewise, a consensus approach to recipe development has the potential to introduce greater precision in dietary exposure assessment, particularly in a regulatory context.

PS 3340 Application of the Threshold of Toxicological Concern (TTC) to Evaluate New Chemicals under the Toxic Substances Control Act (TSCA)

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Background and Purpose: The US Environmental Protection Agency (EPA) is responsible for conducting human health and environmental risk assessments for new chemicals submitted under the Toxic Substances Control Act (TSCA) prior to their entry into US commerce. For every new chemical submission, EPA must make

an affirmative finding on the safety of the new chemical substance. There are no minimum data requirements for a new chemical submission, so human health risk assessments typically employ a number of approaches to fill data gaps such as *in silico* predictions, read-across from analogues, and chemical categorizations. For some chemistries, these methods may not provide sufficient information to derive a point of departure (POD) to quantify risk. The Threshold of Toxicological Concern (TTC) method provides a conservative estimate of human exposure limits for chemicals with limited toxicological data, ensuring minimal risk to human health at or below specific exposure thresholds. The proposed TTC approach addresses a type of new chemical submission, known as Low Volume Exemptions (LVEs) under TSCA, for chemicals characterized by low production volumes, a limited number of use sites, and short review periods (30 days). Building upon recognized practices from the U.S. EPA Office of Pesticide Programs, U.S. Food and Drug Administration, European Food Safety Authority, and Health Canada, use of the TTC approach in new chemical review offers an alternative method for risk determination for chemical submissions with limited exposures and no quantitative data. **Methods:** The proposed framework contains a decision tree and step by step instructions to facilitate rapid and consistent evaluation. The framework begins by evaluating whether the new chemical substance lacks sufficient information to quantify oral routes of exposure based on read-across from analogues or chemical category. Next, if the substance's structure is defined, a decision tree framework is applied, considering chemical class (e.g., inorganic metals, high-toxicity substances, or endocrine disruptors) and predicted bioaccumulative properties to exclude substances from the TTC approach. Cramer classification is determined using the OECD QSAR Toolbox. Combining this information with the predicted genotoxicity potential, an oral POD can then be determined with uncertainty factors (interspecies and intraspecies) included to account for variability. **Results:** This approach has been applied to several LVE new chemical submissions since May 2025. By adapting a well-established framework to fit within the constraints of an LVE submission process, this TTC approach helps quickly identify chemicals that can be reliably assessed using a threshold method. By comparing the TTC value with the exposure estimates, EPA can quantify potential risks of the new chemical substance when analogue data lack sufficient quantitative information. The process increases efficiency for LVE reviews, where more complete toxicological testing may not be warranted if TTC thresholds remain protective. **Conclusions:** Implementing a TTC-based workflow for LVE risk assessments can expedite decision-making and reduce testing burdens without compromising public health objectives. By systematically determining whether a chemical meets established criteria (e.g., known structure, non-excluded category, low exposure potential), regulators can apply transparent, consistent, and science-based thresholds. This approach supports the goals of minimizing animal testing while providing clear, and protective benchmarks for risk assessment. *These views are those of the authors and do not reflect views or policies of US EPA.*

PS 3341 A Human Health Risk Assessment Approach for Evaluating Carbon Nanotubes under the Toxic Substances Control Act (TSCA)

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Background and Purpose: The U.S. Environmental Protection Agency (EPA), Office of Pollution Prevention and Toxics (OPPT), New Chemicals Division (NCD) is responsible for conducting human health and environmental risk assessments for new chemical substances submitted under the Toxic Substances Control Act (TSCA) prior to their entry into U.S. commerce. In recent years, there has been an increase in carbon nanotube (CNT) new chemical submissions with high production volume, largely due to their potential use in electric vehicle batteries. Each new CNT has unique morphology and may contain metal impurities which are residual catalysts introduced during CNT production. CNT materials may contain a single layer or multiple layers of graphene. These nanomaterials exhibit high thermal and electrical conductivity, high surface area, and low density, and may differ in number of CNT walls, diameter, length, and agglomerate type. These properties may impact the toxicity of the CNT with respect to human health. CNT-related hazards potentially include respiratory effects, carcinogenicity, eye irritation, genetic toxicity, immunotoxicity, and/or cardiotoxicity. Moreover, metal impurities, (e.g., cobalt and nickel) introduce additional hazards that must be considered during risk assessment. Because TSCA does not mandate a minimum dataset for new chemical submissions, many CNT filings lack adequate case-specific data to address these potential hazards. The approach described here aims to deliver a more efficient, standardized framework for hazard characterization, deriving points of departure (PODs), and risk characterization. **Methods:** The human health risk assessment approach for CNTs includes both standard and fit-for-purpose hazard language based on percent content of specific metals within the new chemical submission. For each route of exposure, there are multiple candidates for CNTs and the individual metals available for POD selection. The criterion for each POD is dependent, in part, on the morphology of the CNT, for inhalation (tangled- vs. straight-type) or oral and dermal exposures (single- vs. multi-walled). Additional metal-specific PODs are considered depending on the percent of metal impurities in the CNT material. This approach for CNT inhalation exposure ensures consistent selection of the following risk calculation parameters: 1) Average Daily Dose (ADD) to reflect need to protect for chronic inflammation in the lungs, and 2) selection of a respirable fraction exposure value, if available, as local lung hazards are dependent on respirability. **Results:** EPA has received multiple CNT material

submissions since October 2023 that cumulatively represent over 400,000 kg/year of CNT materials that would enter US commerce. The application of this approach has resulted in decreased time to complete human health assessment of new CNT material submissions under TSCA. Notably, this approach also includes a POD for the assessment of tangled-type CNT, a component of the approach requested by stakeholders, that EPA also considers protective for cancer following inhalation exposures. **Conclusions:** This approach provides a more clear, consistent, and efficient method to identify hazards, select points of departure, and assess risks for a carbon nanotube submitted under TSCA, that considers both the submitted CNT dimensions and metal content. As additional toxicological information becomes available in this emerging chemical space, this approach will be updated as needed to stay up to date with the latest science. These views are those of the authors and do not reflect views or policies of the U.S. EPA or other respective organizations.

PS 3342 Utilizing Toxicokinetic and Mode of Action Information to Evaluate the Human Relevance of Ovarian Toxicity Data for 1,3-Butadiene

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Background and Purpose: 1,3-butadiene is a widely used industrial chemical associated with various adverse health effects, including cancer. 1,3-butadiene was designated by EPA as a high priority substance subject to risk evaluation in 2019 under the Toxic Substances Control Act (TSCA) and EPA finalized the risk evaluation in 2025. Prior assessments from EPA and other organizations had previously identified ovarian atrophy (observed in mice) as the most sensitive health effect. However, there is increasing evidence that ovarian toxicity may be a mouse-specific health effect. 1,3-Butadiene has a well-characterized metabolic pathway in rodents that demonstrates significant species-specific differences; exposure biomarkers indicate that overall oxidative metabolism is significantly higher in mice compared to rats or humans, resulting in much higher internal levels of the reactive 1,2,3,4-diepoxybutane (DEB) metabolite in mice. Given the evidence and DEB's proposed causal role in ovarian atrophy, in accordance with TSCA's requirements to consider the best available science, EPA re-examined whether ovarian toxicity represents the most appropriate critical effect for characterizing human hazard from repeated-dose exposure to 1,3-butadiene. **Methods:** To support EPA's Risk Evaluation for 1,3-Butadiene under the Toxic Substances Control Act, information on 1,3-butadiene toxicokinetics and mechanistic data for each endpoint were evaluated to determine whether species differences inform the human relevance of these non-cancer endpoints. EPA first considered published assessments for 1,3-butadiene including those by ATSDR, EPA Integrated Risk Information System (IRIS), California Environmental Protection Agency (CalEPA), and Texas Commission on Environmental Quality (TCEQ). EPA also conducted systematic review of the available literature in 2019 that was supplemented with newer information either provided to the Agency through public comments or otherwise identified by the Agency, with a focus on toxicokinetic and mechanistic information relevant to ovarian toxicity. EPA integrated the evidence from all of these sources to evaluate the mechanistic basis for the observed effects in each species (i.e., rats, mice, and humans). This process included comparing mechanistic and toxicokinetic evidence across species, analyzing whether a mode of action (MOA) could be established, and considering whether the mode of action was species-specific. EPA then determined whether the effects were quantitatively relevant to humans using the International Programme on Chemical Safety framework for analyzing the relevance of a noncancer mode of action for humans (IPCS, 2008). **Results:** Ovarian toxicity, observed exclusively in mice, acts through a well-defined MOA based primarily on the bifunctional metabolite DEB. While specific estimates of metabolite levels are variable, increased oxidative metabolism in mice and greater hydrolysis rates in rats and humans results in DEB levels up to two orders of magnitude greater in mice compared to rats and humans, respectively. Additionally, evidence on 1,3-butadiene and an analog suggests that mice are more toxicodynamically sensitive than rats to similar doses of epoxide metabolites. **Conclusions:** Therefore, EPA determined that while physiological, mechanistic, and toxicokinetic pathways are all qualitatively similar across species, the hazard values for ovarian atrophy in mice cannot be extrapolated to humans with reasonable certainty and it is not an appropriate endpoint for use in human health risk assessment.

PS 3342a Mechanistic In Silico Model of Global and Local Reactivity to Predict Ready Biodegradation in Small Molecules

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Background and Purpose: Readily biodegradable (RB) compounds degrade rapidly under aerobic conditions, typically within 28 or 10 days in standardized assays (OECD 301 and 302, respectively), indicating low environmental persistence. In regulatory risk assessments, RB substances do not undergo further testing, while non-RB classifications require additional evaluation, as they do not inherently indicate persistence. Computational models have emerged as complementary tools to enable rapid screening of large libraries, identifying potentially non-RB chemicals while accounting for physicochemical and mechanistic factors in microbial degradation. Although Structure-Activity Relationships (SARs) exist for predicting

biodegradation, current models have key limitations such as biases towards non-RB predictions; limited structural diversity in training data; and descriptors lacking mechanistic interpretability, impacting accuracy. To address this, the goal was to develop a Random Forest (RF) algorithm to classify compounds as RB or non-RB based on descriptors more closely aligned with the biodegradation process. This approach estimates degradation potential using curated 3D representations of neutral species with known classifications as inputs for QM/MM/MC simulations and linear-response (LR) calculations in aqueous solution; and a physics-based framework using Density Functional Theory (DFT) to capture molecular response to electron-density flux via frontier molecular orbital theory (FMOT) and an atomistic approach integrating FMOT with the Fukui function. **Methods:** Solute-solvent energetics for 919 molecules (460 RB and 459 non-RB) were computed using QM/MM/MC simulations in water and propane. Representative ground-state conformations were extracted from ensemble averages and saved as PDB files for further analysis. Physicochemical and pharmacokinetic properties were calculated via linear response methods and a rapid prediction tool. DFT optimizations and single-point calculations were performed using the mPW1PW91 functional with the MIDIX+ basis set. Atomistic charges, used to evaluate the Fukui index across atomistic positions, were performed with the Hirshfeld and natural population analyses (NPA). A binary decision tree classifier was developed using Random Forest (RF) with nested 5×3 cross-validation, combining global and site-specific SARs to emulate enzyme oxidation/reduction with a physics-oriented model based on DFT. RF models were saved as pickle files for reuse and distribution. **Results:** The Fukui f^+ and f^- indices were computed as local reactivity descriptors, indicating the propensity of atoms to accept or donate electron density and thereby identifying potential sites of nucleophilic or electrophilic attack. These descriptors, derived from QM/MM/MC simulations and DFT calculations, provide a mechanistic framework linking oxidation potential and molecular stability to biodegradation formed the computational basis of the predictive model, with a balanced test accuracy of 71% from a decision tree model based on Electron Affinity, HOMO-LUMO Gap, Electrophilicity Index, and solute-solvent interactions described by Coulombic and Lennard-Jones terms. **Conclusions:** A robust model can be developed using global and local electrophilic/nucleophilic susceptibilities to estimate RB potential more accurately than existing SAR tools by quantifying molecular response to electron-density flux, a proxy for redox processes in Phase I/II transformations *in vivo*. Descriptors related to electron loss, such as HOMO energy and localized Fukui indices (f^+ , f^-), are particularly relevant as they capture oxidation processes, which are critical because biodegradation often begins with electron withdrawal from electron-rich sites. Global descriptors like softness, chemical potential, and electrophilicity provide key indicators of susceptibility to electron transfer, while solvent accessibility descriptors such as localized surface area or volume ensure vulnerable sites are sterically accessible to enzymatic nucleophiles.

PS 3343 A New Approach Methodology (NAM) Based Workflow to Address Systemic Toxicity for Chemicals Including Fragrance Materials

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Background and Purpose: With increasing global restrictions on animal testing, New Approach Methodologies (NAMs) have gained traction for chemical safety assessment. While NAMs are effective for acute endpoints like skin and eye irritation, their application to systemic toxicity—requiring whole-organism context—remains limited. The European Partnership for Alternative Approaches (EPAA) recently started the NAM designation challenge which is a research exercise aiming to develop an appropriate risk management action based on the level of concern (high, medium and low) for systemic toxicity for chemicals based on NAM's. This study explores a NAM-based hazard classification system for chemicals, including fragrances, aiming to assign a level of concern (high, medium, low) based on toxicodynamic and toxicokinetic data. A secondary goal was to evaluate High-throughput Transcriptomics (HTTr) as a standalone assay for broad biological screening. **Methods:** A hazard classification system was developed using DNEL/ADI/RfD values for ~150 chemicals. The system categorizes chemicals into low, medium, and high concern. Low concern implies unrestricted use, medium requires refinement, and high concern indicates a need for risk management. An IVIVE workflow was built using HTTK PBPK models and ToxCast assay data to calculate Equivalent Administered Dose (EAD), which informs the concern level. Separately, HTTr using HepaRG cells was tested as a standalone bioactivity assay, integrated with PBPK modeling, and compared to ToxCast data. **Results:** Case studies showed >80% accuracy in predicting concern levels for industrial and fragrance chemicals. The workflow also performed well for unknown chemicals. HTTr results showed >95% concordance with ToxCast data, supporting its use as a standalone tool. **Conclusions:** This NAM-based workflow effectively categorizes chemicals by systemic toxicity concern level. HTTr shows promise as a standalone assay. The approach offers a screening-level assessment and a foundation for future NAM-based safety evaluations.

PS 3344 Modernizing Inhalation Testing and Assessment: Bridging Exposure Modeling with Regulatory Acceptance

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Background and Purpose: Evaluation of inhalation toxicity is a requirement in the regulation of agrochemicals by both state and federal government. New approach methodologies (NAMs) including *in vitro* and *in silico* methods are being utilized to assess inhalation (IHL) safety and to determine relevant human endpoints without the use of animals. Syngenta recently developed a decision framework, which incorporated US EPA's risk-based waiver guidance with NAMs to replace 28/90-day IHL toxicity studies. This framework was applied to a case study active ingredient (AI) and combined with exposure modelling in a weight of evidence assessment (WoE) to demonstrate a low level of concern via the IHL route. **Methods:** An IHL hazard assessment was conducted using a combination of *in vitro* data (MucilAir), *in vivo* acute IHL toxicity data, *in vivo* 5-day IHL tolerability studies, and comparative TK/PBK modeling, to identify the potential for IHL toxicity. Exposure was modeled using human occupational IHL exposures from the US EPA exposure activity worksheet, then forward dosimetry was used to generate a margin of exposure from the identified hazard data. **Results:** MucilAir and *in vivo* data demonstrated no concern for IHL portal of entry effects. PBK modeling predicted highest systemic exposure would occur from oral routes, which were adequately assessed in the toxicity database. Worst-case occupational IHL exposure of 5.2×10^{-3} mg/kg-day was estimated for handheld application to greenhouse crops, with standard PPE. This exposure was converted to an animal equivalent internal exposure using rat body weight and minute volume, resulting in the animal equivalent IHL exposure of ~0.02 mg/m³. In comparison to the IHL database, the margins of exposure were ~5000-fold, demonstrating no concern for IHL toxicity at the worst-case occupational exposure. Further, a NOAEC threshold of 2 mg/m³ passed for risk assessment and is below the minimum concentration that would be used in a 28-day IHL study. **Conclusions:** This case study utilized a WoE approach incorporating PBK modelling, *in vitro* and *in vivo* toxicity data, and reverse dosimetry to identify low IHL risk from the proposed uses of a case study AI. Using an exposure threshold approach, conducting further animal IHL toxicity studies would not provide any additional information to change the risk assessment conclusions, and would be an unnecessary use of animals. Currently, the implementation of these framework approaches and gaining regulatory acceptance remains at a crossroad.

PS 3345 Identification of functional amino acids in a tricellular tight junction modulator, angubindin-1

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Background and Purpose: The growing prevalence of central nervous system (CNS) disorders highlights the urgent need for new therapies. However, CNS drug development is severely hampered by the blood-brain barrier (BBB). While a major role of the BBB is to safeguard the CNS by preventing harmful or toxic substances from entering the brain, this protective function also limits the penetration of therapeutic agents into the CNS. Thus, the successful development of CNS therapeutics depends on strategies that enable efficient BBB translocation. At the same time, because BBB opening or dysfunction is implicated in CNS pathology, rigorous safety and efficacy evaluations are essential when attempting to cross or modulate the BBB. To overcome this barrier, our approach focuses on the modulation of tight junctions (TJs) between cerebral endothelial cells. TJs comprise two architectural subtypes: bicellular TJs (bTJs) formed at the contacts between two cells, and tricellular TJs (tTJs) formed where three cells meet. Although we have developed and evaluated more than ten TJ modulators, most of our modulators act on bTJs, and only one modulator, angubindin-1, specifically targets tTJs. However, angubindin-1 exhibits insufficient selectivity, binding not only to angulin-1 but also to angulin-3. In this study, we identified the essential amino acids of angubindin-1 that bind to angulins in order to improve binding specificity, reduce toxicity risk, and support the development of safer BBB-modulating therapeutics. **Methods:** We predicted the specific amino acids of angubindin-1 that bind to angulins by comparing the binding component of *Clostridium difficile* transferase (CDTb), which was reported to bind angulin-1 (Nat Microbiol, 2020:102-107). Therefore, we first estimated the angulin-binding amino acids of angubindin-1 based on the structural information of CDTb and performed alanine scanning. The angulin-binding ability was assessed by flow cytometry using cells expressing angulin-1/-2/-3. Furthermore, the effects on barrier function were evaluated by measuring the transepithelial electrical resistance (TER) and paracellular permeability using fluorescein (332 Da) and FITC-dextran (4 kDa, FD-4) in monolayer cultures of angulin-expressing cells. **Results:** First, we performed BLAST search based on the amino acid sequence of angubindin-1, namely binary iota toxin binding subunit Ib 421-664, and found its amino acid sequence of angubindin-1 was the most similar to that of CDTb, whose crystal structure has been solved. Among a total of 244 amino acids of angubindin-1, 197 amino acids (80.7 %) were identical to CDTb. Also, it is reported that CDTb binds to angulin-1. Then, we tried to estimate the amino acids residues involved in the binding of angubindin-1 to angulin-1 using CDTb 3D structure and performed functional domain mapping on 24 amino acids in angubindin-1 by alanine substitution. Flow cytometry analyses of angulin-1 and angulin-3-expressing cells demonstrated that reduced binding activity for the mutants L562A, L598A, E638A, V640A, Y643A, and K644A. Notably, the combined L562A/L598A/

E638A/V640A/Y643A/K644A (6A) mutant completely abolished its binding activity to angulin-3, but still had slight binding activity to angulin-1. In contrast, L562D/L598A/E638A/V640E/Y643A/K644A (4ADE) mutant showed a complete loss of binding to both angulin-1 and angulin-3. Furthermore, unlike angubindin-1, these mutants no longer induced a decrease in TER or an increase in paracellular permeability to small molecules such as fluorescein and FD-4 in angulin-1/-3 expressing cells. **Conclusions:** Our results suggest that the six amino acids mutated in the 6A and 4ADE mutants are involved in the mechanism by which angubindin-1 affects the barrier function of tTJ. Furthermore, the modification of these identified amino acid residues may enable the development of novel modulators that selectively target angulin-1 or angulin-3. Such modulators could serve as valuable tools for the precise regulation and assessment of barrier function in *in vitro* BBB models, thereby facilitating the elucidation of disease mechanisms associated with BBB dysfunction and the development of new therapeutic agents. In addition, these modulators are expected to contribute to the establishment of a reliable safety-evaluation platform, paving the way for their potential translation into real-world biomedical applications.

PS 3346 Understanding Mechanisms of Acute Iron Toxicity and Ferroptosis on *C. elegans* Energetic Activity

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Background and Purpose: Ferroptosis is an increasingly important mechanism for studying cell death in chronic diseases such as cancer and neurodegeneration. Iron(II) is the main iron species driving ferroptosis, and its role in generating lipid peroxides has been well-characterized. Iron(II) is mainly trafficked to mitochondria for functional use in heme biosynthesis and iron-sulfur cluster formation. These constructs are not only essential for respiratory function but are also necessary for other cellular enzymes to function. This implicates mitochondrial iron trafficking as an essential part of cellular health. **Methods:** We approached this by exposing *C. elegans* to ferroptosis inducers (RSL3) and suppressors (N-acetylcysteine and Ferritin II) for 24 hours. Worms were then assessed for pharyngeal pumping (feeding) rate as an energetic behavioral readout. We then used iron (2 mM) to induce ferroptosis and assess its impact on worm pharyngeal pumping rate. Next, we used pharmacological interventions (N-acetyl cysteine and Ferritin II) to rescue iron-induced ferroptosis. Next, we measured whole worm respiration in Clark electrode to recapitulate conditions observed with pumping rates. We followed this with an assessment of mitochondrial function with acute iron(II) exposure to better characterize the mechanisms of these behavioral deficits. We tested the hypothesis that iron(II) exposure impairs bioenergetic function in a dose-dependent manner. Mitochondrial isolates were exposed to a range of iron(II) doses (0, 1, 5, 10, 20, 50, 100 μM) and respiration measured in a Clark-type electrode chamber. After exposure and respiration measurement, mitochondria were collected for iron burden quantification by ICP-MS. To determine the mechanism for iron inhibition, isolated mitochondria were incubated with iron doses selected from the dose response (1 μM, 50 μM) according to different apparent effects on the mitochondrial respiration states. Incubated mitochondria were assessed by enzyme activity measurements of metabolic enzymes (citrate synthase and respiratory complexes I and II). **Results:** Ferroptosis inducers decreased pumping rate while suppressors rescued the behavioral phenotype back to baseline. Iron exposure decreased whole worm respiratory rate. Acutely exposing isolated mitochondria resulted in a dose dependent increase in state 4 leak respiration and a biphasic impairment of state 3 respiration. Two doses (1 μM, 50 μM) were selected for the enzyme activity assays to represent two potentially different mechanisms of iron toxicity. Metabolic enzyme activity showed impaired function with iron incubation. Mitochondrial iron burden was not different from the control. **Conclusions:** Overall, we report a biphasic dose-response of iron toxicity on mitochondrial bioenergetics that likely impacted ETC function and a role for ferroptosis in mitochondrial dysfunction.

PS 3347 Integrating high-content technologies with exposure and toxicokinetics for screening applications

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Background and Purpose: Screening prioritized molecules using high-content (HC) technologies can help identify potential toxicological concern at earlier stages in the development pipeline. Leveraging HC technologies such as cell painting, transcriptomics, and metabolomics applied to *in vitro* test systems allows for broad evaluation of bioactivity elicited by pesticidal MOA-unknown or data-poor chemicals. To integrate these bioactivity screening data with predictive exposure information, bioactivity:exposure ratios (BERs) were applied allowing for risk-based prioritization. **Methods:** Two case studies are presented herein summarizing the evaluation of novel chemicals currently in the development pipeline. Chemicals X and Y are unregistered pesticide active ingredient candidates with limited toxicology data. Prior to *in vitro* testing, a cheminformatics approach to evaluate the properties of chemicals for any characteristics that may confound aqueous based *in vitro* screening in plasticware was conducted. Cell painting, transcriptomics, and metabolomics assays

in addition to cytotoxicity evaluation were conducted after 24 hours of treatment in six human cell lines: U20S (bone sarcoma), SHSY5Y (neuroblastoma), iCell (cardiomyocyte), HepaRG (hepatocyte), HEK293 (embryonic kidney), and H295R (adrenal carcinoma). All datasets generated were analyzed using the appropriate, assay-specific analysis pipeline to derive a representative point of departure (POD). External exposure predictions were generated for dietary (food and , drinking water) and occupational scenarios using standard regulatory models using predictive parameters. Physiologically based kinetic (PBK) modeling was conducted to translate external exposures into systemic organ-level internal doses, accounting for potential clearance and accumulation following repeat dosing and population variability. **Results:** The two case studies combining exposure predictions and bioactivity data from several HC assays were developed with the goal of establishing an adaptable approach for addressing chemicals with limited toxicity data and exposure information. The HC assay results were generally in agreement generating PODs within one order of magnitude. Exposure predictions also spanned at least one order of magnitude given the different scenarios. PBK modeling to evaluate organ-level BERs helped narrow down where any toxicological concern may need follow-up. For the most part the exposure ranges were sufficiently lower than the bioactivity dose ranges supporting safety margins for our case studies. The case studies also demonstrate how to overcome challenges whether it be chemical-properties that confound exposure modeling due to limited solubility or limited bioactivity data due to minimal biological response in the assay systems. Overall, by addressing these challenges, we demonstrate that a BER approach can not only help candidate selection but also provide actionable insights regarding which toxicological concerns should be prioritized as follow-up. **Conclusions:** Our use of *in vitro* test systems paired with HC technologies and computational modeling support the development of a predictive risk assessment framework. This approach helps provide insights into potential hazard(s), inform the development of risk-based modernized testing programs, rationalize animal studies earlier in the research and development pipeline, and add confidence in regulatory use of NAMs approaches.

PS 3348 Enabling single-cell transcriptomics from samples with degraded and low-input RNA using TempO-LINC

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Background and Purpose: Single-cell and single-nucleus RNA sequencing (scRNA-seq/snRNA-seq) technologies enable detailed characterization of cellular heterogeneity, identification of rare cell types, and mapping of transcriptional states in complex tissues. However, their application to many biomedical and clinical samples is limited by RNA degradation, fragmentation, or inherently low RNA abundance. Peripheral blood mononuclear cells (PBMCs) are a common example of low RNA and highly heterogeneous cell populations, while formalin-fixed paraffin-embedded (FFPE) tissues, despite representing an invaluable archive of human samples, often contain degraded RNA of poor quality and quantity due to crosslinking during fixation and embedding. To overcome these limitations, we developed TempO-LINC, a targeted, instrument-free single-cell transcriptomics assay designed for high-throughput gene expression profiling with strong tolerance to low RNA integrity. Here, we report that TempO-LINC enables robust gene expression profiling and rare cell subtype detection in cryopreserved human PBMCs and dissociated FFPE tissues from human and mouse. **Methods:** The TempO-LINC assay utilizes a combinatorial split-pool barcoding approach to uniquely index RNA molecules in hundreds to thousands of fixed cells or nuclei. Cryopreserved PBMCs from three donors were fixed using the TempO-LINC Fixation Kit and profiled with the human whole transcriptome v2.1 probe set. FFPE blocks from human lung and mouse brain were sectioned, two 30 μm thick sections per block, deparaffinized, enzymatically dissociated, filtered, and then processed with the TempO-LINC Fixation Kit. TempO-LINC single cell expression profiling was performed on FFPE lung and brain using the TempO-Seq human whole transcriptome v2.1 and mouse whole transcriptome v1.1 probe set, respectively. Data processing and visualization were performed using the open-source Python Scanpy and R Bioconductor packages for quality control, clustering, and cell type annotation. **Results:** Analysis of 12,027 PBMCs revealed 16 transcriptionally distinct cell populations. In addition to the major classes of blood and immune cell subsets identified, such as CD14+ and CD16+ monocytes, T and B lymphocytes, TempO-LINC also identified rare blood cell populations including hematopoietic stem/progenitor cells (HPSCs) and proliferating CD4+ T cells, demonstrating high sensitivity in low-RNA contexts. For FFPE samples, TempO-LINC successfully generated single cell expression profiles with competitive sequencing performance: average mapping rate ranges from 71% to 93% with close to 1,000 genes detected per cell. UMAP data demonstrated clearly defined cell subtypes from dissociated FFPE human lung and mouse brain tissues. **Conclusions:** The TempO-LINC single-cell transcriptomics assay enables high-resolution gene expression profiling across heterogeneous, low-RNA PBMCs and FFPE samples without the need for specialized instrumentation. These results highlight the assay's compatibility with degraded RNA and its ability to recover rare cell types, establishing TempO-LINC as a powerful scRNA-seq platform for translational, toxicological, and clinical research. By extending single-cell analysis to billions of archived FFPE specimens and previously inaccessible sample types, TempO-LINC unlocks new opportunities in precision medicine, biomarker discovery, and mechanistic toxicology.

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Background and Purpose: Traditional *in vivo* bioassays, while standard, are resource-intensive and ethically challenging. *In vitro* assays offer efficiency but lack physiological realism. *In vitro* to *in vivo* extrapolation (IVIVE) bridges this gap by converting *in vitro* activity concentrations into *in vivo* dose estimates. This study explores the potential of using the IVIVE method, supported by physiologically based kinetic (PBK) modeling, to derive oral equivalent doses (OEDs) that can serve as points of departure (PoDs) in chemical risk assessment. The objective of this study was to determine whether the OED derived using the IVIVE approach can serve as an alternative to the PoD for assessing short-term chemical effects. **Methods:** We evaluated the applicability of the IVIVE approach in quantitative chemical risk assessment by comparing OEDs with No/Lowest Observed Effect levels (N/LOELs) and Benchmark Doses (BMDs) from mouse uterotrophic bioassays conducted by the Japanese Ministry of Health, Labour and Welfare (MHLW). We selected five chemicals: 2,2',4,4'-tetrahydroxybenzophenone (THBP), daidzein (DZ), 4-cumylphenol (4CP), heptyl 4-hydroxybenzoate (HHB), and 2-ethylhexyl 4-hydroxybenzoate (2EHB) from the MHLW Priority List for endocrine activity. These chemicals were carefully chosen based on the availability of chemical-specific parameters, high-throughput *in vitro* estrogen receptor (ER) pathway assays, and mouse uterotrophic bioassays. The chemical-specific parameters included molecular weight, topological polar surface area (TPSA), intrinsic hepatic clearance (CL_{int}), and octanol/water partition coefficient (log K_{ow}). Absorption rate constants were estimated from TPSA. Tissue partition coefficients and clearance rates were derived from log K_{ow} and CL_{int}, respectively. The mouse six-compartment PBK model was validated using bisphenol A (BPA) data. OEDs were calculated by converting *in vitro* activity concentrations at half maximum response (AC₅₀) and activity concentrations at cutoff (ACC) values using IVIVE conversion factors. Critical key events (receptor dimerization, dimer-DNA binding, RNA transcription, protein production, or ER-induced proliferation) were statistically identified by comparing OED distributions across ER pathway steps to derive OED_{crit} values (OED for critical key event). The BMDs were calculated from the mean and standard deviation of uterine weights for each treatment group using the PROAST web application. **Results:** Of the five chemicals, three (THBP, DZ, and 4CP) showed positive estrogenic activity in mouse uterotrophic bioassays, while two (HHB and 2EHB) were negative. The PBK model validated using BPA data showed acceptable predictive performance. The critical key events were identified as protein production for THBP, HHB, and 2EHB, DNA binding for DZ, and receptor dimerization for 4CP. For the three chemicals that exhibited positive estrogenic activity, the BMD₅₀s were calculated as 480 mg/kg/day (THBP), 550 mg/kg/day (DZ), and 1000 mg/kg/day (4CP). The average and absolute average fold errors of the ratio of OED_{crit} values from the *in vitro* AC₅₀ values to the LOEL and BMD₅₀ of uterotrophic bioassays were less than 2. Moreover, the OEDs of HHB and 2EHB were higher than 1000 mg/kg/day, the highest dose of the bioassay. The results support the potential of IVIVE-derived OEDs as alternatives to PoDs in risk assessment. Variability in assay data and uncertainty in some parameters highlight the need for further refinement. **Conclusions:** The study demonstrates that the IVIVE approach, using a PBK mouse model, can successfully convert activity concentrations from *in vitro* assays into OEDs that are comparable to *in vivo* bioassay endpoints such as LOEL, NOEL, and BMDs. The study concludes that while IVIVE methodology is largely established, further research is needed to validate the approach across a broader range of chemicals, improve identification of critical key events, and improve the reliability of OEDs derived from *in vitro* data for quantitative human health risk assessment.

PS 3350 Multi-Omics Integration of Transcriptomics and Phenomics Reveals Complementary Molecular and Morphological Signatures of Compound Response and Toxicity

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Background and Purpose: Understanding how bioactive compounds affect cellular systems is critical for developing safer, more effective chemical products and for reducing late-stage failures in drug discovery pipelines. Cost-efficient morphological and gene expression profiling enable high-throughput phenotypic analysis for genetic and small-molecule perturbation screens, offering early clues into compound mechanism of action and toxicity. Importantly, these complementary approaches capture distinct yet interrelated layers of cellular response, with great promise to enhance biological interpretation when combined. Historically, comparative studies have relied on legacy probe-based transcriptomic platforms such as L1000. Here, we introduce Total DRUG-seq, an enhanced gene expression profiling method that captures the whole transcriptome with full-length coverage, paired-end alignment, and high multiplexing capability—delivering deeper biological insights and expanding discovery in large-scale screens. **Methods:** We applied Total DRUG-seq and Cell Painting to profile gene expression and cell morphology across two datasets comprising three human cell lines U2OS (osteosarcoma), and hepatocarcinoma derive HepG2 and HepaRG, key models for investigating *in-vitro* drug-induced liver

injury (DILI). In the first dataset, 82 compounds from the JUMP-MoA reference collection were tested to assess the ability of each assay to uncover mechanistic biological insights. The second dataset included 58 treatments with balanced DILI-positive and -negative outcomes. To assess the combined potential of Total DRUG-seq and Cell Painting, we implemented data integration strategies and compared the added value of multi-omics integration against single-modality analyses for clustering and classification of treatments, mechanisms of action, and DILI outcomes. **Results:** Our findings show that combining transcriptomic and morphological profiles provides more biologically coherent insights into drug activity than either modality alone. Unsupervised analyses revealed that integration enhances clustering quality across cell types, outperforming individual assays. Total DRUG-seq showed superior treatment clustering for HepG2 cells, while Cell Painting performed better in U2OS cells. Supervised models further confirmed that integrated multi-omics data consistently outperformed individual assays in predicting compound mechanisms of action and DILI outcome. **Conclusions:** These results underscore the synergy between transcriptomic and morphological profiling and highlight the value of multi-omics strategies for characterizing cellular responses and enabling early detection of toxicity across diverse compound classes.

PS 3351 A Weight of Evidence Framework for the Application of New Approach Methodologies (NAMs) in Genotoxicity Risk Assessment

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Background and Purpose: Genotoxicity assessments are essential for public health, especially for data-poor chemicals where traditional *in vivo* testing is limited by cost and ethics. Standard assays (e.g., Ames, micronucleus) often lack mechanistic and dose-response insights. New Approach Methodologies (NAMs), including QSAR, read-across, high-throughput *in vitro* screening, DNA damage biomarkers (e.g., γH2AX, p53), and targeted *in vivo* endpoints, offer efficient alternatives. This framework integrates NAMs into a weight-of-evidence (WoE) approach to assess mutagenicity, aneugenicity, and clastogenicity, while supporting potency and mode-of-action (MoA)-based dose-response analysis. It addresses key adverse outcome pathways (AOPs), such as oxidative damage, alkylation, bulky adducts, spindle disruption, and topoisomerase inhibition, emphasizing DNA repair and threshold potential for non-mutagenic genotoxins. **Methods:** Tier 1 (Data Evaluation) reviewed existing studies for GLP compliance, reliability, and relevance to parent compounds, major metabolites (>10%), or degradants. Endpoint consistency and biological plausibility guided conclusions. Tier 2 (NAMs Prediction) aimed to fill data gaps via: (1) *in silico* QSAR tools (OECD Toolbox, Derek Nexus) to identify structural alerts; (2) read-across from analogs (Tanimoto ≥0.7, shared MoA); (3) integration of high-throughput *in vitro* data (Tox21/ToxCast) with IVIVE using PBK modeling to derive human equivalent administered doses (EADs). Tier 3 (Testing Recommendation) prioritized dose-responsive *in vitro* assays (e.g., MultiFlow®, ToxTracker, 3D HepaRG comet assay) before *in vivo* confirmation. Tier 4 (Dose-Response Evaluation) established points of departure (PoD) using BMD, NOGEL, or TTC. MoA analysis distinguished linear extrapolation for DNA-reactive mutagens (e.g., aflatoxin B1) from threshold models for aneugens (e.g., colchicine) and indirect clastogens (e.g., EMS, MMS). **Results:** The framework robustly identified genotoxic potential and derived protective EADs. For carbendazim, *in silico* analyses flagged benzimidazole alerts linked to tubulin binding; read-across to thiophanate-methyl confirmed aneugenicity via spindle poison MoA; and IVIVE of Tox21 microtubule disruption data yielded an EAD within 3-fold of EPA's chronic reference dose. For diuron, urea structural alerts suggested clastogenicity; read-across to linuron and monuron supported DNA damage via reactive metabolites; and the Tox21-derived EAD was 5-fold below the EPA chronic reference dose, ensuring protectiveness. In a case study using spirotetramat as a negative control, no structural alerts for genotoxicity were identified, and the read-across analysis indicated a non-mutagenic profile. NAMs integration achieved >90% concordance with known outcomes in a blinded validation. Dose-response analyses supported non-linear thresholds for both compounds: carbendazim via mitotic checkpoint saturation, diuron via repair-saturable oxidative stress. Underlying model performance (mutagenicity QSAR sensitivity = 88%, specificity = 92%; Tox21 DNA damage pathway specificity >85%) validated predictive reliability. The framework correctly waived further testing for low-concern analogs and prioritized mechanistic follow-up for equivocal cases. **Conclusions:** This transparent, iterative, and mechanistically anchored framework enables defensible, animal-sparing genotoxicity assessments for data-poor chemicals. By integrating exposure context, NAMs predictability, and MoA-driven dose-response, it guides testing waivers, prioritization, and risk characterization with regulatory rigor. The case studies demonstrate practical utility in deriving human-relevant, protective potency estimates using only publicly available NAMs data. This approach supports global efforts to reduce vertebrate testing under ICH, EPA, and EFSA NAM initiatives, while ensuring robust public health protection through scientifically sound, efficient decision-making.

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Background and Purpose: Our goal is to develop a simple non-animal approach to evaluate the fate of synthetic vitreous fibers (SVF) in the GI tract. SVF are used in insulation and building products as components of thermal and acoustic insulation systems. The SVF used in these applications include glass wool fibers (GW), mineral wool fibers (MW) and alkaline-earth silicate (AES) fibers. Dissolution testing of SVF are typically directed toward evaluating the biopersistence of SVF in the lungs to estimate the clearance half time. Fiber entry into the GI system begets a second question, that of what quantity of dissolved ions may become bioavailable after ingestion? Means of developing this data using dissolution studies can be a facilitator for evaluating risk. In this study, we test static and dynamic dissolution methods to evaluate the extent of dissolution and quantify ion release, leveraging established GI simulant fluids and test procedures designed to evaluate pharmaceutical release rates. **Methods:** A sample of each fiber type (GW, MW, AES) was obtained from commercial sources. Fibers were prepared for testing by low-temperature ashing in oxygen plasma to remove organic material, and in some cases (MW, AES) passed through a sieve to remove gross non-fibrous material. A static dissolution test was conducted with both simulated fasting state intestinal and gastric fluids (FaSSIF, FaSSGF). In all cases ~100 mg of fiber was exposed to 100 mL of simulant fluid at 37 C, for either 2 hrs (FaSSIF) or 6 hrs (FaSSGF). After the exposure time, the supernatant was extracted, filtered and analyzed for pH and ion content (ICP-OES). The dynamic test was based on the USP Apparatus 4, operated in open circuit mode. Either 50 mg or 200 mg of each fiber was exposed first to the FaSSGF fluid (2 hrs exposure) and then FaSSIF fluid (6 hrs exposure). Fluids were introduced to the fiber at 2 mL/minute at 37 Celsius. A 0.22 µm filter in line on the discharge side of the cell to ensure no fiber carryover. There was no flush between the fluids which allowed some mixing. The discharged fluid was periodically sampled via an automated fraction collector and analyzed for pH and ion content (ICP-OES). **Results:** The static dissolution tests showed differences in the extent of fiber dissolution. In the low pH (1.6) FaSSGF, less than 5% of the mass of the GW and AES samples dissolved, with > 70% of the stone wool dissolved. Slight increase in pH occurred with the SW sample (+ ~0.3 pH units) concurrent with significant dissolution of the fibers, suggesting slight neutralization of the fluid by the dissolved ions. In the neutral pH (6.5) FaSSIF fluid, less than 5% by mass was dissolved from any samples over the 6 hour exposure. The dynamic test showed that dissolution of the SW (~30% by mass) was significantly higher than the GW or AES. Sensitivity was noted to sample mass as the lower sample load showed a higher extent of dissolution (~2x). Solution pH, in contrast to the static test, was stable for all fibers and fluids. B₂O₃ release from GW was observed in both FaSSIF and FaSSGF with the total release (~10% of the total) almost 5x greater than the SiO₂ release. **Conclusions:** We demonstrated successful proof of concept around combining biomimetic GI fluids, a static dissolution test, and a dynamic, flow-through test, to develop understanding of the extent of dissolution, and ion release from SVF. Results align well with classic understanding of the impact fiber chemistry, pH, and surface area/volume ratio on SVF dissolution profiles. These methods have potential to inform the fate of fibers entering the GI tract as well as the quantity and rate of elution of ions of potential interest, facilitating weight of evidence and similar evaluations of hazard and risk.

PS 3353 Integrating *in Vitro*, *in Vivo*, and Biomimetic Chromatography Data to Enhance Chemical Toxicity Predictions

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Background and Purpose: Accurate toxicity prediction is essential for drug development, as highlighted by the urgent need for rapid screening during the COVID-19 pandemic. Traditional toxicity assessment relies on *in vitro* and *in vivo* assays, but these often yield discordant results for the same compound due to differences in methodological metabolic competency and in receptor or pathway expression profiles. This leads to unnecessary animal testing or premature elimination of safe candidates. To bridge these gaps, we integrated biomimetic chromatography—a technique that uses reverse-phase liquid chromatography coupled with biologically relevant stationary phases to estimate *in vivo* partitioning and transport—into conventional screening approaches to improve mechanistic understanding and accelerate drug safety assessment. **Methods:** We screened 322 compounds from the Cayman Antiviral Screening Library using 3 complementary approaches. *In vitro* assessment used the AsedaSciences SYSTEMETRIC Cell Health Screen (CHS) with HL60 cells to measure 12 acute cellular stress readouts (including ROS, and mitochondrial function) across 10 concentrations (5 nM - 100 µM) with machine learning predicted human toxicity risk from biological fingerprints. *In vivo* screening exposed individual zebrafish embryos to compounds at 100 µM starting at 6 hours post fertilization (hpf), assessing mortality and embryonic photomotor response at 24 hpf, plus 16 morphological endpoints and larval photomotor response at 120 hpf. Active under-went definitive testing across 10 concentrations (5 nM - 100 µM) and was further screened for morphological and behavioral effects, reported as benchmark doses or lowest effect levels, respectively. Biomimetic chromatography measured compound partitioning using a C18 column at 3 pH values, immobilized artificial membrane

(IAM), human serum albumin (HSA) and α-1 acid glycoprotein (AGP) columns. Retention was reported as the chromatographic hydrophobicity index (CHI) for C18 and IAM and partitioning coefficients for HSA and AGP (log_{K_{HSA/AGP}}, respectively). **Results:** Of the 322 antiviral compounds, 27% (87) were positive hits in the CHS, and 31% (101) showed morphological effects in zebrafish, with only 44 compounds positive in both assays. Notably, 43 CHS hits were non-toxic in zebrafish, while 57 compounds that were negative in CHS caused zebrafish morphological changes, highlighting discordance between the two platforms. Biomimetic chromatography helped explain these differences. Binding to IAM, HSA and AGP columns positively correlated with Cell Health Index, and a multiple linear regression model using biomimetic binding coefficients for these parameters predicted CHS toxicity (p < 0.05) For zebrafish endpoints, CHI at pH 7.4 (p < 0.005) and 10.5 (p < 0.0005), and log K_{HSA} (p < 0.05) were negatively correlated with any-effect BMD₁₀ and LELs in the embryonic photomotor assay. In larval photomotor responses, CHI at pH 10.5 and log K_{AGP} (p < 0.05) correlated with LELs during the light phase. **Conclusions:** Biomimetic chromatography effectively bridges the gap between *in vitro* and *in vivo* toxicity data, helping explain why 27% of compounds show discordant results between assays. Integrating biomimetic partitioning data with *in vitro* screening significantly improved the prediction of *in vivo* zebrafish toxicity. This combined approach offers a practical strategy to reduce reliance on animal testing while advancing the development of safer compounds, with applications across pharmaceuticals and environmental toxicology.

PS 3354 Extraction-Free TempO-Seq Profiling of Dried Blood Spots for Comprehensive Transcriptome Analysis

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Background and Purpose: TempO-Seq gene expression signatures provide a robust approach for evaluating mode of action, and numerous studies have demonstrated their use in high-throughput transcriptomic dose-response analyses for safety and efficacy assessment *in vitro* and *in vivo*. Building on these applications, we recognized that a fingerstick dried blood spot (DBS) assay capable of measuring gene expression signatures, miRNAs, and alternatively spliced isoforms would enhance the platform's diagnostic and monitoring potential. We therefore developed a TempO-Seq workflow to quantify mRNA, miRNA, and splicing isoforms from DBS samples. **Methods:** Whole transcriptome and alternative splicing analyses were performed using the TempO-DBS assay, while the TempO-Seq whole miRNome miRNA assay was used to profile miRNA expression. Whole blood-specific attenuators were incorporated to reduce background signal from highly expressed blood genes. To demonstrate the capabilities of this multi-omic platform, we analyzed samples from Alzheimer's disease (AD) patients and from a donor receiving Zepbound, a weight-loss drug. All samples were collected under IRB-approved protocols. **Results:** Using the whole transcriptome V2.1 panel, we not only identified a classifier of AD, but also identified key genes known to be associated with AD; S100A8, MMP9, and MME. This demonstrates the disease relevance of blood-derived data for a neurodegenerative disease. Using the TempO-Seq whole miRNome miRNA assay, we profiled DBS samples from AD patients and controls. Functional enrichment analysis revealed significant differences in miRNAs associated with aging and inflammation pathways, both linked to AD pathophysiology. DBS samples from three donors were also analyzed using the TempO-Seq DBS assay run with a sentinel splicing panel, which successfully distinguished donors based on alternative splicing profiles alone. Finally, a longitudinal study of a donor self-administering Zepbound injections revealed treatment-associated gene expression changes detectable with the TempO-Seq-DBS assay. Collectively, these results demonstrate that the TempO-Seq platform enables simultaneous measurement of mRNA, splicing isoforms, and miRNA from a single DBS sample. **Conclusions:** Fingerstick DBS collection combined with TempO-Seq multi-omic assays enables self-sampling, eliminates biohazard concerns upon drying, and allows specimens to be safely mailed for analysis. This approach supports remote monitoring of safety, exposure, and disease incidence or risk. Because the TempO-Seq DBS mRNA and miRNA assays can be performed from the same DBS sample, they provide a practical and scalable solution for diagnosis of diseases, evaluating individual responses to drugs, disease progression, and potential adverse effects following exposure.

PS 3355 Phenotype-Anchored Transcriptomic Signatures of Developmental Neurotoxicity (DNT) Compounds in Zebrafish

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Background and Purpose: The NIEHS Division of Translational Toxicology established a 91-compound library to support the development of a battery of assays aimed at elucidating pathways underlying developmental neurotoxicity (DNT). Zebrafish (*Danio rerio*) is a valuable *in vivo* model that offers vertebrate-level biological complexity while reducing reliance on mammalian testing. In this study, we evaluated a subset of 27 compounds from the DNT library, spanning diverse chemical classes including polycyclic aromatic hydrocarbons (PAHs), organophosphates, and

pyrethroid pesticides. Using whole transcriptome sequencing of exposed embryos at concentrations known to cause developmental malformations, we sought to characterize early transcriptional responses that may contribute to the initiation of adverse developmental outcomes. **Methods:** Embryonic zebrafish were statically exposed to DNT compounds at concentrations ranging from 0 to 100 μM in 96-well plates (3 replicate plates, 8 concentrations). For each compound, a concentration-response curve was generated to determine the concentration at which approximately 80% of larvae exhibited morphological malformations at 120 hpf (EC_{80}). Next, embryos were exposed to each compound at its estimated EC_{80} . At 48 hpf, embryo samples (5 pools of 8 embryos) were collected for RNA sequencing using Lexogen's CORALL Total RNA-Seq V2 platform. In parallel, holdback embryos ($n = 16$) were allowed to develop to 120 hpf for assessment of mortality and nine additional predefined phenotypic endpoints. Differential expression and enrichment analyses were performed to identify disrupted biological pathways. Sciome's Transcriptomic Read-Across (TRA) tool was then used to compare the differentially expressed gene (DEG) profiles of the DNT compounds against a large, curated zebrafish dataset from the NCBI Sequence Read Archive. This approach identified additional chemicals outside the current study with statistically similar expression patterns and also enabled comparisons within the current dataset, revealing transcriptional similarities among the 27 DNT compounds. **Results:** The 27 DNT compounds exhibited a wide range of potencies, with estimated EC_{80} values spanning from 0.1 to 100 μM . For example, TCDD at 0.1 μM induced malformations in 62% of zebrafish larvae, whereas dibenz[a,c]anthracene required 100 μM to produce a similar level of malformation. When compared with vehicle control (0.5% DMSO), the number of differentially expressed genes also varied considerably among compounds, ranging from 33 to 994. Pathway analysis revealed distinct enrichment patterns across chemical classes. For the five organophosphates, pathways related to retinoic acid signaling and skeletal development were highly enriched. Among the five PAHs, enriched pathways were primarily associated with xenobiotic metabolism and epidermal development. In the seven biocides, most pathways were linked to immune responses and tissue remodeling processes. TRA analysis identified additional chemicals outside this study that share similar transcriptional profiles. For instance, benzo[k]fluoranthene (a PAH not included in the DNT library) showed a high similarity score with dibenz[a,c]anthracene, dibenz[a,h]anthracene, and TCDD, suggesting a potential common mode of action with these three DNT compounds. The analysis also revealed transcriptional similarities within the 27 DNT compounds. Permethrin and deltamethrin, for example, are both pyrethroid insecticides and grouped together based on their TRA scores. **Conclusions:** Overall, this study demonstrates the utility of phenotype-anchored transcriptomics for identifying early molecular events underlying developmental neurotoxicity in zebrafish across 27 compounds. This approach highlights the value of comprehensive zebrafish transcriptomic databases as tools for discovering chemical-induced adverse effects and elucidating mechanisms of toxicity.

PS 3356 Statistical analysis of multi-endpoint phenotypic screening in the planarian *Dugesia japonica* provides unique value for developmental neurotoxicity testing

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Background and Purpose: There is an urgent need to replace, refine, and/or reduce ("3Rs") vertebrate toxicity testing. This is especially challenging for neurotoxicity and developmental neurotoxicity (DNT) where the functional relevancy of adverse outcomes needs to be assessed on the whole organism. Invertebrates can provide organismal context and promise to augment existing *in vitro* approaches. To evaluate whether planarian phenotypic screening provides complementary information to existing DNT models, we used new statistical analysis methods to re-analyze published planarian data on the NTP 87-compound library (NTP87). The NTP87 library consisted of known and suspected developmental neurotoxicants from five toxicity domains (pesticides, industrials, drugs, flame retardants, polycyclic aromatic hydrocarbons) and five *in vitro* negative controls. **Methods:** We quantified eight additional behavioral endpoints from the original planarian data for a total of 26 readouts on days 7 and 12 of exposure, evaluated at 5 log-scale concentrations (10 nM-100 μM). Benchmark concentration (BMC) modeling replaced lowest-observed-effect-level analysis. To provide insight into systems-level toxicity, we also calculated a concentration-independent multi-readout summary measure using weighted Aggregate Entropy (wAggE). We compared the planarian BMC data to mammalian *in vitro* data and to developing zebrafish data from independent screens of the NTP87 library that were analyzed using the same BMC pipeline. **Results:** The addition of new endpoints provided additional sensitivity without the cost of more experiments, allowing for the detection of more chemical hits with increased potency and more developmentally-specific hits than in our original analysis. The adaptation of BMC modeling increased screen robustness and allowed for direct comparison of our results with existing *in vitro* and developing zebrafish data available on DNT-DIVER. The wAggE analysis provided contextualization of the severity of behavioral phenotypes and thus complemented the potency determinations from BMC. Planarian and developing zebrafish screens showed similar sensitivity. Regenerating planarian hits helped correctly identify known neurotoxicants of the NTP87 library. Hierarchical clustering showed that organismal models, human neuron outgrowth models, and rat primary cortical neuron firing models were the main contributors to the NTP87 DNT battery's information content, while other human cell culture models added no

extra information. **Conclusions:** Neurotoxicity and DNT testing requires a battery approach, leveraging complementary strengths of multiple systems to approximate the complexity of human brain development, function, and metabolism. The systems comparison presented here emphasizes the importance of including organismal models, such as developing zebrafish and planarians, within a DNT test battery to measure DNT on a systems level while also accounting for xenobiotic metabolism. Using this battery approach, chemicals with more NT/DNT hits across new approach methods from various levels (*in vivo*, *in vitro*, *in silico*) can be prioritized as chemicals of higher interest for further study.

PS 3357 Characterizing lineage specific drug induced injury responses and sensitivity using a mechanism based gene co-expression network approaches and fluorescent reporters.

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Background and Purpose: Current chemical risk assessment relies heavily on animal testing. There is an increasing desire to replace animal based safety testing with safer, cheaper and more human relevant *in vitro* methods. Induced pluripotent stem cell (iPSC) derived *in vitro* models show great promise to replace or supplement animal testing as a human relevant test systems. However, uncertainty remains on the accurate prediction of human toxicity. We have used a systematic toxicogenomics screening of iPSC-derived hepatocyte, renal proximal tubular and cardiomyocyte lineages to define common and different transcriptional programs that represent adverse response with respect to clinical liabilities for hepato-, nephro- and cardio-toxicity. **Methods:** More than fifty compounds were selected from FDA rank lists based on known relation to liver, kidney and/or heart adverse events and covering a wide range of toxicological mechanisms. iPSCs were differentiated into hepatocyte-like cells (HLC), proximal tubule-like cells (PTLC) and cardiomyocyte-like cells (CMLC). All lineages were exposed to the full selection of compounds in identical concentration ranges based on simulated human Cmax values. Cell death was measured as a cell-fate readout through confocal imaging 24 hours after exposure, directly followed by sample collection for high throughput whole transcriptome sequencing (TempO-Seq). Differential gene expression analysis was performed using DESeq2 followed by weighted gene co-expression network analysis (WGCNA) to identify gene networks to facilitate biological interpretation. Sensitivity per compound was determined by concentration response modeling and subsequent benchmark concentration analysis using BMD express. Furthermore, the defined co-regulated gene networks were used to identify potential stress response pathway related biomarkers to be used to generate fluorescently tagged reporter cells for quick insight into stress pathway activation. Several identified reporter options for the oxidative stress response pathway, inflammation pathway, unfolded protein response pathway and DNA damage response pathway were generated and validated using known pathway activating compounds. Following differentiation cells were exposed to a wide dose range of pathway activators and fluorescent protein expression was captured after 24 hours through confocal microscopy. **Results:** Evaluation of the transcriptomics results showed variations in transcriptional responses between lineages. Compounds showed concentration responsive transcriptional changes that differed between HLC, PTLC and CMLC. Several compounds showed transcriptional changes in all lineages, although differences in gene networks and point-of-departure were observed. From the data, 13 representative genes have been identified representing four toxicity-relevant stress response pathways based on dose dependency and specificity. Using CRISPR-based genome engineering and stringent selection and validation, we established nine functional iPSC reporter lines. From these 9 we selected one reporter per pathway was as most representative for use in follow up experiments. ICAM1 for inflammation, CDKN1A for DNA damage response, HSPA5 for unfolded protein response and SRXN1 for oxidative stress response. These reporters showed a dose dependent increase in fluorescent signal in response to activation of the corresponding pathway in the HLC, PTLC and CMLC lineages. Initial data on the functionality of these reporters to assess the activity of selected DILI, DIKI and DICI compounds will be presented. **Conclusions:** Using toxicogenomics, we demonstrate commonalities and differences in the response of DILI, DIKI and DICI compounds in HLC, PTLC and CMLC lineages. Selected biomarkers for critical toxicological pathways were successfully integrated in iPSC reporters and demonstrated functionality with respect to drug-induced adversity liability detection.

PS 3358 *C. elegans* as a model to detect negative-feedback effects on conserved developmental signal transduction pathways

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Background and Purpose: Chemical effects on apical endpoints such as the timing of developmental milestones and locomotion, and the reversibility of these effects, are important components of chemical safety assessments. Signal transduction pathways critical to organismal growth and development, as well as neuromuscular architecture and neurotransmitter function are well conserved across phyla, supporting the use of small model organisms (SMOs) such as invertebrate *Caenorhabditis elegans* for 3Rs-compliant developmental toxicity screening. Additionally, an SMO

assay for the detection of chemicals that modulate developmental pathway feedback regulation and thereby make critical signaling processes less responsive to endogenous signals would be a useful addition to developmental toxicity test batteries. **Methods:** At 20°C, *C. elegans* reach the mid-third larval stage (mL3) at about 36 h post-initiation of feeding after hatching (first feeding, FF), and chemically induced changes in time to developmental stage or locomotor activity are indications of developmental toxicity. Wild-type *C. elegans* monitored for developmental stage acquisition timing and locomotor activity were exposed from FF either continuously or for 24 h. *C. elegans* data from three representative chemicals was analyzed and compared: 5-fluorouracil (5FU), which has irreversible effects on fetal growth and induces hypoactivity in mammals, ribavirin (RV), which has partially reversible effects on mammalian development, and cannabidiol (CBD), which modulates endocannabinoid signaling, a pathway critical for mammalian embryonic, fetal, and pubertal development. **Results:** In *C. elegans*, all three chemicals induced dose-responsive delays to mL3. Continuous exposures to 5FU and CBD also induced hypoactivity at mL3, albeit at ~100-fold higher concentrations for CBD relative to 5FU. RV induced a non-significant trend towards slight hyperactivity at mL3 in both continuous and 24 h exposure groups. Relative to continuous exposures, removal of 5FU 24 h after FF did not alter subsequent delays to mL3 or 5FU-induced hypoactivity, consistent with irreversible developmental effects. Removal of RV at 24 h post-FF reduced the delays to mL3 by about 50% relative to continuous exposures, consistent with partially reversible effects on developmental timing. Removal of CBD at 24 h post-FF nearly doubled the delays to mL3 relative to the delays induced by continuous CBD exposures. In contrast, the mL3 hypoactivity observed during continuous CBD exposures returned to normal levels after removal of CBD. **Conclusions:** *C. elegans* juvenile growth patterns were able to distinguish between two anti-proliferative drugs with irreversible vs. partially reversible developmental effects in mammals. The increase in developmental delays after removal of CBD suggests that *C. elegans* growth patterns can also detect desensitization of critical developmental pathway(s) that induce a refractory period resulting in delayed development. Additionally, the resumption of normal locomotor activity levels after removal of CBD indicates that CBD influences developmental timing and locomotion via different pathways.

PS 3359 Reproducibility of points of departure derived from high throughput transcriptomic assays with four species of aquatic organisms.

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Background and Purpose: US EPA is evaluating ecological high throughput transcriptomics (Eco-HTT) assays as a new approach methodology for estimating concentrations of chemicals or mixtures that would not be expected to cause adverse ecological effects. An important aspect of assay performance is the inter-assay reproducibility and variability of points of departure (PODs) derived from the assay. An understanding of assay reproducibility is important for determining suitability for risk assessment applications and informing data interpretation. **Methods:** Larval *Pimephales promelas* (a fish), *Daphnia magna* (a crustacean), *Chironomus dilutus* (an insect), and *Raphidocelis subcapitata* (a green algae) were exposed to seven concentrations of cyanazine (0.016-100 µM; fish and daphnia), imidacloprid (16-7200 ng/L; insect), or atrazine (6-32,350 µg/L; algae) for 24 h in a 96-well plate format. A single replicate consisting of RNA pooled from three individuals (or populations of cells in the case of algae) exposed in separate wells, was generated per concentration, per assay (five replicates for controls) and each assay was repeated 9-12 times over a period of weeks. Upon completion, all the collected samples, per species were sequenced on a single sequencing lane (minimizing technical variation associated with the sequencing process) and PODs were derived from concentration-response modeling of either targeted TempO-Seq (fish and daphnia) or whole transcriptome 3' RNAseq (insect and algae) data. **Results:** The number of concentration responsive genes (CRGs) detected, relative to plate-matched controls, varied among the various repeats of the assay. For cyanazine, the number CRGs ranged from 17-678 (40-fold; n=10) for *P. promelas* and 27-760 (28-fold; n=11) for *D. magna*. For *C. dilutus* exposed to imidacloprid the number of CRGs ranged from 6-34 (5.7-fold; n=9), while those for *R. subcapitata* exposed to atrazine ranged from 237-900 (3.8-fold; n=12). It could not be determined whether the greater variability in the number of CRGs identified for *P. promelas* and *D. magna* was due to the species, reference chemical selected, the use of the targeted TempO-Seq method or other factors. Regardless, the PODs derived based on the 10th percentile of the distribution of benchmark concentrations for the CRGs ranged from 5.6-97 µM cyanazine for *P. promelas*, 7.7-67 µM cyanazine for *D. magna*, 22-293 ng imidacloprid/L for *C. dilutus*, and 65-879 µg atrazine/L for *R. subcapitata*. Overall, across all the repeated assays for the four species, the PODs varied by a factor of 13.2, or 1.12 log units. **Conclusions:** The current results indicate that PODs derived from ecological high throughput transcriptomics assays have around an order of magnitude of uncertainty. The ability to calculate a POD based on one replicate per concentration for these transcriptomic data sets, as demonstrated in the current study, provides a computationally tractable approach for calculating and reporting variability estimates for PODs that incorporate both uncertainty of the curve fitting and variability in the CRGs identified into the estimate, which has not been common practice to date. Additional *in silico* re-sampling of both the current

and new data sets may provide even more robust estimates of variability and reproducibility that better reflect the study designs commonly used for testing. *Contents of this abstract neither constitute, nor necessarily reflect US EPA policy.*

PS 3360 Transcriptomic Points of Departure from Whole vs Targeted Transcriptome Sequencing

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Background and Purpose: Transcriptomic points of departure (tPODs) derived from analysis of global gene expression change after short-term assays have been proposed as an alternative to long-term animal studies for evaluating chemical toxicity and risk. Targeted RNA sequencing (RNA-seq) is an attractive alternative to whole-transcriptome RNA-seq because it focuses sequencing depth on a subset of transcripts selected a priori, which reduces cost per sample and streamlines the bioinformatic analysis. However, it is unclear if targeted RNA-seq, examining just a fraction of the overall transcriptome, will provide comparable tPOD estimates to that of whole transcriptome RNA-seq. **Methods:** To address this, we analyzed whole transcriptome data from previous high-throughput exposure studies with *Chironomus dilutus* (an aquatic insect) and *Raphidocelis subcapitata* (a microalgae). In each study, the test species was exposed to multiple concentrations of chemicals with various modes of action for 24 hours in 96 well plate format. To simulate the data that would be obtained from a targeted transcriptome sequencing approach, subsets of the whole-transcriptome data were created *in silico* in three ways: 1) randomly selecting gene targets; 2) using the gene IDs from a commercial targeted sequencing platform based on KEGG pathway coverage; 3) deriving species-specific, effects-driven gene lists with an approach similar to that used to create the human S1500+ targeted RNA-seq gene list. Each of the three types of subsets were equal in number of genes included, each having 1,450 and 856 genes for *C. dilutus* and *R. subcapitata*, respectively. Using a standard analysis pipeline, benchmark concentration-response modeling was applied and tPODs were calculated from both whole and simulated reduced transcriptome data for each chemical. **Results:** Results suggest that while targeted approaches tended to produce tPODs similar to the whole transcriptome, the effects-based target selection tended to perform better than the random and pathway-based selections, having a tPOD within a 10-fold difference to the whole transcriptome for 85% and 72% of exposures, respectively. The effects-based targets also had on average 2.5-fold and 4.4-fold more genes responsive to the exposure than the random and pathway-based targets, respectively. With increased concentration-responsive genes, the tPOD agreement between the subsets and the whole transcriptome tend to improve. In cases of large tPOD differences greater than 1 order of magnitude, the majority (83%) also have 10 or fewer responsive genes. **Conclusions:** A species-specific, effects-driven gene selection subset tends to perform better than both the commercial subset based on pathway coverage and random subsets, which is likely driven by quantity of responsive genes detected. For species with largely unannotated genomes, such as the present test species, there may be insufficient annotated pathways to produce a reliable set of sequencing targets. Future analyses will explore these approaches in species with better annotated genomes, such as *Pimephales promelas* and *Daphnia magna*. Overall, this study demonstrated that targeted sequencing is a viable alternative to whole-transcriptome sequencing that could expedite chemical hazard screening.

PS 3361 Implementation of a New Approach Methods (NAMs)-based Framework to Evaluate Skin Irritation of New Chemicals under TSCA

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Background and Purpose: US EPA Office of Pollution Prevention and Toxics, New Chemicals Division (NCD), is responsible for making human health and environmental safety determinations for new chemicals submitted under the Toxic Substances Control Act (TSCA) prior to their entry into US commerce. During each new chemical human health assessment, the potential to cause skin irritation/corrosion is determined to support evaluation of chemicals by regulatory authorities. This determination has historically been performed using animal testing. As new approach methodologies (NAMs) have been developed and accepted by national and international entities, there has been significant work across the risk assessment community to implement NAMs into regulatory frameworks and encourage adoption by industry stakeholders. Several publications support this transition from animal (*in vivo*) studies to NAMs for evaluating skin irritation/corrosion. These publications demonstrate that NAMs provide superior reproducibility and greater biological relevance to mechanisms of human skin irritation/corrosion when compared to testing using animals. TSCA section 4 (h)(2)(A) states that EPA must "develop a strategic plan to promote the development and implementation of alternative test methods and strategies to reduce, refine, or replace vertebrate animal testing and provide

information of equivalent or better scientific quality and relevance for assessing risks of injury to health or the environment [...]. In support of this legal directive, NCD has collaborated with colleagues at PETA Science Consortium International and the Institute for In Vitro Sciences to develop an approach that prioritizes and encourages the submission of human-relevant reproducible NAMs data for the evaluation of skin irritation/corrosion for new chemicals. **Methods:** The key principle of this approach is the use of the best available science for the prediction of skin irritation/corrosion hazard in humans. In applying this framework to a new chemical review, NCD first prioritizes human cell-based tissue model NAMs that have been demonstrated to be reproducible and relevant to skin irritation/corrosion. If data from human cell-based tissue model NAMs are not available or there are data gaps, NCD then prioritizes data from other skin irritation/corrosion NAMs that have been demonstrated to be reproducible and provide information on the mechanisms of toxicity relevant to skin irritation/corrosion. If no acceptable information on skin irritation is available, the framework allows for consideration of other information, such as physicochemical properties. *In vivo* data are only used in the absence of sufficient NAMs data. **Results:** This decision framework has been implemented for assessment of skin irritation/corrosion potential within NCD, including for 42 skin NAMs studies provided by submitters in 2024. The application of this framework has resulted in clear, consistent prioritization of reproducible, human-relevant data from NAMs, as well as facilitated the assessment of this hazard for new chemical substances, resulting in increased efficiency and consistency in approaches across new chemical assessments. **Conclusions:** This approach prioritizes reproducible and human-relevant data for assessing skin irritation/corrosion potential and is expected to encourage the submission of these data from members of industry. In addition, this approach has improved the efficiency, consistency, and transparency of the assessment of skin irritation/corrosion potential within NCD. Once finalized, it will serve as a resource for stakeholders preparing new chemical submissions to EPA under TSCA. These views are those of the authors and do not reflect the views or policies of the U.S. EPA or other respective organizations.

PS 3362 Longitudinal high-throughput phenotypic profiling using live-cell staining in U-2 OS cells over a 72-hour period.

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Background and Purpose: Cell Painting is a high-throughput phenotypic profiling assay that uses fluorescent cytochemistry to visualize a variety of organelles and high-content imaging to derive a large number of morphological features at the single-cell level. Most Cell Painting studies have used the U-2 OS cell line for chemical or functional genomics screening. The assay can be used with many human-derived cell types, given that it is based on the use of fluorophores that label organelles present in most human cells. Rather than assay fixed specimens, an alternative would be to employ a longitudinal study design that assays Cell Painting endpoints in living cells using a combination of organelle stains and endogenously expressed fluorescent protein(s) at prescribed intervals over a 72-hour period. Using non-toxic organelle labels alleviates the requirement for separate assay plates and reagents for each time point. This provides the opportunity to ascertain a more precise time to measure benchmark concentrations or tipping points in the cellular response to chemical exposure. **Methods:** U-2 OS cells were labeled using two live-cell labeling schemes. The first uses a multiplexed combination of NucleoLive (Saguaro) for the nucleus, Biotracker 405 (Sigma) for mitochondria, wheat germ agglutinin (WGA) 555 (Sigma) for the cell membrane, and LysoTracker 633 (Sigma) to label lysosomes. The second uses engineered U-2 OS cells containing endogenously tagged Blue Fluorescent Protein LaminB1 (Sigma) for the nuclear membrane, MitoTracker Green FM (Sigma) for mitochondria, WGA 555 and LysoTracker 633. For each assay, a stain mixture was combined with U-2 OS cells, which were plated in wells of a 384 well plate using a Certus Flex microdispenser. Following an 18-hour incubation for labeling and attachment, cells were rinsed twice with growth medium and imaged using a Revvity Opera Phenix Plus Imager. Immediately following the initial scan, chemical treatments were applied using an Echo 550 acoustic liquid handler (Beckman). Cells were returned to incubation and imaged at 6, 24, 30, 48 and 72 hours. After image acquisition, 1019 features were analyzed. Following feature extraction, data were normalized to vehicle controls using median and median absolute deviation (MAD). Cell-level data were aggregated to the well-level and used for concentration response modeling with the R package tcplfit2 (v.0.1.9). Normalized cell counts were calculated to evaluate any cytotoxic or cytostatic effects and used to exclude cytotoxic treatments from further analysis. Concentration response modeling was performed using Mahalanobis distance calculations of the well-level data. In addition, 34 separate 'Category-Level Mahalanobis' distance values, representing features categorized by channel, measurement type, and cellular compartment (e.g. DNA_Intensity_Nuclei) were also calculated. These distances were modeled in R to determine benchmark concentration (BMC) values for treatments where the bioactivity exceeded the benchmark response (BMR) of 1 standard deviation from the median of vehicle controls. A Phenotype Altering Concentration (PAC) was defined as the minimal of the global BMC and most-sensitive category-level BMC. **Results:** In this study, 17 chemicals were assayed. Cells were exposed to eight concentrations of Amperozide, Berberine Chloride, Ca-074-Me, Cucurbitacin I, Etoposide, Fenbendazole, Fluphenazine, Latrunculin B, Lys05, Metochlopramide, Oxibendazole, Rapamycin, Rotenone, Taxol, Tetrandine, Saccharin and one concentration of Staurosporine. For

all chemicals assayed, phenotypic profiles generated from the data largely agree with expected cellular effects. For example changes in mitochondria associated cellular features were evident at several time points following exposure to Berberine Chloride. Derived PAC values were compared to some of those found in previous Cell Painting time course studies (e.g Nyffeler et al, 2020) and found to be within expected ranges. **Conclusions:** The use of non-toxic fluorescent organelle labels in a longitudinal high-throughput phenotypic profiling assay is a potentially viable method for Tier 1 screening, and can provide time-course type data from a single exposure experiment. *This abstract does not represent US EPA policy.*

PS 3363 Evaluating an *in vitro* Human-Based Blood-Milk-Barrier Model for Drug Transport During Lactation

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Background and Purpose: Breastfeeding provides well-established maternal, infant, and economic benefits, yet the use of medications during lactation remains a major barrier due to limited data on drug transfer into human milk. Because lactating women have historically been excluded from clinical drug studies, significant knowledge gaps persist, complicating treatment decisions for breastfeeding mothers. Existing approaches, such as targeted clinical studies, animal models, and traditional 2D cell cultures, offer useful insights but are limited by slow enrollment, ethical constraints, and reduced ability to recapitulate complex 3D human breast tissue structure and function. These challenges highlight the need for new approach methodologies (NAMs) to study drug transport across the blood-milk barrier. In this study we evaluate a microphysiological system (MPS) that models the human blood-milk barrier *in vitro* as a tool to predict drug transfer into breast milk. **Methods:** Here, we utilize a blood-milk barrier MPS composed of primary human mammary epithelial and endothelial cells. The MPS is designed to recreate key features of the human mammary epithelium and its microenvironment *in vitro*. Primary mammary epithelial cells were exposed to lactogenic media and characterized by qPCR for milk-associated and transporter gene expression, as well as by immunostaining of tight-junction markers pre- and post-differentiation. Nonspecific drug binding was assessed through an LC-MS/MS-based adsorption study to quantify nonspecific interactions within the MPS. Evaluation of drug transfer using a panel of medications with diverse physicochemical properties is planned and will be incorporated as the next phase of the study. **Results:** Characterization of the blood-milk barrier MPS confirmed formation of a continuous epithelial monolayer expressing tight junction markers, indicating functional barrier integrity. qPCR analysis demonstrated induction of milk-related proteins following exposure to lactogenic media, supporting the physiological relevance of the model. Preliminary assessments of nonspecific drug binding enabled differentiation between compound loss and true permeability. A panel of medications with diverse LogP values and passive permeability characteristics is currently being evaluated to determine drug transfer across the barrier. Drug-transfer metrics generated by the MPS will be compared with existing human lactation data to assess concordance and the predictive performance of the model. **Conclusions:** Critical physiological features and performance criteria of the MPS have been demonstrated, and validation of this platform for predicting drug transfer into human milk is ongoing. This tool has the potential to advance toxicology assessment and regulatory science by addressing data gaps and improving the safety assessment of medications used during breastfeeding. Ultimately, this approach may inform on the use of medications in breastfeeding mothers and enhance maternal-infant health. **Disclaimer:** This abstract reflects the views of the authors and should not be construed to represent FDA's views or policies.

PS 3364 CAMERA: A Centralized Public Resource for Validated Alternative Methods to Support Regulatory Decision-Making

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Background and Purpose: The Collection of Alternative Methods for Regulatory Application (CAMERA) is a publicly accessible online resource that provides streamlined access to validated and qualified new approach methodologies (NAMs) for chemical and biological safety assessment. Agencies within the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) identified a critical need for a unified platform that compiles validated alternative test methods to support the transition toward reduced animal use, consistent with the recent initiatives of the National Institute of Health (NIH) and the US Food and Drug Administration (FDA). The CAMERA initiative aims to integrate alternative methods into a cohesive workflow to better support and inform regulatory decision-making. **Methods:** Validated NAMs for human relevant endpoints, including skin sensitization, skin irritation, eye irritation, and corrosion, were identified from OECD-accepted test

guidelines. Validation reports for these methods were reviewed to extract and curate key information such as test method type, endpoint, test system, mode of action, applicability domain, validating organization, reference chemicals, and performance metrics. In addition, relevant supporting documents, regulatory guidance/guideline and literature were identified. **Results:** CAMERA illustrates the effective consolidation of validated NAMs into a single, accessible platform, improving the accessibility of methods and their underlying validation data that was previously scattered across multiple agencies and organizational websites. The platform's architecture enables user-driven, filtered searches by method type, endpoint, and regulatory agency. The platform also allows side-by-side comparisons of method information, including a Venn-diagram that visualizes reference chemicals across methods, with downloadable tables detailing chemical properties and performance results. Chemicals can be further explored for their characteristics and toxicity information through the Integrated Chemicals Environment. CAMERA integrates supporting materials such as standardized method descriptions, performance metrics, links to validation study reports, protocols, curated datasets, regulatory acceptance documents, adverse outcome pathways, and access to publications via PubMed query. User testing indicated strong support for CAMERA's ability to streamline the identification of regulatory-accepted NAMs, enable comparison of method applicability, validation status, and performance. Initial feedback emphasized its value in meeting agency needs for transparency, harmonization, and improved access to NAMs that advance the principles of the 3Rs in replacing, reducing and refining animal tests. **Conclusions:** CAMERA provides an efficient, user-friendly platform that significantly enhances access to validated and qualified NAMs, supporting broader federal goals to reduce animal use and modernize toxicology. By integrating study reports, protocols, performance data, and regulatory guidance into a unified interface, CAMERA enhances transparency and facilitates consistent application of NAMs in chemical safety evaluation. Ongoing development will expand method domains, strengthen interoperability with ICE, integrate new tools for enhanced interactive capabilities, and continue to integrate user feedback to strengthen CAMERA's utility. This research was supported by the Intramural Research Program (ES# 103386) of the National Institute of Environmental Health Sciences, National Institutes of Health under contract HHSN273201500010C. Information technology service associated with this project was provided under contract 75N93025F00252. The findings and conclusions presented in this abstract are those of the authors and do not necessarily reflect the views of the National Institutes of Health or the US Department of Health and Human Services.

PS 3365 **hiPSC-derived Sensory Neuron and Schwann Cell Co-culture as a NAM for Peripheral Neuropathy Risk Assessment**

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Background and Purpose: Peripheral neuropathy (PN) is a common and dose-limiting side effect of chemotherapy including antibody drug conjugates (ADCs). Traditional animal models are limited in throughput and translational relevance. Human induced pluripotent stem cell (hiPSC)-derived sensory neurons (SN) offer a promising *in vitro* alternative but lack the cellular complexity of the peripheral nervous system. This study aims to evaluate a co-culture model of hiPSC-derived sensory neurons and Schwann cells (SN+SC) as a new approach methodology (NAM) for PN risk assessment, hypothesizing that the inclusion of Schwann cells enhances sensitivity and physiological relevance. **Methods:** Cryopreserved hiPSC-derived SNs and SCs were obtained from a vendor following approved regulatory and ethical protocols. The SNs and SCs were derived from the same healthy male donor which eliminated cross-individual variations. The culture was maintained for 3 weeks before 48-hour treatments with vincristine (VCR) or paclitaxel (PTX). Response to the compounds of the co-culture were compared to SN monoculture and neonatal rat primary dorsal root ganglia (DRG) neurons. Endpoint assessments included morphological evaluations via phase contrast and high-content fluorescent imaging, multi-electrode array (MEA) recording, RNA sequencing, and bead-based chemokine/cytokine release quantification. **Results:** Both the mono- and co-culture displayed PTX-specific swollen and retracted neurites like rat primary DRG neurons reported in our prior study, supporting its physiological relevance. This neurodegenerative phenotype was observed at a PTX concentration as low as 3 μ M. The SN+SC co-culture demonstrated a ~20% greater neurite loss compared with SN-only cultures under VCR (30 nM) or PTX (30 μ M) treatment. RNA sequencing results further supported the sensitivity of the co-culture by significant enrichments in microtubule function-related pathways reflecting leading mechanisms of VCR-induced PN. The top three impacted pathways in the co-culture at 10 nM VCR were microtubule motor activity, microtubule binding, and tubulin binding. These disruptions were not observed in the SN monoculture at the same VCR concentration. MEA data revealed that the spontaneous firing rate in both mono- and co-culture were sensitive to VCR exposures. At 3 nM, VCR induced a rise in mean firing rate while neurite retraction was not significant, indicating that functional changes can occur prior to noticeable structural damage. In addition, elevated monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), interleukin-8 (IL-8), and interleukin-18 (IL-18) was detected at the same VCR level, indicating the responsiveness of functional readouts to VCR exposure. **Conclusions:** The hiPSC-derived SN+SC co-culture model offers equal or superior sensitivity and functional potentials

over SN-only and rat primary DRG systems. Its ability to replicate key morphological, functional, and molecular features of PN positions it as a promising NAM for peripheral neurotoxicity risk evaluation in oncology drug development.

PS 3366 **Development of Microfluidic Chip-Based Multi-Cellular Model for Local Toxicity Test**

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Background and Purpose: In alignment with the U.S. Food and Drug Administration (FDA) Modernization Act, New Approach Methodologies (NAMs) have recently gained significant attention. There is a growing demand in the industrial sector for NAMs that not only emulate human physiology but also enable high-throughput-like screening strategies. However, conventional single-cell culture models for assessing local toxicity, such as skin, exhibit limited biological relevance and often fail to replicate the layered structure and barrier function of human skin. To address these limitations, an advanced tri-culture horizontal platform within a microphysiological system (MPS) is developed in this study. **Methods:** The microfluidic chip was fabricated using a 3D printer and treated with air plasma to induce a hydrophilic surface. A simultaneous skin and eye irritation test model was designed using human keratinocytes (HaCaT), human umbilical vein endothelial cells (HUVEC) and rabbit cornea epithelial cells (SIRC). Additionally, alternative combinations of cells, including HaCaT, HUVEC and human dermal fibroblasts (NHDF), were employed to replicate more advanced skin structures, enabling enhanced skin toxicity tests. The model was evaluated using proficiency test materials, including cyclamen aldehyde and glycerol, which are recommended under the Organisation for Economic Co-operation and Development (OECD) Test Guidelines 439 and 492. Test materials were applied for 5 minutes at concentrations of 0.05% and 5%. After 72 hours of incubation, cell viability (CV) was evaluated using a dual-modality approach: an absorbance-based cholecystokinin octapeptide (CCK-8) assay and a fluorescence-based LIVE/DEAD assay. Imaging analysis was conducted automatically using Python-based graphic user interface. **Results:** The jig was additionally designed to accommodate the chip devices and facilitate high-throughput screening using a standardized spectrophotometer during the study. The fibrin hydrogel matrix was optimized at a concentration of 1.0 mg/ml to support diffusion and interaction of test samples across channels during treatment. Based on CV measurements from three different channels, gradient graphs at each concentration were illustrated and the standard deviation among all test materials was calculated to below 15% (n=3). A scoring criteria system (0 to 3) was established based on CV significance between three cell channels: score 0 for non-irritating (CV >80%), score 1 for mild irritating (50% < CV ≤80%) and score 2 for severe irritating (CV ≤50%). The model demonstrated its ability to distinguish between irritants and non-irritants, aligning with the Globally Harmonized System (GHS) categories. The results indicate that the model successfully has its potential as a preliminary screening tool. **Conclusions:** The multi-cellular model was designed in a standardized 96-well plate format, incorporating chip devices and a corresponding analytical jig to ensure compatibility with conventional colorimetric assays. This chip-based assay represents a cost-effective and scalable screening tool within the MPS for safety evaluation, utilizing standardized absorbance and spatially resolved analysis. Furthermore, the model is expected to be applicable for assessing additional aspects of toxicity and efficacy in future studies.

PS 3367 **Unveiling Oral Vulnerability: A Human 3D Model of Sulfur Mustard-Induced Epithelial Injury**

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Background and Purpose: Sulfur mustard (SM) is a highly reactive alkylating agent that penetrates epithelial barriers and causes severe tissue injury through DNA and protein adduction, inflammation, and cytotoxicity. While SM effects on skin, eyes, and lungs are well documented, the oral mucosa remains an understudied target despite clinical reports of acute necrosis, persistent ulceration, and epidemiologic associations with oral cancers. Mechanistic insights into oral SM toxicity are limited and rely primarily on retrospective human case reports with variable exposure histories. Commercial oral organoids exhibit deficits for toxicology studies, including poor epithelial stratification, lack of a proliferative basal layer, and short culture lifespan. These deficits are important for oral SM studies, as proliferating basal epithelial cells are particularly susceptible to SM-induced interstrand DNA crosslinks. To overcome these limitations, we developed a long-lived, human oral mucosal tissue equivalent (OMTE) composed of primary oral keratinocytes, fibroblasts, and endothelial cells. **Methods:** Primary human oral keratinocytes were seeded atop a fibrin hydrogel embedded with fibroblasts and endothelial cells. Cultures were grown at an air-liquid interface under conditions optimized for long-term viability and epithelial stratification. Tissue architecture was validated by histology (H&E), and phenotypic maturation was assessed via IHC for epithelial, stromal, and endothelial markers. SM vapor exposures were applied apically and quantified as concentration × time (CT), spanning subthreshold to overtly cytotoxic doses. Post-exposure analyses included H&E, IHC for DNA damage (γH2AX) and cell death markers, and quantification of

lactate dehydrogenase (LDH) release in basolateral media as a measure of cytotoxicity. **Results:** OMTEs maintained a proliferative basal epithelium and stratified architecture through at least 42 days. H&E revealed a non-keratinized epithelium with a columnar basal layer, polygonal stratum spinosum, and outer stratum superficialis, supported by a fibroblast-rich stroma with robust neovessel formation. IHC confirmed epithelial maturation (p63+, CK5+, CK13+), basal epithelial cell proliferation (ki67+), and stromal (vimentin+) and endothelial (CD31+) identity. SM exposure induced dose-dependent epithelial separation, progressing to widespread detachment and stromal disruption. IHC revealed activation of multiple cell death pathways: cleaved caspase-9 (intrinsic apoptosis) was present, while caspase-8 (extrinsic apoptosis) and phosphorylated RIPK3 (necroptosis) were absent. Cleaved gasdermin D indicated pyroptotic pore formation, and nuclear AIF co-localized with γH2AX foci, consistent with parthanatos and DNA double-strand breaks, while GPX4 downregulation suggested lipid peroxidation and ferroptosis. LDH release correlated with histologic injury severity, reflecting progressive cytotoxicity and barrier compromise. **Conclusions:** The OMTE model recapitulates SM-induced, dose-dependent DNA damage and reveals selective activation of intrinsic apoptosis, pyroptosis, ferroptosis, and parthanatos, with minimal evidence for extrinsic apoptosis or necroptosis. These mechanistic insights support quantitative dose response analysis and comparative injury profiling, positioning the OMTE as a robust, human-relevant platform for oral toxicology by vesicants and other toxicants. This work enables high-resolution mapping of epithelial vulnerability in a translationally relevant system. Future studies will explore targeted inhibition of cell death pathways to mitigate injury progression and improve tissue outcomes and extend to other oral toxicants.

PS 3368 Dual NR4A1 and NR4A2 Ligands Promote Ferroptosis and Inhibit Rhabdomyosarcoma Growth through CD71 Activation

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Background and Purpose: Rhabdomyosarcoma (RMS) is the most prevalent soft tissue sarcoma in children and adolescents, with poor prognosis in metastatic or treatment-resistant cases. Conventional therapies often fail to eliminate aggressive tumor populations. Ferroptosis, a regulated form of cell death driven by iron accumulation and lipid peroxidation, has emerged as a promising therapeutic strategy for targeting RMS cells. This study investigates the potential of bis-indole-derived C-DIM compounds to induce ferroptosis in RMS cells by targeting the NR4A1/2-Sp1-CD71 axis, which regulates iron uptake and oxidative stress. **Methods:** Human RMS cell lines (RD and RH30) were treated with C-DIM compounds, and cell viability was assessed using the XTT assay. Intracellular ROS levels and lipid peroxidation were evaluated using H₂DCFDA and BODIPY™ 581/591 staining, respectively, while malondialdehyde (MDA) assays quantified oxidative damage. CD71 mRNA and protein levels were measured by qPCR and Western blot. siRNA-mediated knockdowns of NR4A1, NR4A2, and Sp1 were performed to examine their roles in regulating CD71 expression and ferroptosis. A CD71 promoter luciferase assay was used to assess Sp1-dependent transcriptional activity. **Results:** C-DIM treatment significantly reduced RMS cell viability by enhancing ferroptosis, as evidenced by increased ROS, MDA production, and lipid peroxidation. CD71 expression was markedly upregulated following treatment. Knockdown of NR4A1/2 or Sp1 led to a decrease in CD71 expression and protected RMS cells from ferroptosis. CD71 promoter assays confirmed that Sp1 regulates its transcription in response to C-DIMs. **Conclusions:** C-DIM compounds promote ferroptosis in RMS cells through an NR4A1/2-Sp1-mediated upregulation of CD71. These findings highlight a novel therapeutic strategy targeting the NR4A1/2-Sp1-CD71 axis to induce ferroptosis and overcome treatment resistance in rhabdomyosarcoma.

PS 3369 Beyond phthalates: cytotoxicity and obesogenic potential of emerging alternative plasticizers

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Background and Purpose: Plasticizers are widely used polymer additives, historically dominated by phthalates and bisphenols, both of which are now well recognized for their endocrine-disrupting and obesogenic properties. In response, the industry has shifted toward alternative plasticizers such as 2,2,4 trimethyl 1,3-pentanediol diisobutyrate (TPIB), di(2-ethylhexyl) adipate (DEHA), dipropylene glycol dibenzoate (DPGDB), tris(2-ethylhexyl) trimellitate (TOTM), polyethylene adipate (PEA), and diocetyl adipate (DOA). Despite their increasing use in food packaging, consumer products, and medical devices, toxicological information on these alternatives remains scarce. **Methods:** Here, these plasticizers were evaluated for their cytotoxicity in human mesenchymal stem cells (MSCs), mature human adipocytes, and hepatocytes (HepG2 cell line) following 24 h and 72 h exposures. Cytotoxicity was quantified using the MTT assay, a colorimetric technique that measures mitochondrial dehydrogenase activity as an indicator of cell viability. Dose-response curves were generated from the data to calculate half-maximal effective concentration (EC₅₀) values for each compound and cell type, providing a comparative assessment of their relative cytotoxic potency over time. **Results:** Most compounds showed very low acute toxicity, with EC₅₀ values >1,000 mM in differentiated adipocytes and hepatocytes (for DEHA, PEA, DOA). However, selective effects were evident in MSCs: TPIB and DPGDB induced moderate toxicity (EC₅₀ ~16-18 mM), and TOTM showed marked potency at

prolonged exposure (EC₅₀ = 0.4 mM). **Conclusions:** The lack of cytotoxicity in mature adipocytes suggests these compounds may exert non-lethal, metabolic effects. Ongoing studies are exploring whether alternative plasticizers disrupt lipid metabolism or promote adipogenesis, potentially contributing to obesity-related outcomes. These findings emphasize the need to evaluate such compounds beyond viability to understand their long-term metabolic impacts and prevent regrettable substitutions.

PS 3370 Identification of clozapine mediated protein modification with a novel strategy: welding click chemistry to advance clozapine toxicity research

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Background and Purpose: Schizophrenia affects a significant proportion of individuals, wherein a subset of patients is described as treatment-resistant. Clozapine is an atypical antipsychotic that targets dopaminergic and 5-HT_{2A} receptors, which is reserved for these patients and is superior in its anti-suicidal activity. However, it carries numerous serious warnings, and is well-known for its risk of drug-induced agranulocytosis (severe neutropenia). The exact mechanism of toxicity remains unclear but may involve clozapine's covalent binding to proteins and off-target effects caused by its reactive metabolites. These metabolites are produced by neutrophil myeloperoxidase (MPO), the most prevalent neutrophil enzyme that supports immune responses and also generates reactive species metabolites. Therefore, the identification and protein network analysis of the protein-clozapine adducts could contribute to the current understanding of toxicity pathways. **Methods:** For protein identification, a novel clickable clozapine (Click-CLOZ) derivative was developed and the following click reaction protocol was designed for Click-CLOZ pull down. The Click-CLOZ covalent bound proteins were then captured, allowing for proteomic analysis. The HL-60 (human promyelocytic leukemia) cell line and isolated human neutrophils significantly express myeloperoxidase and were used to identify the protein covalent targets of Click-CLOZ. **Results:** Click-CLOZ and clozapine showed similar cytotoxic effects in HL-60 cells, with comparable reactive metabolite production in an isolated myeloperoxidase system. In HL-60 cells, liquid chromatography-mass spectrometry (LC/MS) analysis identified numerous proteins bound by Click-CLOZ in comparison to the vehicle control. Some captured proteins were known for their roles in DNA replication, immune responses and oxidative stress, such as cathepsin G, myeloperoxidase, ribophorin I and P1-MCM3. In neutrophils, Click-CLOZ bound proteins included myeloperoxidase, S100, and DEFA1B, which are also associated with neutrophil-mediated oxidative stress and immune responses. Interestingly, in both HL-60 cells and neutrophils, myeloperoxidase protein binding was common. **Conclusions:** In conclusion, the application of click chemistry proteomics has facilitated a novel approach to identify multiple clozapine-bound protein targets that will be used to advance the understanding of the toxicity of clozapine in future experimental models.

PS 3371 From Barrier Integrity to Clinical Outcome: A Machine Learning -Enhanced Human Intestinal Model for Diarrhea Risk Assessment Across Small Molecules and ADCs

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Background and Purpose: Drug-induced diarrhea remains a frequent dose-limiting toxicity with multifactorial mechanisms. Traditional animal models often fail to recapitulate human-specific gastrointestinal (GI) risk, and conventional 2D *in vitro* systems lack the complexity to model barrier function and microenvironmental interactions. Predictive tools that integrate barrier biology and computational analytics are needed to anticipate risk early across modalities. To address this gap, we validated a predictive platform that leverages a machine learning-enhanced 3D human intestinal barrier model for mechanism-aware assessment and early prediction of diarrhea risk across small molecules and antibody-drug conjugates (ADCs). **Methods:** We developed a 3D human intestinal barrier model (NAMkind™ intestine) for ileum and colon by incorporating epithelial and stromal cell components to preserve polarity and tight junctions. Assays measured barrier integrity (trans-epithelial electrical resistance) and cell viability/redox state (ATP, and Alamar Blue) under repeat dosing. A machine learning (ML) model was trained on assay features and clinical incidence and diarrhea severity data using 5-fold cross-validation with thresholds calibrated for high sensitivity. For ADC assessment, we performed comparative arms—intact ADC, antibody-only, and payload-only—using the same exposure schedule and endpoints to enable modality-aware interpretation. **Results:** The *in vitro* model achieved 95% accuracy across a diverse reference set, including NSAIDs, PPIs, TKIs, EGFR inhibitors, and GLP-1 agents. Mechanistic differentiation was evident within NSAID series (COX-1 vs. COX-2 selectivity) and PPI series. ADC comparative testing (intact vs antibody vs payload) demonstrates the model's ability to capture on-target off-site and off-target off-site liabilities, supporting preliminary risk ranking of conjugates and components. ML integration improved prediction granularity, enabling cross-tissue generalization (colon and ileum) and ranking of compounds by projected diarrhea incidence and severity. **Conclusions:** Inclusion of

fibroblast, endothelial, and smooth-muscle cells enables detection of broader micro-environmental stress beyond epithelial injury. ADC arm comparisons further parse barrier-centric versus systemic cytotoxic liabilities. Coupling a physiologically relevant intestinal barrier assay with ML calibration provides a translational framework for early GI safety assessment across small molecules and ADCs. This approach using our NAMkind™ intestine supports mechanism-aware risk stratification and informs candidate selection across therapeutic classes and modalities.

PS 3372 Recreating Scalp Inflammation Ex Vivo: A Human Hair Follicle-Preserving Model for Immunotoxicological and Therapeutic Assessment

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Background and Purpose: Inflammation of the human scalp plays a critical role in various dermatological disorders, including seborrheic dermatitis, psoriasis, and alopecia. Reproducing these complex immune responses in a physiologically relevant system remains challenging. We present a novel inflammation model, a bio-stabilized ex vivo human scalp model, designed to mimic bacterial-induced inflammatory conditions and assess their impact on hair follicle integrity and immune response. **Methods:** The ex vivo skin models consist of 6 mm diameter human scalp biopsies obtained from five female donors (ages 51-72) with consistent hair density. Biopsies were embedded in a proprietary solid matrix, exposing the epidermis to an air-liquid interface, and maintained under standard cell culture conditions using serum-free medium. Inflammation was induced by 24-hour topical application of lipopolysaccharide (LPS), mimicking Gram-negative bacterial components. PBS served as a control. Tissue integrity was assessed via H&E staining on days 1, 2, and 5. A 36-plex cytokine panel was used to quantify pro-inflammatory mediators. Co-immunostaining of Ki67 and active Caspase-3 enabled automated quantification of proliferative and apoptotic responses within hair follicles, respectively. **Results:** At baseline (day 0), all samples exhibited preserved epidermal, dermal, and follicular architecture. LPS treatment led to mild pyknosis in the superficial epidermis in one donor. Cytokine profiling revealed that LPS induced a measurable inflammatory response characterized by elevated levels of GM-CSF, IL-8, and MIP-1β ($p = 0.062$). LPS exposure resulted in decreased proliferation as measured by Ki67 expression (notably in donor 2 at day 2) and increased apoptosis by day 5 (significant in donor 4). Despite these changes, overall tissue and follicle morphology remained intact throughout the study. **Conclusions:** The platform successfully models scalp inflammation induced by topically applied LPS, triggering a localized immune response and modulating hair follicle cell turnover. This ex vivo system offers a robust and reproducible tool for investigating scalp inflammation mechanisms and evaluating the efficacy of anti-inflammatory scalp therapies under physiologically relevant conditions.

PS 3373 Quantitative Estimates of Toxicodynamic Variability for New Approach Methodologies-Based Systemic Safety Toolbox Using a Population-Based Human *in Vitro* Model

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Background and Purpose: Next-Generation Risk Assessment (NGRA) frameworks leverage New Approach Methodologies (NAM) to support regulatory decision-making without animal testing. While NAM-based methods for hazard and dose-response assessment are widely used, considerations of chemical-specific evidence for inter-individual variability are still largely reliant on the default uncertainty factors for toxicokinetic and toxicodynamic variability. However, chemical-specific estimates of inter-individual variability can be derived using human cell-based *in vitro* models. Accordingly, this study tested the utility of a NAM-based approach to derive toxicodynamic variability factors in the context of NGRA. We tested a hypothesis that by including chemical-specific data on toxicodynamic variability into risk characterization step of NGRA, more protective estimates of human risk will be derived. **Methods:** We used 131 human lymphoblastoid cells (LCLs) from four European and African subpopulations as an *in vitro* model of inter-individual variability. A broad range of substances ($n = 53$) to which humans are exposed in a variety of ways, including consumer use and industrial chemicals, pharmaceuticals, and pesticides were tested in concentration-response (0.3 nM to 300 μM) and viability measurements were collected. To derive quantitative estimates of cytotoxicity of each substance, Bayesian concentration-response modeling was conducted and points of departure calculated for each cell line. **Results:** Overall, 18 out of 53 tested substances had effects below the highest concentration tested and toxicodynamic variability factors 5% (TDVF05) were derived. The median TDVF05 was 3.8 [range from 1 to 46], which is in line with the default TDVF. In addition, we conducted a genome-wide association

study (GWAS) to explore potential genetic drivers of inter-individual variability and to further characterize potential mechanisms of toxicity. This analysis showed that for most substances with inter-individual differences in cytotoxicity, genomic loci containing transporters or metabolism genes conferred genome-wide suggestive effects underlying such variability. **Conclusions:** Overall, this study demonstrated that human LCLs can be used as an *in vitro* model to quantify inter-individual variability in the context of NGRA. These data will improve confidence in the overall risk predictions using NAM data and enable the development of testable hypotheses regarding the mechanisms of inter-individual variability.

PS 3374 Development of a Recirculating Liver-on-a-Chip Platform for Toxicological Application: Evaluation of Hepatotoxicity of Triphenyl Phosphate

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Background and Purpose: Due to limitations of conventional animal testing, toxicity data of thousands of industrial and consumer chemicals remain insufficient, underscoring the need to introduce new approach methodologies (NAMs). Organ-on-a-chip (OOC) platforms are promising for toxicity assessment under human-relevant environments, but deployment has been constrained by usability, incomplete exposure profiling under flow (perfusion/recirculation), small working volumes, and limited biological replication. These hurdles impede their establishment as reliable tools for chemical toxicity assessment. In this study, we developed a recirculating, tubing-free, clamping-cartridge liver-on-a-chip (LOC) using human HepG2 cells to deliver uniform flow, parallel biological replicates, and quantitative exposure profiling. Using triphenyl phosphate (TPHP) as a case study, we assessed hepatotoxicity and biotransformation under physiologically relevant flow with the aim of evaluating the platform's applicability as a toxicological tool. **Methods:** A tubing-free recirculating clamping-cartridge enabled 12 parallel, time-resolved exposures (1.5 mL). HepG2 were stabilized 24 h, exposed to 10 μM TPHP for 48 h; LC-MS/MS quantified TPHP and metabolites (DHP, 4HO-DHP, 4HO-TPHP), hepatic biomarkers (albumin, urea, LDH, AST) were assayed, ten liver-function genes profiled, and RNA-seq performed. LOC results were benchmarked to 24-well plates; acellular recirculation quantified system-level recovery. **Results:** The LOC is a clamping-cartridge platform that allows 12 parallel biological replicates under software-controlled, unidirectional recirculating laminar flow. For chemical-recovery profiling, 10 μM TPHP was analyzed under acellular conditions in both systems. The LC-MS/MS analysis showed that about ~30% of nominal TPHP in the LOC was maintained with low variation among cartridges. The LOC showed reproducible exposure kinetics under recirculating flow (coefficient of variation, CV, 5.2% vs 14% in plates), demonstrating stable mechanical flow performance. The static 24-well plates retained ~70% after 48 h. At 10 μM TPHP, albumin remained largely stable with a mild decline, urea and LDH were altered, and AST rose ~24 h earlier than in plates. Basal hepatic function was higher on the LOC, with mRNA levels of CYP3A4, CYP2E1, and CYP1A2 increased by ~24-, ~5-, and ~30-fold, respectively. Of the three targeted metabolites (DHP, 4HO-DHP, 4HO-TPHP), only DHP—the hydrolyzed product—was detected, showing more active conversion in the LOC despite lower cellular exposure. RNA-seq revealed liver-injury-related pathways, including spliceosome and lipid metabolism. Overall, the LOC produced earlier and stronger toxicological responses, indicating greater sensitivity under uniformly controlled flow with high inter-chip reproducibility. **Conclusions:** The newly proposed modular recirculating LOC achieved the uniform flow, high reproducibility, and greater sensitivity to TPHP than plate-based assays, with desirable RNA-seq results providing mechanistic confirmation of hepatotoxic pathways at lower concentrations than previously reported. Collectively, this platform overcomes key hurdles and advances OOC toward practical toxicological application. The platform enables an exposure-contextualized linkage from mechanism to phenotype in the liver. Future work will refine exposure calibration and enhance the physiological fidelity through surface engineering and endothelial-epithelial co-culture. In this context, the platform is well positioned to serve as a NAMs for predicting complex hepatotoxicity poorly captured by static assays.

PS 3375 A Human Airway-on-a-Chip Microphysiological System for Modeling Chlorine Gas Toxicity

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Background and Purpose: There is an urgent need to understand the physiological mechanisms involved in the initiation and downstream events of injury from acute exposure to chlorine gas (Cl₂) and to identify medical countermeasures (MCMs) that can be applied across the exposure spectrum. Limited information is available regarding the time course and mechanisms of injury after acute Cl₂ exposure due to the lack of human clinical data and ethical, cost and throughput challenges with large and small animal models. Our group has recently developed a 3D airway-on-a-chip *in vitro* model for the study of mechanisms of acute Cl₂ pulmonary toxicity and for identification and testing of MCMs. The purpose of this study was to develop and validate the airway-on-a-chip platform as a model of pulmonary toxicity resulting from exposure to Cl₂ and for identification and testing of potential MCMs. **Methods:**

The airway-on-a-chip model was developed and exposed to a range of Cl₂ doses, exposure times and evaluated across multiple post-exposure durations. Exposure outcomes were validated with numerous chemical, biological, and functional assays to evaluate physiological airway-on-a-chip responses to Cl₂ exposure, and transcriptomic, proteomic and metabolomic analyses were performed to identify candidate MCMs. MCM screening was performed to identify candidates that mitigated the detrimental impacts of Cl₂ exposure. **Results:** The airway-on-a-chip platform was validated to generate and deliver precise concentrations of Cl₂ across a wide range of doses, flow rates and exposure durations. Airway surface pH, ROS production, viability, barrier formation and inflammatory/fibrotic airway changes were evaluated. Extensive multi-omic analysis delineated expected and unique molecular, cellular, and physiological pathways involved in the toxic response to acute Cl₂ exposure. Finally integrated bioinformatic analysis of this data identified candidate MCMs for efficacy testing. MCM screening identified several promising candidate MCMs that could successfully mitigate airway-on-a-chip cell toxicity caused by Cl₂ exposure. **Conclusions:** This work represents a significant step towards application of the airway-on-a-chip platform for the rapid characterization of pulmonary toxins and informing the targeted screening of potential countermeasures

PS 3376 Utilizing Human Small Intestinal Organoids for Quantitative Prediction of Oncology Therapy Induced Diarrhea

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Background and Purpose: Diarrhea is a common adverse event associated with oncology therapies, significantly affecting patient quality of life and treatment adherence. Conventional preclinical models often fail to accurately predict these gastrointestinal toxicities before clinical trials. **Methods:** To address this limitation, we have developed an organoid-based platform for early safety assessment of GI toxicity risk, bridging the gap between simple *in vitro* studies and clinical diarrhea outcomes. **Results:** Using clinical back-translation, we show that a high-throughput 384-well organoid assay differentiates compounds that pose a diarrhea risk with high sensitivity (97%) and specificity (94%) across diverse small-molecule mechanisms of action, enabling improved hazard identification in early discovery. The model also detects mechanistic synergy in combination regimens, predicting clinically relevant exacerbation of diarrhea. By integrating clinical exposure data, we established two models validated against clinical positive and negative controls. A binary hazard identification model and a logistic regression model enabling the prediction of risk including the incidence and severity of human diarrhea risk. Beyond risk prediction, the organoid platform supports target safety deconvolution via genetic perturbation (e.g., shRNA), multi-omics profiling, and high-throughput, high-content imaging. Ongoing work extends validation to monoclonal antibodies and ADCs and operationalizes the screening workflow in early development, aiming to inform program strategy and combination design. **Conclusions:** Our efforts to predict and minimize clinical GI toxicity aim to improve outcomes for patients with advanced cancer and maximize the combinability of therapeutic options.

PS 3377 Optimizing Air-Liquid Interface Respiratory NAM Models for Acute Exposure Scenarios to Support Regulatory Screening and Safety Assessment

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Background and Purpose: New Approach Methodologies (NAMs) are increasingly at the forefront of inhalation toxicity assessment, supporting regulatory safety evaluation. In alignment with the FDA Modernization Act 2.0 and its April 2025 roadmap to reduce, refine, and decrease animal studies, advanced human-relevant approaches—such as organ-on-a-chip systems, 3D tissue models, computational modeling, and *in vitro* assays—are being prioritized for investigational new drug (IND) applications and beyond. Creating aerosols for inhalation studies requires specialized systems tailored to the physicochemical properties of the test article. For gases, dynamic dilution and continuous-flow exposure systems are commonly used to deliver controlled concentrations under physiologically relevant conditions. Complex mixtures often employ whole-mixture generation chambers or nebulization systems that maintain chemical integrity while ensuring reproducible particle size distribution. Nanoparticles typically require high-precision aerosolizers such as electrospray or dry powder dispersers, which prevent agglomeration and allow control over particle size and number concentration. For fibers, rotating brush generators or fluidized bed systems are used to suspend fibers in air without altering their morphology, ensuring accurate representation of inhalation exposure scenarios. These technologies consistently deliver aerosols to *in vitro* platforms, supporting mechanistic studies and regulatory screening under NAM frameworks. **Methods:** This study leveraged air-liquid interface (ALI) 3D lung model MucilAir™ in combination with a continuous-flow gaseous exposure system (Vitrocell® 24/4) to examine acute inhalation hazards. Ethylene oxide (EtO), nitric oxide (NO), and sulfur hexafluoride (SF₆) were delivered for 60 minutes at defined ppm levels. Endpoints assessed after a 24-hour recovery included cell viability (WST-8), membrane integrity (LDH), barrier function (TEER), cilia beat frequency (CBF), and pro-inflammatory cytokine release. **Results:** EtO exposure produced

>50% reductions in WST-8 and LDH relative to controls, accompanied by significant TEER disruption, CBF alterations, and distinct cytokine responses—outcomes not seen with SF₆ and only moderate effects from NO. These findings demonstrate the discriminatory power of continuous-flow NAM platforms in detecting acute gaseous toxicities and elucidating mechanistic pathways. **Conclusions:** By integrating 3D ALI models with dynamic exposure technologies, this study supports the FDA's roadmap to reduce animal use and reinforces NAM-based frameworks important for modern, ethically responsible inhalation toxicity screening.

PS 3378 Benzalkonium Chlorides Alter Gene Networks Governing Cell Proliferation in Human Enteroid Monolayers

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Background and Purpose: Benzalkonium chlorides (BACs) are a subclass of quaternary ammonium compounds widely used as disinfectants in consumer and occupational settings. BAC usage has increased since the COVID-19 pandemic, resulting in elevated levels in human blood-serum and feces. Humans are exposed orally through unintended hand-to-mouth transfer and food processing, with both the general population and workers handling BACs for extended periods being at risk. The small intestine serves as the primary site of contact for ingested xenobiotics and is highly susceptible to toxicity. However, the behavior of the BACs in the gut remains understudied. The purpose of this study is to evaluate human intestinal uptake of BACs and characterize their potential toxicity at concentrations reflective of those detected in human feces. **Methods:** Human duodenal crypt-derived enteroids were first cultured and expanded in 3D spheroids and later seeded as single cells onto Transwell™ inserts (6.5mm) to form polarized monolayers. Confluent monolayers were differentiated with Intesticult® Organoid Differentiation Media (ODM) for 14 days to reach stable trans-epithelial electrical resistance (TEER) values. On day 14, monolayers were exposed to either DMSO or a BAC cocktail (40% C₁₂, 50% C₁₄, 10% C₁₆-BAC) at 50nM, 500nM, 3µM. Bidirectional transport was assessed by dosing apically and basally with 10 µM lucifer yellow in HBSS++ for 60 minutes, followed by collection of media and lysates for UPLC-MS/MS quantification of BACs and potential metabolites, normalized to a deuterated internal standard. Additional monolayers were exposed apically to the same BAC cocktails for 16 hours for RNA-sequencing analysis. Monolayer viability and cytotoxicity were measured by performing MTS and LDH assays on ODM collected after 16-hour exposure to DMSO or individual C₁₄- or C₁₆-BACs (0.05-40µM). **Results:** Monolayer integrity was confirmed with stable TEER values (1030-1680 Ω*cm²) and minimal lucifer yellow permeability (<1% diffusion). Targeted UPLC-MS/MS detected parent BACs in cell lysates, while metabolites were not quantifiable. Significant increased intracellular uptake of all BAC chain lengths was observed in 500nM and 3µM treatments compared to the control, whereas 50nM had no effect. MTS and LDH assays demonstrated no significant change in cell viability and cytotoxicity, respectively, for both treatments (C₁₄- and C₁₆-) compared to the control. RNA-sequencing revealed differential expression of 400, 492, and 355 genes (p < 0.05) at 50nM, 500nM, and 3µM, respectively. Compared to the control, all conditions showed significant upregulation in genes related to the canonical Wnt and PI3K/Akt signaling pathways, and downregulation of TGF-β related genes. Other processes significantly affected were bile secretion, glycosphingolipid biosynthesis, and tight junction organization. *CLDN1* and *CLDN23*, encoding tight junction proteins, were downregulated, whereas thiamine transporter genes *SLC19A3* and *SLC25A19* displayed upregulation across all treatments. **Conclusions:** These findings indicate that BACs can be absorbed following oral exposure and accumulate intracellularly, driving transcriptional changes linked to cell proliferation and barrier regulation. Notably, the most significantly altered biological processes were related to cell cycle progression, cell proliferation, and cell signaling. The transcriptional profile indicates upregulation of Wnt and PI3K/Akt signaling pathways, suggesting that the cells are in a proliferative state, potentially as a stress-induced adaptive response. Overall, C₁₄- and C₁₆-BAC exposures did not significantly affect cell viability or induce cytotoxicity after 16 hours. To understand long-term impacts, ongoing studies are examining the effects of two-week BAC exposure on monolayer integrity, including lucifer yellow permeability, TEER, tight junction protein localization via immunocytochemistry, and mRNA expression.

PS 3379 In Vitro Safety Profiling of ADCs Using an iPSC-Derived Human Tissue Screen to Support FDA Roadmap for Biologics

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Background and Purpose: Antibody-drug conjugates (ADCs) are a rapidly expanding class of targeted cancer therapies designed to enhance the therapeutic index of cytotoxic agents. Despite their specificity, many ADCs encounter clinical setbacks due to dose-limiting or off-target toxicities. Recent analyses of phase 1 ADC trials report grade ≥ 3 treatment-related adverse events in up to 58 % of patients [1]. More broadly, approximately half of investigational biologics fail in late-stage (phase 3)

development, with safety concerns contributing substantially to these failures [2, 3]. These observations highlight the limitations of traditional preclinical models and the need for predictive, human-relevant *in vitro* systems to better anticipate toxicity risks. The U.S. FDA's roadmap for biologics emphasizes functional safety profiling using mechanistically relevant assays that can assess tissue-specific toxicity risks early in development and support IND submissions. **Methods:** A novel *in vitro* safety profiling platform was developed and validated using seven fully human induced pluripotent stem cell (hiPSC)-derived cell types representing key tissues including lung, liver, heart, CNS, skin, intestine, and vascular endothelium. The cell types were selected based on target antigen expression and relevance to known ADC toxicities. Multiple ADCs, including *cetuximab-MMAE* (anti-EGFR) and *trastuzumab-emtansine* (anti-HER2), were evaluated for off-tumor toxicity using real-time impedance (xCELLigence). EGFR and HER2 expression were confirmed by High-Content imaging. **Results:** The platform demonstrated reproducible, tissue-specific cytotoxicity profiles across ADCs at concentrations up to 100 µg/mL. *Cetuximab-MMAE* induced dose-dependent toxicity in EGFR-low alveolar epithelial cells, melanocytes, and hepatocytes with up to 61%, 58% and 30% killing, respectively. *Trastuzumab-emtansine* caused moderate off-tumor effects with up to 17% cell death in HER2-low ventricular cardiomyocytes. Neurons and other antigen-negative cell types were minimally affected with less than 6% cell death, confirming assay specificity and sensitivity. **Conclusions:** This fully hiPSC-derived assay panel offers a human-relevant, functional approach for *in vitro* safety profiling of ADCs and other biologics. The impedance-based cytotoxicity readout, combined with imaging and cytokine profiling, enables early identification of tissue-specific risks and supports FDA guidance on functional safety assessment. The platform's reproducibility, scalability, and mechanistic relevance make it a powerful tool for de-risking targeted therapies in preclinical development.

PS 3380 Utilization of *In Vitro* Liver Models for the Toxicology Testing of Rat and Dog Hepatocytes

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Background and Purpose: Global regulatory requirements for the registration of agrochemical compounds are a complex process that relies on extensive animal testing that includes a 90-day non-rodent toxicity study, for which the dog is the selected species. To reduce the need for animal testing in regulatory submissions and early discovery programs, a suite of *in vitro* assays potentially could provide critical data on relative species sensitivity, to inform when animal studies, namely dogs, could be waived. **Methods:** Hepatocytes were co-cultured with fibroblasts in 2D and 3D *in vitro* liver models before being treated with either ketoconazole, cyclophosphamide, or triazolam in both acute (24-hour) and repeat dose (6-day with treatment refresh every 48 hours) testing paradigms. Treated media was collected for measurement of albumin by ELISA, as well as parent and metabolite quantification through LC MS/MS to assess metabolic competency. RNA was isolated from treated cells for sequencing to determine the transcriptional points of departure (tPODs). *In vitro*-derived endpoints were included in *In Vitro* to *In Vivo* Extrapolation (IVIVE) calculations, to compare *in vitro* outcomes to measured *in vivo* liver-specific PODs. **Results:** Albumin measured from treated media for both 2D and 3D models when using the administered equivalent dose 95 in IVIVE calculations resulted in a similar species sensitivity compared to the measured *in vivo* effects. Using RNA sequencing from treated cells to derive a transcription point of departure (tPOD) for IVIVE resulted in recapitulation of relative species sensitivity for all three chemistries for the 2D *in vitro* platform. **Conclusions:** This proof-of-concept study provides justification for the use of *in vitro* liver co-culture models (either 2D or 3D) to assess relative species sensitivity. While analysis using both albumin and RNA resulted in outcomes that recapitulated the measured species sensitivities of the molecules tested, RNA sequencing potentially provides a more comprehensive toxicity characterization, as it encompasses the effects on the entire transcriptome, instead of a single captured data point, which likely will provide greater use for a broader chemical test set. These data could be vital to demonstrate when dog studies currently required for agrochemical registration, would not add value to, or enhance human health risk assessment. There is a global push to further new approach methodologies that allow for the reduction of animal use for regulatory testing, and methods like this bring us closer to reducing and one day eliminating the need for such testing.

PS 3381 *In vitro* imaging and safety profiling of siRNA RyR2 delivered via inhalation in human models

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Background and Purpose: Small interfering RNAs (siRNAs) are emerging therapeutics able to selectively silence gene expression. Among them, siRNA targeting ryanodine receptor 2 (RyR2) is a promising strategy for treating dominant catechol-

aminergic polymorphic ventricular tachycardia (CPVT). Inhalation represents the administration route, as oxygenated blood and drug molecules carried through the pulmonary circulation rapidly reach the heart. The aim of the study is to set up a new approach methodology (NAM) based on *in vitro* human cells model to evaluate the potential immune and respiratory toxicity of siRNA RyR2. **Methods:** Normal human bronchial epithelial (NHBE) cells differentiated at the air-liquid interface were employed as a 3D *in vitro* model to investigate respiratory exposure to siRNA RyR2. Cells were exposed for 48 h to 400 nM siRNA RyR2, scramble, and calcium phosphate nanoparticles (CaP NPs). Cellular uptake and internalization were assessed using Cy5-labeled siRNA and confocal microscopy. Gene expression analysis was conducted via rt-PCR to evaluate the modulation of key toxicity-related pathways, including mitochondrial stress, oxidative stress, endoplasmic reticulum stress, and autophagy. Pro-inflammatory cytokines release (TNF-α, IFN-γ, IL-8, and IL-6) was quantified from the cell culture supernatant. Immune cell interactions were further explored using human monocyte-derived dendritic cells and macrophages. Following 48 h exposure to 400 nM siRNA RyR2, scramble, and CaP NP, uptake was visualized via confocal imaging, and flow cytometry allows to characterize phenotypic characteristics and activation markers of the immune cells. **Results:** Confocal microscopy confirmed successful internalization of siRNA RyR2 in both epithelial and immune cells. Cytokines analysis showed a slight increase in IL-8 release induced by both siRNA and scramble, while IL-6 remains undetectable. Additional investigations are ongoing to profile gene expression associated with stress-related pathways, including oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress, and autophagy. Regarding the immune assessment, analyses focus on the secretion of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-8, and IL-6) are also ongoing. **Conclusions:** Overall, these results provide initial insights into the safety and toxicological profile of inhaled siRNA RyR2 and support the application of human-relevant, animal-free *in vitro* systems for preclinical evaluation of nucleic acid-based therapeutics. This work is funded by the European Union - Next-Generation EU ("PNRR M4C2-Investimento 1.4-CN00000041").

PS 3382 Integrating *in vitro* and *in silico* approaches for assessing local respiratory effects in inhalation exposure safety evaluations

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Background and Purpose: There is increasing interest in advancing non-animal approaches for inhalation exposure safety assessment. However, defined methodologies for predicting local respiratory effects from irritant exposures are lacking. Fragrance materials, which are typically organic and semi-volatile with low aqueous solubility, present additional challenges, as sensory irritation often complicates the interpretation of respiratory responses. Inhalation safety evaluations can be strengthened by integrating human odor and odor irritation thresholds with No Observed Adverse Effect Concentrations (NOAECs) derived from fit-for-purpose human *in vitro* models. The current fragrance safety assessment framework applies a weight-of-evidence approach that prioritizes existing data, read-across from structurally appropriate analogues, and, when data gaps exist, the inhalation Threshold of Toxicological Concern (TTC). For substances that exceed TTC values and lack adequate toxicological information, additional considerations include evaluating physicochemical properties, applying *in silico* exposure modeling, and using *in vitro* models to identify points of departure for risk assessment. The present study employed an integrated *in silico* and *in vitro* strategy to characterize the local respiratory effects of the fragrance ingredient acetic acid, evaluating both human-relevant exposure concentrations and levels associated with cytotoxicity. **Methods:** Local respiratory effects were examined using reconstructed human bronchial epithelium (RhBE) tissues (MucilAir™) maintained at an air-liquid interface. Tissues derived from five healthy adult donors were used to capture interindividual variability. Acetic acid dosing solutions (20, 40, 120, and 240 µg/cm²) were applied apically (25 µL), and endpoints were assessed at 2, 6, and 24 hours (h) post-exposure. The dose range reflects predicted tracheobronchial deposition levels, estimated using a modified Multiple-Path Particle Dosimetry (MPPD) model. Simulations incorporated chronic aggregate inhalation exposure of 0.001 mg/person/day and odor-based sensory effect thresholds of 12.3 mg/m³ (NOAEC) and 24.6 mg/m³ (LOAEC) during a 120-minute resting exposure. Endpoints included ciliary function (ciliary beat frequency and active area), barrier integrity (transepithelial electrical resistance), tissue viability, and inflammatory signaling (IL-6 and IL-8 release). The treatment groups were statistically compared to respective vehicle control groups using ordinary one-way ANOVA and Dunnett's multiple comparisons test. **Results:** Baseline variation across donors was evident in ciliary function, barrier integrity, and cytokine levels, reflecting biological diversity. At 120 µg/cm², reductions in barrier integrity were detected within 2 h, with increases in IL-6 observed within 6 h and IL-8 at 24 h. Significant cell viability loss occurred only at 240 µg/cm², where corresponding changes in ciliary activity and barrier integrity are likely attributable to generalized cytotoxicity. No adverse effects were observed at 20 and 40 µg/cm². Based on these responses, the NOAEC was determined to be 40 µg/cm², and the LOAEC was 120 µg/cm². Comparison of the *in vitro* NOAEC to the human deposition estimate of 0.000098 µg/cm² yielded a Bioactivity Exposure Ratio (BER) greater than 100, after applying conservative uncertainty factors for population variability and *in vitro* to *in vivo* extrapolation. **Conclusions:** The combined *in vitro* and *in silico* approach provided a human-relevant evaluation of acetic acid respiratory bioactivity. The *in vitro* NOAEC aligned with the sensory irritation threshold for acetic acid, and the high BER indicates a substantial margin of safety under typical

fragrance use conditions. While the RhBE model effectively represents the bronchial epithelium, a comprehensive assessment should also incorporate upper respiratory tract models coupled with Computational Fluid Dynamics deposition modeling. Further work is also needed to define the optimal number of donors required to represent population variability. Despite these considerations, the findings support the use of integrated non-animal approaches for inhalation safety assessment of fragrance ingredients.

PS 3383 Development and Characterization of Non-Human Primate iPSC-Derived Ventricular Cardiomyocytes for *in vitro* Safety and Toxicity Screening

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Background and Purpose: Human induced pluripotent stem cell-derived cardiomyocytes (Hu CM) are widely used in nonclinical cardiac safety studies but interspecies translation remains a limitation in early-stage pharmacology and toxicology. Non-human primates (NHPs) are physiologically close to humans, are often used as secondary species for *in vivo* studies, and can provide translational insights. However, there are limited *in vitro* models using primary NHP cardiomyocytes. We aimed to establish and characterize an *in vitro* platform of NHP iPSC-derived ventricular cardiomyocytes (NHP CM) suitable for translational *in vitro* safety and toxicity applications focusing on consistency, functional responsiveness, and pharmacodynamic sensitivity to known reference compounds. **Methods:** iPSCs from cynomolgus macaques were sourced from the German Primate Center (Tereshchenko, Y et al. *Cells* 2024, doi.org/10.3390/cells13060558) and differentiated into ventricular cardiomyocytes in stirred tank bioreactors. Expression of cTnT, α -actinin, and MLC2V was quantified via immunostaining. Cardiomyocyte function was assessed using micro-electrode arrays (MEA) and impedance-based contractility assays, and the response to cardioactive compounds was tested in these assays. **Results:** NHP CM showed >93% cTnT-positive cells post-thaw with co-expression of α -actinin and MLC2V, similar to their human counterparts (Hu CM). Spontaneous beating emerged by day 3 post-thaw and persisted for at least 10 days post-thaw with beat rates ranging from 28±4.8bpm and FPD of 528±57.8ms. MEA analysis revealed intra-well FPD variability of 2.19% and inter-well variability of 2.98%, indicating strong functional consistency across batches. Upon compound exposure, NHP CM revealed differences to Hu CM: in MEA, 100nM isoproterenol caused 28±7% vs 73±32% increase in beat rate; 10nM dofetilide caused more severe arrhythmia; and nifedipine caused beat arrest at all concentrations, but only > 100nM in Hu CM. In impedance contractility assay, 10µM pentamidine caused beat arrest at d3 vs d4 in Hu CM; 1µM blebbistatin caused 50% reduction in contraction for 48h in NHP CM but > 70% reduction in Hu CM for 7 days; and sunitinib caused time- and dose-dependent cytotoxicity and increased beat duration in Hu CM but no signs of toxicity in NHP CM. **Conclusions:** NHP CM can be differentiated at large scale, display high structural fidelity, stable function in MEA and impedance assays, and respond to cardioactive compounds. The observed species-specific differences (e.g., arrhythmia signatures, chronic sensitivity) highlight the translational value of the model for *in vitro* toxicity and safety studies.

PS 3384 Building Confidence in MPS Use in Regulatory Applications: The 3Rs Collaborative's & FDA-CDER's Cross-Platform DILI Project

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Background and Purpose: New Approach Methodologies (NAMs), combined with expanding interest in reducing reliance on animal testing in favor of more human-centric, physiologically relevant models, there is great potential for NAMs such as microphysiological systems (MPS) to address gaps in preclinical testing, improving drug discovery and development, and patient outcomes. However, there is a need to establish confidence in the performance of such methods to increase widespread integration of MPS in drug discovery and development including toxicological risk assessment. This may be achieved through standardized assessment of their accuracy, reliability, and technical characterization. **Methods:** The FDA's Center for Drug Evaluation Research (FDA-CDER) and the 3Rs Collaborative's MPS Initiative (3RsC-MPS) are conducting a collaborative project under a consortium agreement with involvement from the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Critical Path Institute (C-PATH). The project's main goal is to perform an evaluation across 9 companies' liver MPS using standard conditions from a similar baseline protocol to evaluate drug induced liver injury (DILI) for a focused panel of hepatotoxicants using endpoints of toxicity. The choice to use liver MPS is based on their increasing use in drug discovery, and the fact that many MPS manufacturers have liver platforms which are considered relatively advanced in their development. The design and performance differences in each platform are expected to highlight the expected variabilities that might be observed in study reports which are ultimately submitted to regulatory agencies, for those endpoints of interest in the prediction of DILI risk in

humans. There is a need to understand the extent of this expected variability, such that the study results can be accurately interpreted. **Results:** The chosen context of use for the project was use of liver MPS as a tool for retrospective analysis of treatment-related liver injury signals observed in clinical trials. Across the nine platforms, five included only primary human hepatocytes (PHH), two had PHH + Kupffer cells (KC), one had iPSC-derived hepatocytes, one had HepG2 + TW + NT1 + THP1, and a finally had PHH, KC, and sinusoidal endothelial cells. Developers were provided 4 pairs of more and less toxic compounds analogues in a blinded fashion that spanned across 4 distinct indications and drug classes within the small molecule designation. Compounds were tested at least 3 dose levels with a maximum concentration of 10 times Cmax. At least 7 out of 9 technologies providers measured ALT, LDH, Albumin and ATP while only 5 measured CYP3A4 based on their optimized protocols. Initial results showed 5 providers correctly distinguished all 4 blinded hepatotoxicants from their less toxic analogues. Additionally, 2 providers correctly distinguished 2 hepatotoxicants. Results are still pending for 2 providers. **Conclusions:** This project is expected to support increased confidence in the use of MPS across platforms and study sites. In turn, it may lead to broader use of NAMs, including MPS into drug development and ultimately contribute to increased submissions of MPS-based studies in regulatory submissions, and decision making. Such projects are a step towards reaching the 3Rs (replace, reduce and refine) in the use of animals for the safety assessment of drugs before marketing.

PS 3385 Assessment of a 3D Primary Human Thyroid Microtissue Assay for Identifying Thyroid-Disrupting Chemicals

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Background and Purpose: The European Chemicals Agency (ECHA) and the European Food Safety Authority (EFSA) have jointly published guidance for identifying endocrine disruptors. Together with the U.S. EPA's Endocrine Disruptor Screening Program (EDSP), these initiatives emphasize the need for high-throughput screening (HTS) approaches to efficiently evaluate potential thyroid-disrupting chemicals (TDCs) and minimize reliance on traditional, low-throughput, animal-based methods. Deisenroth et al. previously developed a novel 3D human thyrocyte microtissue platform to identify TDCs that act through various mechanisms relevant to thyroid hormone synthesis. This study was designed to evaluate the within-laboratory reproducibility and performance of the assay using primary human thyrocytes from one donor lot, and to assess the applicability of the 3D model for characterizing responses to known TDCs using thyrocytes from a different single donor. **Methods:** Thyrocytes were derived from healthy donors (≤55 years, BMI ≤35) and cultured in a 96-well 3D format. Control groups included baseline (CT-1), TSHR agonism (CT-2), and TSHR antagonism (CT-3) conditions to assess model sensitivity and specificity. Cells were maintained for 14 days with medium changes every 2 days. Culture media were collected on day 14 for T3 and T4 quantification by ELISA, and microtissue viability was determined using the CellTiter-Glo® assay. Model performance was evaluated by dynamic range, precision, and screening quality metrics. For evaluation of the model with known TDCs, medium samples were collected on day 14 after a 6-day exposure (0.001-100µM) for T4 quantification by ELISA. The half-maximal inhibitory concentration (IC₅₀) for each compound was calculated using GraphPad Prism. **Results:** The thyrocyte lots met all specification criteria for screening applications. Microtissues were formed within 4 days and evenly distributed. Microtissues produced T4 concentrations exceeding 1.0 ng/mL at day 14, with robust dynamic ranges (RS/B >2.5-fold), coefficients of variation <30%, and Z'-factors >0.6 for T3 and T4. Dose-dependent inhibition of T4 synthesis was observed with 6-day exposures (Days 8-14) to three reference TDCs, methimazole (MMI), propylthiouracil (PTU), and sodium perchlorate (NaClO₄), yielding average IC₅₀ values of 0.50 (SE 0.01) µM, 0.96 (SE 0.18) µM, and 32.89 (SE 5.46) µM, respectively, while the negative control (methomyl) showed no effect. No cytotoxicity was observed for any of the reference compounds. **Conclusions:** In summary, the primary human thyrocyte 3D microtissue assay using ELISA represents a promising strategy for evaluating and prioritizing potential TDCs based on human hazard-related bioactivity. Moreover, the model enables the identification of compounds acting directly on the thyroid and supports simultaneous evaluation of multiple MIEs (e.g., TPO, NIS, TSHR) for small-molecule screening in both agriscience and therapeutic research.

PS 3386 Evaluating toxicity of polycyclic aromatic hydrocarbons from wildfire smoke in primary human bronchial epithelial cells in the presence and absence of THP-1 macrophages

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Background and Purpose: Wildfires are a growing public health crisis with wildfire occurrence, duration and intensity increasing in recent years; however, little is known about toxicity associated with inhalation of wildfire smoke, which are chemically complex emissions containing polycyclic aromatic hydrocarbons (PAHs) among other pollutants. In the respiratory system, induction of airway inflammation is orchestrated through signaling between airway epithelial cells and macrophages, which are two of the most abundant cell types in the respiratory tract contributing to the innate immune response. Activation of airway immune response and inflammation are critical events leading to adverse health effects and damage to the respiratory system after chemical exposure. To evaluate the combined contribution of airway epithelial-macrophage response to PAHs from wildfires, we established a co-culture model utilizing organotypic primary human bronchial epithelial cells (HBEC) differentiated at the air-liquid interface and THP-1 macrophages. **Methods:** HBEC in the presence and absence of THP-1 macrophages were exposed for 24 hours to mixtures of PAHs (0-600 μ M) previously identified from wildfires in the Pacific Northwest by passive air sampling. These mixtures were comprised of PAHs detected at the highest concentrations at sites in the PNW with an AQI > 100, with one mixture being based on abundance (SW12) and the other mixture only including abundant PAHs with 4 or more aromatic rings (SFM). Cultures were assessed for cytotoxicity, cytokine protein secretion, apoptosis and cell immunophenotype following treatment with wildfire mixtures. In addition, a panel of biomarkers were evaluated by qPCR for CYP450 induction and cytokine response in HBEC. To further explore pathways impacted by each mixture, global gene expression was assessed by RNA sequencing. **Results:** Each wildfire mixture resulted in unique patterns of toxicity and inflammatory response that was specific to each mixture and dependent on the presence of macrophages. For example, only SW12 caused significant cytotoxicity (p -adj<0.05) and only in the co-culture. SW12 and SFM also resulted in distinct responses for CYP1A1, CYP1B1, and inflammatory cytokine (IL-1 β , IL-6, IL-8, and TNF- α) gene expression in HBEC. Of interest, only SFM significantly (p -adj<0.05) induced both CYP1A1/1B1 transcripts in both models in a dose-dependent manner compared to SW12. **Conclusions:** Overall, it appears that signaling between epithelial and macrophage cells is necessary for a more sensitive cytotoxic response in HBEC. Furthermore, data show that unique mechanisms likely mediate toxicity by SW12 and SFM and that the macrophages have a distinct role in response to each mixture. These studies fill critical knowledge gaps regarding the role of inflammation in toxicity of inhaled PAHs from wildfires in an organotypic co-culture model.

PS 3387 The comprehensive cardiovascular toxicology assessment in multi-level of *in vitro* cell models

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Background and Purpose: Cardiovascular toxicity remains a leading cause of drug attrition during development and post-market withdrawal. The limitations of traditional, siloed testing strategies—which often fail to capture the integrated physiology of the human heart—necessitate a more holistic and predictive *in vitro* approach. To establish and validate a comprehensive, multi-level *in vitro* platform that enables a mechanistic and functional assessment of cardiovascular toxicity across a spectrum of key biological processes, from molecular targets to integrated cellular responses. **Methods:** Our platform integrates a suite of complementary assays using recombinant systems, 2D and 3D human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs): 1. Molecular Level (Ion Channel Function): Comprehensive profiling of compound interactions with key cardiac ion channels (e.g., hERG, Nav1.5, Cav1.2) utilizing a combination of manual and automated patch clamp, fluorescence-based assays, and radio-ligand binding techniques. 2. Protein-Level (Channel Trafficking): Assessment of chronic toxicity via hERG channel trafficking inhibition to identify compounds that cause channel mislocalization. 3. Integrated Cardiac Function in 2D Models: Functional phenotyping in 2D iPSC-CM monolayers, including action potential (AP) and field potential (FP) recordings, calcium transient analysis, contractility measurements and cell viability assay. 4. Integrated Cardiac Function in 3D Models: Assessment of compound effects in more physiologically relevant 3D cardiac organoids, evaluating cell viability, calcium transient dynamics, and field potential profiles. **Results:** We demonstrate that this integrated platform successfully provides a multi-parametric dataset for candidate compounds. Isoproterenol showed weak acute hERG block but shortened field potential duration. Geldanamycin had no effect on hERG current by patch clamp recording but caused cardiac toxicity by chronic effect of hERG trafficking inhibition. By correlating data across levels, we can deconstruct complex toxicological phenotypes. **Conclusions:** This multi-level *in vitro* platform offers a powerful and translational strategy for the comprehensive assessment of cardiovascular toxicity. It moves beyond standalone hERG testing to provide a systems-level understanding of compound effects, enabling earlier

and more informed decision-making in drug safety evaluation. This approach holds significant promise for reducing late-stage attrition and enhancing the cardiac safety profile of new therapeutic agents.

PS 3388 *In Vitro* Human Bronchiole Models as Promising Tools for Drug Screening with Potential Pulmonary Toxicity

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Background and Purpose: Human bronchus is the key structure of the lower respiratory tract, which serves as the main passageway for air to travel from the trachea into the alveoli for gas exchange. Its wall is lined with a ciliated pseudostratified epithelium, which is composed of mucus-producing goblet cells. These cilia rhythmically beat to propel trapped dust and pathogens upward, which is essential for lung defense. Pulmonary toxicity is lung damage that can be caused by cancer treatment, environmental exposures, or genetics. Drug-induced lung disease (DILD) has been reported in many compounds, for example amiodarone and afatinib, as a key factor for discontinuation of drug therapy. To better identify potential drug toxicity, *in vitro* pulmonary models are crucial tools for assessing the toxicity of compounds and pharmaceuticals. Among the *in vitro* pulmonary models, three-dimensional (3D) organoid model grown in Matrigel and air-liquid interface (ALI) culture model are commonly used, which better mimic the human bronchial structure, allowing screening of cytotoxicity of various compounds and pharmaceuticals. In this study, 3D human bronchial organoid model (HBO) and human ALI airway model, both static and perfused (bronchi-on-a-chip), were established with the differentiation capacity into mature mucociliary airway structure, which served as tools for *in vitro* toxicity screening. **Methods:** HBO model was established under expansion state for model characterization and drug screening purpose. Three-dimensional CellTitre Glo (CTG) experiment was conducted for drug screening using compounds with reported pulmonary toxicity. HBO was differentiated for 14 days into mature airway organoids, which were characterized using immunofluorescence staining, immunohistochemical staining and RT-qPCR analysis. Normal human bronchial epithelial (NHBE) cell ALI model was established by transwell-based ALI culture for 21-28 days and characterized with immunofluorescence staining and RT-qPCR analysis. CNBio Physiomimix system was used for establishment of perfused bronchi-on-chip model using NHBE cells for 14 days, which was compared with static NHBE model in terms of differentiation outcome by RT-qPCR analysis and histological staining. Drug screening on reference compounds with reported pulmonary toxicity and with no reported pulmonary toxicity was conducted in static and perfused NHBE ALI model for parallel comparison. **Results:** HBOs under expansion state were mainly composed of basal cells, with minimal goblet cell composition. HBOs showed dose-responsiveness to Afatinib, Doxorubicin, STS and Amiodarone, with reported pulmonary toxicity, in terms of cell viability. After 14 days of differentiation, HBOs were capable of differentiating into goblet cells and ciliated cells, which can serve as a potential model for dome-based 3D mature airway model. NHBE cells were capable of forming mucociliary barrier model by day 21 post static ALI culture, which differentiation capability showed donor variation and passage limitation. Under perfused culture condition using bronchi-on-chip model, NHBE cells are capable to differentiate into mature mucociliary structure by day 14 post ALI, which showed superior differentiation outcome compared to static culture. Perfused bronchus-on-a-chip model showed dose-responsiveness to reference compounds with known pulmonary toxicity. **Conclusions:** Three-dimensional human bronchial *in vitro* model can be established as both 3D organoid model and transwell-based barrier model. Both models showed dose-responsiveness to compounds with reported pulmonary toxicity, which serve as potential tool for drug screening purpose for identification of drug side-effects.

PS 3389 An Engineered 3D Liver Tissue Model (in vivo) for Long-Term Culture Without Medium Exchange

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Background and Purpose: Accurate preclinical evaluation of drug safety requires *in vitro* models that recapitulate key functional attributes of the human liver. Although primary human hepatocytes are widely used, maintaining essential functions for extended periods remains challenging. This study aimed to construct a three-dimensional human hepatic model capable of sustaining long-term functional stability without medium exchange and to evaluate its hepatic functionality, including metabolic competence, transcriptional characteristics, bile canaliculus formation, and responsiveness to prototypical hepatotoxicants. **Methods:** Primary human hepatocytes prepared from humanized mice with chimeric liver (PXB-cells) were treated with heparin and collagen to induce the formation of a cohesive viscous cellular body. This viscous body was then embedded into a hydrogel matrix that supports cellular accumulation and promotes stable cell-cell adhesion, thereby enabling the organized formation of bile canaliculi and the emergence of enhanced hepatic functionality. The resulting three-dimensional tissue construct (in vivo) was subjected

to a series of evaluations encompassing metabolic function, transcriptomic signatures, bile canaliculus formation, and responsiveness to benchmark hepatotoxic compounds. Long-term culture stability without medium exchange was examined from day 8 onward. Additionally, the model was continuously exposed to carbamazepine for three weeks without medium exchange, and metabolite formation and concentration-dependent cytotoxicity were assessed using mass spectrometry and cell viability assays. **Results:** The invivo model exhibited hepatic functions that exceeded those of spheroid and sandwich cultures. Confocal imaging and bile acid transport assays showed a well-developed bile canaliculus network with clear MRP2/BSEP localization and CDFDA/CLF excretion. Transcriptomic profiling demonstrated comparable or greater expression of key metabolic genes, including CYP3A4, CYP2C9, CYP1A2, and MRP2. PCA of 61 ADME-related genes positioned invivo closer to human total liver than the comparator models, and pathway enrichment analyses highlighted metabolic and transporter-related processes. Functionally, invivo showed greater sensitivity to hepatotoxicants, particularly cholestasis-associated compounds. IC_{50} values for compounds such as bosentan, cyclosporine A, and danazol were substantially lower than in spheroids, indicating enhanced detection of cholestatic hepatotoxicity. Long-term culture evaluation demonstrated that invivo maintained viability, bile canaliculus function, hepatic gene expression, and metabolic activity for at least two weeks without medium exchange. Only limited numbers of differentially expressed genes were detected between medium-change and no-medium-change samples. Furthermore, induction of CYP1A2, 2B6, and 3A by omeprazole, CITCO and rifampicin, respectively, was retained, confirming functional robustness. Additionally, invivo enabled extended testing of low-clearance compounds. Three-week exposure to carbamazepine resulted in concentration-dependent toxicity and produced an S-cysteine conjugated metabolite detectable by LC-MS/MS, whereas such metabolites were not observed in prior spheroid studies. These findings indicate that invivo supports long-term metabolic evaluations and reveals metabolic profiles and metabolite-driven hepatotoxicity not captured by conventional *in vitro* systems. **Conclusions:** The invivo model exhibits higher liver functional performance and superior toxicity-prediction sensitivity compared with conventional *in vitro* systems. It supports long-term hepatic function and enables extended low-clearance compound exposure without medium exchange. These results indicate that the model is highly suitable for evaluating metabolite-driven toxicity, pharmacokinetic clearance behavior, and *in vitro-in vivo* extrapolation (IVIVE) in drug safety research.

PS 3390 Implementation of the LEAP assay in stem cell-derived cardiomyocyte to detect sodium channel blockers in pre-clinical safety

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Background and Purpose: Drug-induced QRS interval widening on an ECG is an uncommon but concerning effect as it typically is associated with a sodium channel blocker which results in a disruption of ventricular depolarization. QRS prolongation was identified on an anesthetized dog CV study as part of the screening progression for a discovery project. Sodium channel blockage is highly correlated with sudden death; therefore, the team evaluated and adapted the screening strategy to be able to detect this unwanted effect prior to running a dog CV study. **Methods:** First, the team established that the compound-associated QRS widening was consistent with other sodium channel blockers and could be detected in a modified anesthetized rat CV model that added ECG collection and analysis. This was a first key step as it allowed for screening for new compounds in rats and removed the dependence on using dog CV. Second, the team validated a new capability on our existing stem cell-derived cardiomyocyte (hiPSC-CM) platform, Local Extracellular Action Potential (LEAP), which allowed us to screen compounds using a plate-based *in vitro* system. The key endpoint in the hiPSC-CM *in vitro* platform assesses the rise time of the cardiac action potential which is sodium channel-dependent. **Results:** The team demonstrated that compounds that produce QRS widening *in vivo* also increase rise time in the LEAP assay, while compounds that did not impact QRS *in vivo* had no effect on rise time in the LEAP assay. **Conclusions:** This provided valuable proof-of-concept data to the project team that convinced them to run all additional compounds through the *in vitro* LEAP assay before progressing into an *in vivo* model. Furthermore, those compounds that do progress to the *in vivo* rat CV model are now run with ECG analysis to ensure this liability will not be an issue in a dog CV study.

PS 3391 Bridging In Vitro and In Vivo Inhalation Toxicity: Dosimetry and Deposition Assessment of Organic Solvents and Nanoparticles in Air-Liquid Interface Systems

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Background and Purpose: Developing new approach methodologies (NAMs) is essential to reduce animal testing, driven by ethical and cost considerations, particularly in safety assessment. Reliable NAMs for inhalation toxicity are needed to ensure

consistency with traditional *in vivo* studies. This study evaluated key dosimetry and dose-metric parameters in *in vitro* inhalation toxicity tests for organic solvents and particles using an air-liquid interface (ALI) system, to align *in vitro* data with *in vivo* outcomes. **Methods:** Isopropyl alcohol (IPA) and acetone vapors were generated and exposed to the ALI system for 1-2 hours at various generation rates. Protein nanoparticles were also aerosolized for ALI exposure. Key dose metrics—including particle size, number, vapor, and particle concentration stability, and deposition variability—were assessed. Central deposition mechanisms (diffusion, impaction, and gravitational settling) were also analyzed. **Results:** *In vitro* ALI deposition experiments showed average coefficients of variation (SD/mean) below 10% for IPA and acetone vapors across 6-transwells, and below 20% for protein particles. Deposition across 6-transwell plates was uniform for the vapors, with no statistically significant variation among wells. For particles, deposition showed some variability across experimental series. **Conclusions:** Diffusion was identified as the dominant deposition mechanism for both organic solvent vapors and nanoparticles under *in vitro* ALI conditions. When *in vitro* deposition profiles and dose metrics align with *in vivo* data and regulatory criteria, NAM-based ALI systems can serve as reliable alternatives to traditional animal inhalation studies, supporting global efforts to reduce animal testing in toxicology.

PS 3392 High-Content Mechanistic Profiling in a 3D Mini-Testis Model: Integration of Proliferation and Epigenetic Markers for Comprehensive Toxicity Screening

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Background and Purpose: Increasing evidence linking environmental chemical exposures to the global decline in male reproductive health underscores an urgent need to develop efficient and mechanistically informed New Approach Methodologies (NAMs) for reproductive toxicity screening. Addressing this challenge requires advanced *in vitro* systems that can capture the complex cellular interactions and comprehensive mechanistic pathways disrupted by toxicants, enabling high-throughput, quantitative, and pathway-based evaluation of male reproductive hazards. Our group previously developed a 3D mouse mini-testis model integrated with high-content analysis (HCA), which demonstrated strong concordance with *in vivo* data using key markers of DNA damage (γH2AX) and cytoskeletal integrity (F-actin). To capture a broader spectrum of toxicological mechanisms, we expanded the assay panel to include additional mechanistic endpoints, cell proliferation and cell cycle (BrdU incorporation), and epigenetic modification (Methyl-CpG-binding protein 1, MBD-1), representing critical and distinct molecular targets of reproductive toxicants. **Methods:** A 3D mini-testis model consisting of murine spermatogonia, Sertoli, and Leydig cells embedded in an extracellular matrix (ECM) was used to evaluate 87 compounds from the National Toxicology Program library, including known reproductive toxicants and non-toxicants. Following exposure, 3D mini-testis were sequentially labeled for DNA damage (γH2AX), cytoskeletal structure (F-actin), and DNA Methylation (MBD-1) prior to cell proliferation marker of BrdU labeling. This sequential labeling strategy preserved cellular morphology and antigenicity before DNA denaturation required for BrdU staining. Multi-channel fluorescence images were acquired using an automated high-content imaging system (Arrayscan VTI, Thermo Scientific) and analyzed with AI-assisted pipelines to quantify nuclear intensity, cytoskeletal organization, proliferation, and co-localization metrics. Mechanistic endpoint scores were integrated into a composite toxicity index, and hierarchical clustering with ToxPi visualization classified compound-specific mechanistic fingerprints. **Results:** The newly incorporated proliferation (BrdU) and epigenetic (MBD-1) were successfully validated, showing robust, dose-dependent responses to known reproductive toxicants. MBD-1 chromatin signature, observed with methylmercuric chloride and tris (2-chloroethyl) phosphate (TCEP), revealed altered chromatin organization and DNA methylation associated with protein binding preceding overt cytotoxicity. This finding is biologically significant, as early chromatin remodeling in germline precursors may lead to persistent or heritable epigenetic changes. Anti-proliferative compounds such as colchicine and 5-fluorouracil showed significant BrdU suppression at 1-10 μM, consistent with S-phase inhibition in spermatogonial cells. Valinomycin exhibited an early broad cytotoxic profile, concurrently affecting viability, MBD-1, and BrdU endpoints. Notably, MBD-1 response preceded BrdU suppression, identifying epigenetic remodeling as an early mechanistic event. Top-ranked compounds within these distinct phenotypic patterns defined three major toxicity pathways: (1) anti-proliferative (colchicine, 5-FU), (2) broad cytotoxic (valinomycin), and (3) epigenetic/chromatin remodeling (TCEP, methylmercuric chloride). Integration of all five marker classes into a composite toxicological index (ToxPi) enabled higher hierarchical clustering analysis of compounds by their primary mechanism of action, substantially enhancing the platform's descriptive and predictive performance. **Conclusions:** The expanded multi-parametric 3D mini-testis model provides a robust and comprehensive platform for screening, classifying, and prioritizing male reproductive toxicants. Its ability to detect early epigenetic remodeling and mechanistic disruption without requiring long-term animal exposure highlights its value as a rapid and mechanistically informative NAMs. This enhanced platform strengthens predictive power for reproductive toxicity and advances the broader goal of reducing and ultimately replacing traditional animal testing in chemical risk assessment.

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Background and Purpose: A powerful new approach methodology (NAM) for lung research is the human precision-cut lung slice (hPCLS). To prolong hPCLS use, we utilized cryopreservation and tested the hypothesis that cryopreservation retains cellular, transcriptional, and functional regulation of effector cell types/states in hPCLS from fibrotic lungs. **Methods:** Using the 10x Genomics Visium platform, we performed spatial sequencing of preserved cells within cryopreserved (up to 1 year) hPCLS from three healthy and three fibrotic donor lungs. Measurements were performed immediately upon tissue thaw and compared with published spatial transcriptomic datasets generated from never-frozen human lung fibrotic biopsies. **Results:** Enabled by marker gene expression profiles, we identified twenty-three cell populations within the cryopreserved healthy and fibrotic hPCLS. Further comparative analysis revealed enhanced fibroblasts, myofibroblast, and historically under-represented endothelial and rare cell type expression, and reduced AT1, AT2 and basal epithelial cell expression in the cryopreserved fibrotic hPCLS. These cellular changes were spatially co-incident with 1) collagen-rich regions enriched for extracellular matrix (ECM)-related genes (e.g., COL1A1, SPARC), 2) expanded fibroblast-endothelial crosstalk with enhanced chemokine signaling (CXCL12-CXCR4), 3) integrin-driven mesenchymal signaling targets (ANGPTL-integrin), 4) stress-adapted and immune-interacting Alveolar Type 2 cells with impaired surfactant gene expression, and 5) endothelial reprogramming toward a contractile and profibrotic phenotype. At a functional level, these spatially-defined fibrotic niches were characterized by increased immune responses (IGKC and IGHG1), fibrosis (collagen, fibronectin, and DCN), mechanotransduction (MYH11, TPM2, ACTG2, ACTA2), immune cell recruitment (CCL2, IL6, ICAM1), ECM remodeling (e.g. thrombospondin 1, THBS1), and surfactant production (SFTPB, SFTPC). At a molecular level, they coincide with activation of three fibroblast mediating signaling pathways: the matricellular glycoprotein, SPARC, Relaxin signaling, and immunoglobulin transcripts including IGHA1, IGHG1, IGHG3, and IGKC. Notably, these cryopreserved fibrotic hPCLS measurements closely resemble published findings with never-frozen human lung fibrotic biopsies. **Conclusions:** We verify model fidelity, identify known and new pro-fibrotic genes, and promote using cryopreserved human hPCLS for disease modeling (e.g. fibrosis) and more generally, for drug discovery and toxicity testing.

PS 3394 Establishing and Validating an *In Vitro* Seizure Liability Assay with hiPSC-Derived Glutamatergic Neurons and Astrocytes

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Background and Purpose: Drug-induced seizures are serious adverse reactions that can arise during drug development. Current methods for assessing seizure liability primarily rely on animal studies; however, these approaches face limitations including low throughput, variable outcomes due to species differences, and decreasing societal acceptance of animal testing. Emerging new approach methodologies (NAMs) offer promising alternatives for evaluating the seizure liability of drug candidates and have gained increased attention. Human induced pluripotent stem cell (hiPSC)-derived neural cell models have been shown to recapitulate aspects of the central nervous system and predict the seizurogenic potential of test compounds. Nevertheless, establishing a robust assay that consistently delivers reproducible results remains challenging. Furthermore, there is a lack of comprehensive information regarding the applicability and limitations of these assay systems. **Methods:** We developed a co-culture system of hiPSC-derived neurons and astrocytes to record extracellular field potentials using a multi-electrode array (MEA) system. To achieve sufficient throughput for investigational toxicology, we cultured iCell® glutamatergic neurons and iCell® astrocytes (both from FUJIFILM Cellular Dynamics, Inc.) in 96-well Cytoview MEA plates (Axion Biosystems, Inc.) following the instructions published by manufacturer of iCell® products (FUJIFILM Cellular Dynamics, Inc.). We established a dose-escalation protocol to assess the dose-response effects of pharmaceutical compounds using up to four concentration levels. Extracellular field potentials were recorded at each dose, and neural activity parameters, including network burst frequency, were analyzed. **Results:** We confirmed that the system can detect the effects of 12 compounds with diverse pharmacological mechanisms, including GABA antagonists, D2R antagonists, K⁺ channel blockers, M2R agonists, A1R inhibitors, GLR antagonists, and an antipyretic agent as a negative control. The lowest concentrations at which effects on neural activity were observed and reproducible across two to four independent experiments, with variability within a 10-fold range. These findings are consistent with the published data such as the cerebrospinal fluid (CSF) concentrations associated with seizure induction in animal studies. **Conclusions:** In conclusion, we have established a robust and high-throughput *in vitro* assay using a co-culture of hiPSC-derived glutamatergic neurons and astrocytes combined with an MEA system (dose escalation protocol in 96-well plate format). This assay accurately detects known pharmacological effects of seizurogenic drug

compounds. It offers a valuable tool for assessing the safety profiles of candidate drugs at early stages of drug discovery, thereby contributing to more successful drug development by reducing the risk of seizurogenic adverse effects in later phases.

PS 3395 The use of multiple cell culture models for developing quantitative Adverse Outcome Pathways (qAOPs) for genotoxicity

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Background and Purpose: DNA damage can lead to a number of safety issues, and the development of adverse outcome pathways (AOPs) for chemical toxicity has been utilised to assess the risk of carcinogenicity and better understand the mechanisms involved. AOPs typically link a molecular initiating event to an adverse outcome via multiple causally related key events, and current genotoxicity AOPs are generally qualitative, but chemical risk assessment may be better supported by quantitative AOPs (qAOPs). **Methods:** This study aimed to support the development of qAOPs by providing robust data for different KEs on a network of genotoxicity AOPs using the cytokinesis-blocked micronucleus assay, assessing chemicals with different modes of action: electrophiles (MMS & B[a]P), radical species inducers (hydroquinone, sodium arsenite) & a topoisomerase II inhibitor (dexamethasone). **Results:** TK6 cells, HepG2 cells in a 2D model, and 3D HepG2 spheroids were dosed with each compound for 1.5 cell cycles and subsequently analysed for micronucleus induction using the Metafer semi-automated scoring system. MMS gave lowest observed adverse effect levels (LOAELs) of 9.1µM, 68.1µM, and 45.4µM in TK6 cells, the 2D HepG2 cell model and 3D HepG2 spheroids, respectively. When treating the TK6 cells and 2D HepG2 model with hydroquinone, LOAELs of 50µM were determined, although this was reduced to 40µM when treating the 3D HepG2 spheroid model. Further, there was a 1.65-fold increase in micronuclei over background when treating the 2D HepG2 model with 1µM AFB1, but a 1.86-fold increase when treating the 3D HepG2 spheroids with the same concentration. Additionally, treating the 3D HepG2 spheroid model with B[a]P at 15µM yielded a 2.17-fold increase in micronuclei over background levels. **Conclusions:** This data collectively demonstrates clear differences in sensitivity and metabolic activity between the cell culture models in determining chemical cellular toxicity. This demonstrates the clear differences between the cell culture models in determining cellular toxicity and this data will be used to support the development of qAOPs for chemical risk assessment. Moreover, ongoing work aims to perform dose-dependent analysis of reactive oxygen species (ROS) production and γH2AX investigation which will elucidate the mechanisms involved in eliciting genotoxicity and support the development of qAOPs by determining the point at which one key event tips into the next.

PS 3396 Building a predictive structure activity relationship tool to help avoid ion channel related central nervous system liability in novel compounds

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Background and Purpose: Unwanted central nervous system (CNS) liability remains a significant cause of attrition throughout pre-clinical and clinical drug development. Human-based new approach methods are needed to provide an earlier prediction of CNS risks to influence medicinal chemistry and reduce reliance on costly animal studies. Previously we have demonstrated the value of iSLA, an *in vitro* CNS liability assay based on 1) a CNS-related ion channel panel evaluated via electrophysiology, and 2) an human induced pluripotent stem cell (hiPSC)-neuronal model analysed using microelectrode array (MEA) to measure electrical activity in response to known seizurogenic compounds. Therefore, to assist in the development of therapies without CNS liability, we embarked on a study to develop SAR (structure activity relationships) by identifying the structural characteristics of the compounds that were associated with ion channel inhibition. **Methods:** The Enamine REAL database is the largest database of synthetically feasible molecules that can assist in SAR investigations. 78 compounds were selected from the Enamine REAL database based on their structural similarity to the 10 selected test compounds (amoxapine, diphenhydramine, quetiapine, 4-AP, linopirdine, bepridil, NS1619, quinidine, verapamil, XE991). These 88 compounds were screened using cells expressing the human isoforms of Na_v1.2, K_v2.1, GABA-α₂β₂γ₂ or nicotinic-α₄β₂ ion channels with automated electrophysiology (QPatch II, Sophion) and the fingerprint of activity was compared to the parent compound for each ion channel. A subset of these compounds were also screened with hiPSC-neurons to assess whole cell responses across MEA parameters. **Results:** The 78 compounds tested showed a range of activities (IC₅₀ values) across the Na_v1.2, K_v2.1, GABA-α₂β₂γ₂ and nicotinic-α₄β₂ ion channels that were different to the 10 parent molecules. Initial analysis showed some structural features had a statistically significant higher potency at K_v2.1 suggesting these may present a significant risk, which may extend to other channels. Significant differences in MEA parameters were also observed in hiPSC neurons incubated with amoxapine, diphenhydramine and quetiapine compared to selected Enamine REAL compounds. For example, iPSC-neurons dosed with 1 µM amoxapine exhibited a clear seizurogenic

phenotype (increased firing rate and network burst frequency) whereas minimal changes in activity were detected with the derivative compound PV-002730229172. This is reflective of the decreased potency of PV-002730229172 across all 4 ion channels screened (IC_{50} at $Na_v1.2 = 64.8 \mu M$, $K_v2.1 = 15.1 \mu M$, $GABA-\alpha_1\beta_2\gamma_2 > 100 \mu M$ and nicotinic- $\alpha_4\beta_2 = 10.7 \mu M$) compared to the amoxapine parent compound (IC_{50} at $Na_v1.2 = 26.2 \mu M$, $K_v2.1 = 6.7 \mu M$, $GABA-\alpha_1\beta_2\gamma_2 = 2.6 \mu M$ and nicotinic- $\alpha_4\beta_2 = 7.4 \mu M$). The loss of the halogen group and replacement of a hydrogen molecule with a bulkier ethyl chain may contribute to the decreased potency observed with PV-002730229172. **Conclusions:** We have demonstrated that we can develop SAR for compounds that interact with neuronal ion channels. Future work testing the compound set across more ion channels will provide more evidence and help to build a predictive SAR tool. This will help structural chemists avoid CNS liability in the development of novel compounds and reduce attrition during pre-clinical drug development phases. Collectively, this approach can be utilised for early de-risking and optimised drug design using human-relevant models that will save time, money and resources resulting in a more streamlined drug discovery process and ultimately improved patient safety.

PS 3397 Utilising *in vitro* assays to assess drug induced central nervous system liabilities

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Background and Purpose: Central nervous system (CNS) liabilities, such as seizures, sedation, tremors and cognitive impairments, remain a significant cause of attrition during pre-clinical and clinical drug development. However, they are typically not identified until late-stage *in vivo* toxicology studies where adverse findings can result in costly delays or project termination. Further, ~25% of clinical trial failures are due to safety concerns that were undetected in earlier *in vivo* studies highlighting translatability concerns. This emphasises the need for predictive, human-relevant *in vitro* assays that can be implemented earlier in the drug development process to assess CNS safety risks. We have previously developed two complementary *in vitro* CNS safety assays: 1) a CNS-related ion channel panel evaluated via automated electrophysiology, and 2) an human induced pluripotent stem cell (hiPSC)-neuronal model analysed using microelectrode array (MEA) to measure electrical activity in response to known seizurogenic compounds. In addition to seizure risk, sedation is a common adverse finding in preclinical studies. GABAergic signalling plays a crucial role in the sleep-wake cycle, with GABA_A receptor activation resulting in sedation. To address this, we aimed to validate our *in vitro* CNS assay for predicting sedative compound effects. **Methods:** We tested 16 compounds with reported sedative activity (targeting both GABA and non-GABA pathways) and 5 non-sedative controls in the hiPSC-neuronal model using MEA. Changes in activity from baseline across a range of parameters (weighted mean firing rate [WMFR], network burst frequency [NBF], synchrony) were assessed to determine the whole cell phenotypic response. These compounds were further screened with automated patch-clamp electrophysiology (QPatch II, Sophion) using Chinese hamster ovary (CHO-K1) cells expressing the human isoforms of, $K_v1.1$, $K_v2.1$, $K_v3.1$, $K_v7.2/7.3$, $Na_v1.2$, $GABA-\alpha_1\beta_2\gamma_2$, nicotinic- $\alpha_4\beta_2$ or TREK-1. **Results:** MEA analysis showed that GABA_A agonists (muscimol, etomidate, ethanol) and certain ion channel antagonists (valbenazine, dextromethorphan) reduced neural firing and network burst activity, indicating sedative effects. For example, hiPSC-neurons treated with 3 μM muscimol exhibited a 57% decrease in WMFR and 100% decrease in NBF and overall synchrony compared to vehicle treated cells. Similar effects were observed in cells incubated with 500 mM ethanol, 4 μM etomidate, 10 μM valbenazine or 6 μM dextromethorphan. Negative control compounds produced no significant changes in MEA parameters. Of the 21 known compounds screened, 90% were accurately classified. Automated patch-clamp electrophysiology further confirmed these findings by testing compound interactions with GABA_A and other CNS ion channels. For example, $K_v2.1$, nicotinic- $\alpha_4\beta_2$ and TREK-1 were most frequently hit and valbenazine was the most promiscuous compound, inhibiting all but 2 channels screened. **Conclusions:** These results support the use of our *in vitro* CNS models for detecting sedative and seizurogenic effects, thus enhancing translational relevance. These platforms offer valuable human-based tools for early hazard identification and prioritisation of drug candidates, as well as interpreting CNS-related findings from *in vivo* studies thus complying with the principles of 3Rs to minimise and replace the use of animals in scientific testing. Ongoing work is focused on expanding this model to evaluate additional sedation-related ion channels.

PS 3398 Application of a Human Reporter-Based Live-Cell Imaging Platform for Detection of Mitochondrial Toxicity

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Background and Purpose: Impairment of mitochondrial function by drugs and chemicals represents a major mechanism underlying toxicity and a common cause of late-stage drug development failure. Early detection of mitochondrial liabilities is therefore crucial for strengthening chemical safety evaluation; however, many existing

screening strategies lack sensitivity and specificity, limiting their predictive value for chemical safety and human health risk assessment. **Methods:** To address this limitation, we developed a human reporter cell-based assay designed for confocal imaging of mitochondrial networks in living cells. The assay utilizes the HepG2 CYC1-GFP reporter line, where CYC1—part of complex III within the mitochondrial electron transport chain (ETC)—is tagged with Green Fluorescent Protein (GFP), enabling real-time visualization of mitochondrial morphology. Quantitative image analysis was performed using morphological (Symmetry-Threshold Compactness-Axial-Radial; STAR) and texture (Spot-Edge-Ridge; SER) parameters, which together capture distinct features of mitochondrial organization. These metrics reflect changes in mitochondrial network patterns and distribution, such as shifts from tubular to fragmented phenotypes, allowing sensitive detection of structural alterations linked to mitochondrial stress. **Results:** Validation was performed using a reference panel of 20 compounds with well-characterized mitochondrial or non-mitochondrial toxicity profiles (e.g. rotenone, antimycin A, and tunicamycin). The complex I ETC inhibitor rotenone induced pronounced changes in mitochondrial morphology, evident from a marked transition from tubular to punctate mitochondrial networks at a non-cytotoxic concentration (0.09 μM). In contrast, the non-mitochondrial toxicant tunicamycin did not induce changes in mitochondrial morphology, confirming the ability of the assay to distinguish mitochondrial-specific effects. **Conclusions:** In conclusion, these findings demonstrate that morphology-based analysis using a human reporter cell-based model provides a mechanistically informative tool for early and more accurate detection of mitochondrial toxicity.

PS 3399 Self-assembling Peptide Hydrogels As User-defined Synthetic Matrices for *In Vitro* Modelling.

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Background and Purpose: Current 3D *in vitro* models typically rely on animal-derived materials, such as those derived from Engelbreth-Holm-Swarm mouse sarcoma (e.g., Matrigel). However, these materials are ill-defined, have high batch variability, and do not accurately represent human matrices. Researchers require a more reliable, reproducible, and ethical model platform (Nitsche *et al.*, 2025, doi 10.3389/ftox.2025.1649393). **Methods:** A synthetic self-assembling peptide hydrogel (SAPH) platform has been developed that addresses all the current shortcomings of available 3D *in vitro* models. The SAPH is well-defined, has limited batch variability, and is fully customisable, both in terms of stiffness and composition, to mimic the composition of any target tissue type. The end stiffness of the hydrogel can be controlled by adjusting the concentration of gelator peptide used during preparation. This allows the design of different hydrogels which match the stiffness of different tissue types. Customisation with soluble matrix components or choosing a gel with amino acid motif functionalisation can influence end gel stiffness. However, this can be accounted for by adjusting the initial concentration of gelator peptide. **Results:** Brightfield and immunofluorescence images of mouse embryonic stem cells (mES), U937 acute myeloid leukaemia cells, MCF breast cancer cells, and human mammary fibroblasts cultured in unmodified PeptiMatrix™ Core hydrogels will be presented. These results demonstrated the compatibility of PeptiMatrix™ with a wide variety of cell types without matrix modification. The optical clarity of PeptiMatrix™ hydrogels enabled flexible, high-resolution assays of cell growth and pluripotency. Real-time quantification of growth will be presented. End-point immunostaining of mES and MCF7 cells demonstrated compatibility with fluorescence imaging and marker analysis. Pre-customised formulations containing integrin-binding motifs from key extracellular matrix proteins: fibronectin (RGD), laminin (IKVAV), and collagen (RGD+GFOGER) are covalently immobilised within the hydrogel scaffold, providing stable and biologically relevant signals. These pre-customised peptide-functionalised hydrogels influence cell adhesion, behaviour, and differentiation, often promoting the emergence of more physiologically relevant, *in vivo*-like responses. Human induced pluripotent stem cells (hiPSCs) were cultured on PeptiMatrix™ hydrogels. Colonies remained viable across all conditions, with enhanced viability and colony spreading observed in laminin-modified gels. Pluripotency was maintained in all cultures, as confirmed by positive Oct4 staining. A range of human leukemic cell lines remained viable and formed colonies within unmodified SAPH, confirming the suitability of this matrix for 3D culture. U937 cells showed sustained growth across increasing hydrogel concentrations, while phase-contrast imaging demonstrated colony formation at different seeding densities across cell lines. To evaluate translational relevance, THP-1 cells were encapsulated in SAPH and treated with candidate drugs. Distinct responses were observed between early-stage and established models, highlighting the potential of SAPH-based systems to capture treatment efficacy in physiologically relevant conditions. **Conclusions:** The use of a SAPH, which can be selectively customised to mimic the stiffness and composition of different tissues, provides a reliable, reproducible, and ethical platform for modelling a variety of different cell lines.

PS 3400 Delivery of mRNA-based fusion protein reporters in primary hepatocytes for real-time quantitative imaging of mitochondrial morphology and dynamics

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Background and Purpose: Mitochondria are critical sensing organelles essential for metabolism. ROS are common byproducts of oxidative phosphorylation. Mitochondria are the sites for producing the chemical energy molecule ATP via oxidative phosphorylation. Their structure, function, and fusion/fission are dynamically tuned in response to intrinsic and extrinsic stresses. Impact on mitochondria has proven to be an early mechanistic indicator. Their regulation is important because of their potential to cause oxidative damage to DNA, proteins, and lipids. *In vitro* cell-based high-content screening (HCS) technology combining automated microscopy and quantitative image analysis can address biological questions such as mitochondrial stress. However, we previously found that chemical dye-based approaches such as MitoTracker are not practical for primary hepatocyte real-time imaging and texture analysis due to their intrinsic limitations: 1) they permeabilize in less viable cells while fading significantly after approximately 1 hour; 2) they are harmful to cells during extended incubation; 3) they are not flexible for customization and multiplexing. We reasoned that multiplexed mRNA-based reporter (namely, MMR) transfection in primary hepatocytes may overcome those limitations and allow monitoring of mitochondria qualitatively and quantitatively in a robust manner. **Methods:** We engineered four *in vitro* transcribed mRNAs encoding distinct subcellular reporters (mito-MMR hereafter): 1) iRFP670-Fis1¹⁰¹⁻¹⁵² (iRFP670 fused with Fis1 amino acids 101-152, serves as a mitochondrial membrane reporter); 2) 53BP1¹²²⁰⁻¹⁷¹¹-mTagBFP2 (53BP1 amino acids 1220-1711 fused with mTagBFP2, serves as a nucleolus reporter); 3) mApple-LC3B (serves as the reporter of autophagosome/autolysosome); and 4) Cox8-roGFP2-GRX1 (Cox8 mitochondrial inner matrix targeting peptide fused with ROS sensing roGFP2 and GRX1 in tandem, serves as a mitochondrial inner matrix ROS sensor). The mito-MMR was delivered to rat, cynomolgus monkey, and human primary hepatocytes by either electroporation (EP:mRNA) or lipid-based delivery methods (LNP:mRNA). **Results:** Through optimized EP:mRNA and LNP:mRNA procedures, we achieved rapid and efficient expression of mito-MMR simultaneously in rat, cynomolgus monkey, and human primary hepatocytes. The expression of mito-MMR lasts for approximately six days, offering good windows for cell-based imaging assays. Importantly, by testing a series of known mitochondrial toxicants, we demonstrated that mito-MMR achieved single-cell-level real-time quantitative imaging of mitochondrial dynamics—including fusion, fission, and mitophagy. What's more, this approach allows us to multiplex with LDH release assay or other destructive cell viability assays in parallel to correlate the early morphological changes with cellular viability. We observed that mitochondrial morphology disruption by known toxicants at early time points aligned with decreased oxygen consumption rates (OCR) in Seahorse assays and predicted 24-hour viability outcomes. **Conclusions:** This MMR approach has advanced our current *in vitro* hepatotoxicity screening capabilities by providing early mechanistic insights, thus enhancing our understanding of compound-related safety risks. It has the potential to increase technical success rates by derisking potential mitochondrial toxicity and overt disruption of bioenergetic pathways, which supports acceleration of early safety screening and lead optimization involving diverse modalities including small molecules, protein degraders, and gene therapy. In summary, we are the first to report that multiplexed mRNA-based fusion protein reporter (mito-MMR) transfection in primary hepatocytes achieved real-time quantitative imaging of mitochondrial morphology and dynamics. Our collective findings demonstrated that we can use MMR with high-content live-cell imaging to investigate key biological processes in real time, paving the way for endless possibilities of other MMR assays in primary cells and complex culture systems.

PS 3401 Leveraging a Human-Based Microphysiological System for In Vitro Pharmacokinetic Assessment of Oral Drug Formulations

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Background and Purpose: Pharmacokinetics plays a pivotal role in preclinical drug development, yet traditional *in vivo* studies are often limited by cost, complexity, and translational relevance. Microphysiological systems (MPS), which integrate reconstructed human organ models under dynamic flow conditions, offer a promising alternative for simulating absorption, distribution, metabolism, and excretion (ADME) *in vitro*. This study explores the utility of a three-organ MPS platform to evaluate the pharmacokinetics of an active pharmaceutical ingredient (API) delivered via a self-nano-emulsifying drug delivery system (SNEDDS), a formulation strategy designed to enhance oral bioavailability and protect APIs from degradation and first-pass metabolism. **Methods:** A human-based MPS platform comprising EpiIntestinal™ (intestine), TruVivo™ (liver), and HK-2 (kidney) cultures connected through a series of semi-permeable membranes and a micro syringe pump (McKim et al., 2024) was employed to assess the pharmacokinetics of an API formulated in a SNEDDS. Preliminary cytotoxicity (MTT for intestine; ATP, Urea, LDH for liver; ATP, KIM-1, β-NAG for Kidney) and intestinal absorption studies were conducted using three SNEDDS variants (A, B, and

C) at a final concentration of 0.4% v/v. Based on absorption performance and safety profiles, SNEDDS C was selected for full pharmacokinetic evaluation in the MPS. The API (200 µg/mL) was loaded in SNEDDS C in buffer and applied to the apical surface of the intestinal tissue, and media samples were collected from all organ compartments over 72 hours. API concentrations were quantified via liquid chromatography tandem mass spectrometry (LC-MS/MS). **Results:** Minimal to no cytotoxicity was observed in response to 24-hour exposure to the SNEDDS, whether delivered alone or loaded with the API (100, 10, and 1 µg/mL for the intestine mimicking oral exposure; 20, 2, and 0.2 ng/mL for liver and kidney mimicking predicted *in vivo* C_{max}). When compared to the API delivered in buffer, two of the three SNEDDS facilitated slightly increased intestinal absorption of the higher doses of the API (100 and 10 µg/mL) over the 24-hour exposure period, with permeability coefficient equal to 1.50 ± 0.03 x10⁻⁶ cm/s, 1.68 ± 0.05 x10⁻⁶ cm/s and 1.69 ± 0.04 x10⁻⁶ cm/s for API alone, API in SNEDDS B, and API in SNEDDS C, respectively. In the MPS platform, the candidate SNEDDS, SNEDDS C, permitted sustained absorption of the API through the intestinal culture, which was subsequently distributed throughout the liver and kidney compartments. **Conclusions:** This study demonstrates the value of MPS platforms for evaluating formulation-dependent pharmacokinetics in a human-relevant, interconnected organ system. While SNEDDS C modestly improved intestinal absorption, it did not significantly alter downstream pharmacokinetics, supporting its role as a protective oral delivery vehicle. Importantly, the findings underscore the MPS platform's potential as a translational tool for formulation screening, mechanistic ADME analysis, and physiologically based pharmacokinetic (PBPK) modeling, bridging the gap between *in vitro* and *in vivo* pharmacokinetics.

PS 3402 Quantifying signaling-perturbation kinetics for *in vitro* developmental toxicity testing

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Background and Purpose: Embryonic and fetal development is governed by coordinated signaling interactions that regulate cell proliferation, differentiation, tissue formation, and morphogenesis. Traditional animal studies have provided indispensable information through the evaluation of developmental outcomes such as malformations, growth retardation, and embryo-fetal death. These approaches have established the foundation of developmental toxicology, but ensuring reliability and interspecies translation requires substantial resources. While certain developmental processes can be modeled *in vitro*, it remains challenging to comprehensively recapitulate the morphological outcomes observed in animal studies. Therefore, identifying measurable surrogates that reflect developmental toxicity is essential for advancing human-relevant testing. We focused on intercellular signaling, which regulates developmental processes and can be quantitatively captured as dynamic responses to chemical exposure. To address this challenge, we aimed to establish an *in vitro* assay that quantitatively monitors signaling-perturbation kinetics as a surrogate indicator of developmental toxicity using human iPS cells. **Methods:** We established a human induced pluripotent stem (iPS) cell-based live-cell luciferase assay, termed DynaLux/c, to monitor dynamic changes in signaling responses. In this system, NanoLuc luciferase expression is controlled by Serum Response Factor (SRF), which primarily functions downstream of the MAP kinase pathway but can also respond to other developmental signaling inputs. Luminescence was continuously recorded during chemical exposure to quantify changes in SRF activation kinetics as a readout for signaling perturbation. The area between curves (ABC) was calculated between the solvent control and each test concentration, and the resulting ABC values were summed across concentrations to provide a quantitative index of signal disruption. **Results:** The DynaLux/c assay detected compound-dependent changes in SRF activation kinetics, including enhancement or attenuation of pathway activity at different time points. ABC-based analysis quantitatively distinguished known human developmental toxicants from non-toxic reference compounds. These findings suggest that quantifying signaling-perturbation kinetics provides a practical and reproducible approach for assessing chemical effects associated with developmental toxicity. **Conclusions:** The DynaLux/c system enables quantitative evaluation of chemical-induced perturbations in signaling responses using human iPS cells. This approach provides a human-relevant NAMs framework for developmental toxicity testing and supports the establishment of mechanism-based, non-animal assessment strategies.

PS 3403 RNA-seq can be used for gene expression quantification in the GARDskin method for assessment of skin sensitizers

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Background and Purpose: The GARDskin assay is a non-animal method for identifying skin sensitizers. Its development and validation have been described in a series of publications, and it is currently included in the OECD test guideline 442E. The assay infers the sensitization potential of test chemicals based on gene expression

profiles, generated according to the standard GARDskin protocol using NanoString nCounter technology. While the nCounter platform possesses several advantageous properties, including rapid sample acquisition, reproducible results, and easy data handling, it is relevant to explore alternative acquisition platforms. Broader platform compatibility could make the GARDskin technology more accessible and extend the ability to perform secondary exploratory analyses. In this work, we investigated whether RNA-seq could be used as an alternative acquisition platform within the GARDskin assay. **Methods:** The study was conducted in two main parts. Firstly, 24 paired samples were acquired using both Nanostring nCounter and RNA-seq. These were used to establish an RNA-seq quantification pipeline aimed at producing gene expression measurements as similar as possible to the nCounter-derived data. This involved a primary mapping between RNA-seq transcripts and nCounter probe targets based on sequence alignment and a subsequent optimization to reconstruct the nCounter signal. Concordance between the two platforms was evaluated using correlation metrics and linear regression. In the second part of the study, the predictive capacity of RNA-seq-derived data was evaluated. A new set of 24 treatments, including 14 well-recognized skin sensitizers and 10 non-sensitizers, were analyzed using RNA-seq. Based on the established processing pipeline, expression data compatible with the GARDskin assay were generated and analyzed with the standard GARDskin classification pipeline. Classifications were compared with reference labels to generate standard performance statistics (accuracy, sensitivity, specificity), and with historical outcomes obtained using the assay's standard protocol. **Results:** The RNA-seq data were first adapted for compatibility with the GARDskin assays. Transcripts corresponding to the 196 NanoString probes were identified through sequence alignment. All but one probe could be confidently assigned to at least one reference transcript. For the unmatched probe (*COX20*), all transcripts annotated with the same gene symbol were included. The initial mappings were refined by iteratively excluding transcripts to maximize similarity between RNA-seq and NanoString expression profiles. Following the refinement, a strong concordance between the platforms was observed. Normalized expression measurements showed a Pearson correlation of 0.69 and a Spearman correlation of 0.66. Effect estimates comparing the vehicle control with the assay's positive control also showed a strong linear relationship along the identity line. The Pearson and Spearman correlations were estimated to 0.87 and 0.95, respectively. Lin's concordance correlation coefficient was estimated to 0.87. The predictive capacity of data generated with RNA-seq was evaluated on the separate set of 24 treatments (14 sensitizers and 10 non-sensitizers). All samples were accurately classified, yielding accuracy, sensitivity, and specificity of 100%. The classifications also showed 100% concordance with historical GARDskin classifications. **Conclusions:** These findings demonstrated that RNA-seq could be effectively adapted for use within the GARDskin assay. The RNA-seq platform showed high concordance with NanoString nCounter data, and classifications generated from samples quantified with RNA-seq displayed 100% concordance with both historical GARDskin classifications and reference sensitizing categories.

PS 3404 Translational Toxicology: Adapting Liver Microphysiological System for Cross-Species Evaluation of Hepatotoxicity Risk

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Background and Purpose: Drug-induced liver injury (DILI) remains a leading cause of drug candidate attrition during development, underscoring the need for improved translational models that can predict human-specific toxicities. Traditional animal models, while informative for systemic toxicity, often fall short in forecasting human liver responses due to inherent interspecies differences. To address this translational gap, we leveraged the PhysioMimix® Organ-on-Chip (OOC) Core System to develop a robust DILI assay using primary hepatocytes from human, rat, and dog sources. **Methods:** Using the PhysioMimix Core System, human, rat or dog hepatocytes were cultured as 3D liver microtissues under dynamic perfusion in the Liver-12 plate for up to 14 days, maintaining stable species-specific functionality (CYP activity, albumin, urea). A panel of six reference compounds (Troglitazone, Pioglitazone, Nefazodone, Buspirone, Tolcapone and Entacapone) recommended by the IQ MPS Consortium, including high-risk DILI agents and structural analogues with low human liability, was applied across a 7-point dosing regimen over four days. Functional *in vitro* and clinical biomarkers (albumin, urea, LDH, ALT) were monitored to assess hepatocellular health. **Results:** The assay successfully captured known species-specific toxicities. For instance, nefazodone and troglitazone (DILI Rank 8) induced hepatotoxicity in human and rat models, while dog hepatocytes showed reduced sensitivity. Conversely, buspirone and entacapone exhibited minimal toxicity in animal models, though the human MPS detected subtle hepatotoxic signals at higher entacapone concentrations, highlighting its sensitivity to rare DILI events. Albumin consistently emerged as a sensitive cross-species marker, with dose-dependent decreases aligning with known toxic profiles. **Conclusions:** These findings demonstrate the value of integrating human and animal liver MPS models to enhance cross-species interpretation and improve *in vitro-in vivo* extrapolation (IVIVE). The PhysioMimix system can be simply adapted to test multiple species in parallel to determine species-specific toxicity. As regulatory interest in MPS technologies grows, this approach offers a promising path to refine liver safety assessments, reduce reliance on and numbers of animal models used, and better predict human hepatotoxicity risks earlier in the drug development pipeline.

PS 3405 Developing A Repeated Exposure Regimen Using A Buccal Epithelium-Fibroblast Tissue Model

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Background and Purpose: Oral tobacco products (OTPs) are considered potentially reduced-risk alternatives to traditional combustible cigarettes within the tobacco harm reduction paradigm. However, their mechanistic toxicity profiles, particularly local effects on oral tissues, remain insufficiently characterized. Current mechanistic assays have largely focused on single-exposure scenarios, which do not capture the cumulative biological effects associated with repeated use, a pattern that better reflects real-world consumer use behavior. Therefore, evaluating repeated exposure is an essential step toward filling this knowledge gap and understanding oral tissue responses that may more accurately represent human outcomes. In this preliminary study, we established a feasible approach for performing up to five repeated exposures using a human buccal epithelium-fibroblast co-culture tissue model (ORL-300 FT, MatTek Corporation, Ashland, MA, USA). **Methods:** The Kentucky 1R6F reference cigarette condensate (prepared in ethanol) and CRP2.1 moist snuff extract (prepared in complete artificial saliva, CAS) were used as test articles (TAs). The 1R6F condensate was tested at concentrations of 1.5%, 6%, and 10% (v/v) and the CRP2.1 CAS extract was tested at 20%, 60%, and 100% (v/v). ORL-300 FT tissue models were exposed to each TA for 4 hours daily over five consecutive days. After each exposure, tissues were maintained either in the presence or absence of CAS on the apical surface until the next treatment. Cytotoxicity and IL-6 and IL-8 secretion were measured in the culture media after the third (T3), fourth (T4), and fifth (T5) treatments. Following the final exposure (T5), tissues were fixed in 10% formalin and morphological changes assessed by H&E staining. **Results:** Repeated exposure to 1R6F condensate resulted in a time- and concentration-dependent increase in cytotoxicity in ORL-300 FT tissue models, while CRP2.1 extract induced only marginal cytotoxicity, with the highest observed cytotoxicity being less than 25%. No significant difference in cytotoxicity was detected between conditions with or without CAS. 1R6F condensate markedly decreased IL-6 secretion in a concentration-dependent manner at T3 and T4 under both CAS conditions. By T5, IL-6 secretion was completely suppressed at all concentrations, regardless of CAS presence. For IL-8, inhibitory effects of 1R6F condensate were observed at the highest concentration at T3 and T4 and at mid and high concentrations at T5, irrespective of CAS conditions. In contrast, the CRP2.1 extract had minimal effects on IL-6 secretion at T3, regardless of CAS presence. At T4, CAS enhanced the reduction in IL-6 levels induced by CRP2.1 compared to the group without CAS. By T5, CRP2.1 extract reduced IL-6 secretion in a concentration-dependent manner, with CAS having minimal additional impact. The effects of CRP2.1 extract on IL-8 secretion were less pronounced; with decreases not exceeding 50%. Histological evaluation by H&E staining revealed time- and concentration-dependent epithelial and dermal toxicity in tissues exposed to 1R6F condensate and CRP2.1 extract, with significant changes observed at higher concentrations by T3 and moderate toxicity at lower concentrations by T5. CAS-exposed controls exhibited normal morphology at T3 and T4, while extended post-exposure to T5 resulted in compromised tissue health, as evidenced by pronounced epithelial cornification. **Conclusions:** This preliminary study demonstrates the feasibility of employing a repeated exposure regimen using a human buccal epithelium-fibroblast co-culture tissue model to evaluate local toxicity associated with oral tobacco products. Notably, the presence of CAS during the maintenance phase had significant, time-dependent effects on IL-6 secretion and histopathological outcomes, underscoring the need to consider CAS-related effects under extended exposure scenarios. These findings highlight the importance of developing standardized, reproducible protocols capable of capturing cumulative biological responses over extended timeframes.

PS 3406 Modeling CAR-T Cell Migration and Efficacy in a Microphysiological System

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Background and Purpose: Microphysiological systems (MPS) offer a promising *in vitro* platform to evaluate the efficacy and safety of T-cell-based immunotherapies, including chimeric antigen receptor (CAR) T-cell therapies. This study aims to develop an MPS to assess CAR-T cell motility and migration across endothelial and epithelial compartments, simulating the process of tissue homing. By using induced pluripotent stem cell (iPSC)-derived endothelial cells, donor PBMC-derived T cells, and a transwell-based system, the study seeks to investigate CAR-T cell behavior in a controlled, physiologically relevant environment. **Methods:** CAR-T cells were engineered using CRISPR-Cas9-mediated knock-in and expanded for functional testing. Migration was evaluated under various chemokine gradients, and flow cytometry and imaging were employed to quantify both migration and activation of CAR-T cells. Cytotoxicity against CD19-expressing Nalm6 cells was assessed by measuring the relative abundance of wild-type GFP (WT) and knockout RFP (KO) Nalm6 cells in the apical compartment of the MPS model. **Results:** The study found that endothelial cells are essential for facilitating CAR-T cell migration, with chemokine gradients playing a significant role in motility. Allogeneic CAR-T cells exhibited reduced migration compared to autologous donor PBMC-derived T cells. Importantly, while the presence of Nalm6 target cells did not affect CAR-T cell migration, it confirmed robust

cytotoxicity, with selective depletion of CD19-expressing Nalm6 cells in the apical compartment, indicating effective tumor cell killing by CAR-T cells. **Conclusions:** This work highlights the potential of MPS to model CAR-T cell migration and tissue homing, offering a valuable tool for preclinical testing of cell-based immunotherapies for autoimmune diseases. The inclusion of autologous systems enhances the precision of testing, and the platform's ability to replicate key features of immune cell interactions reduces the need for animal models. This approach offers a promising avenue for optimizing CAR-T cell therapies and advancing personalized treatments for autoimmune disorders.

PS 3407 Opportunities in Toxicity Testing of New Drug Modalities with 3D *in vitro* Models

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Background and Purpose: Emerging drug modalities offer new opportunities to treat previously unmet diseases but also introduce complex, unpredictable side effects. Traditional nonclinical safety testing lacks translatability, driving the need for better methodologies. Human 3D *in vitro* models provide more physiologically relevant systems that may improve safety assessment by revealing modality-specific toxicities. **Methods:** The OrganoPlate® platform, a microphysiological system was used to generate organ models known to be affected by different drug modalities including small compounds, antibody drug conjugates (ADCs), and AAVs (adeno-associated virus). The OrganoPlate comprises up to 96 individual chip, patterned underneath a 384-well microtiter plate. This innovative platform allows researchers to explore a wide range of flow and co-culture conditions, in a set-up that is compatible with standard laboratory equipment as microscopes and plate readers. The limited volumes required for the individual models allows the use of a range of starting materials, including primary cells, iPSC (induced pluripotent stem) cells, and ASC (adult stem cell) derived organoids. **Results:** We have developed several organ-on-a-chip models suitable for detecting different forms of toxicity. A neurite outgrowth model based on iPSC motor neurons extending their neurites into an extracellular matrix was leveraged to predict toxicity levels of ADCs. We were able to generate colon tubules using Caco-2 cell line, and ASC colon organoids. Their barrier integrity could be monitored through measuring the transepithelial barrier resistance, which reduced over time upon exposure to a panel of known gastrointestinal toxicants. Finally, we developed a liver MPS which exhibits unprecedented fidelity with the organization and interactions found within the native tissue by embedding primary or stem-cell derived liver cells in a hydrogel. The resulting culture includes polarized hepatocytes, functional vasculature with associated stellate cells and functional resident immune cells. This liver model was used to detect small molecule liver toxicity, as well as AAV transduction efficiency and toxicity. **Conclusions:** In conclusion, the OrganoPlate® platform enables the development of physiologically relevant organ-on-a-chip models that enhance the detection of modality-specific toxicities. These models demonstrate strong potential to improve the predictive power of nonclinical safety testing across diverse drug modalities.

PS 3408 Co-mentoring: A novel approach to improve undergraduate retention in toxicology and other STEM fields

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Background and Purpose: Northern Kentucky University is in the final cohort year of an NSF-funded project to increase STEM retention and success for transfer students and other groups under-represented in STEM disciplines. The TRUE-ACCESS (TRansfer & Underrepresented minority Experiences for Autonomy, Competence, Connection, and Careers: Enhancing Systemic change in STEM) project uses a 4C model with activities focused on Careers, Community-Building, Content-Area Research and Co-mentoring to engage student Fellows and to connect them to the larger campus community. The methods and results presented here will focus on the co-mentoring aspect of the project. **Methods:** There were 72 students admitted to TRUE-ACCESS beginning in Fall 2023 and continuing with new cohorts each semester through Spring 2025. More than one-third of TA Fellows were transfer students. Each cohort participated in an onboarding event that included an introduction to the non-hierarchical nature of co-mentoring. The co-mentoring process provided an opportunity to address challenges that arose. Examples included student deference to older faculty, tensions between traditional-aged students and older transfer students, and inconsistent attendance due to job and family conflicts. The common theme was that all participants are experts in something and are able to contribute to others' growth. **Results:** Retention and STEM persistence varied across cohorts. Cohorts starting in the 2023-2024 academic year achieved 74% overall retention and 68% STEM persistence, indicating a strong alignment between program goals and student trajectories. Cohorts starting in 2024-25 show respectable renewal but weaker STEM persistence ranging from 40-42% across the two cohorts. The results of pre- and post-surveys showed significant improvement in STEM identity ($p = 0.003$). Qualitative data indicated additional improvements in autonomy,

competence and belonging. Examples of positive comments from students about the co-mentoring experience include "I met different types of people...and learning about them made me more confident." "Everyone is equal in the session." "I love my co-mentoring sessions. We built friendships." Modifications included giving second-year Fellows autonomy in developing an on-campus event "College is Hard; Let Us Help" where they shared their experiences and survival tips with the broader campus community. The experience of the faculty co-mentors was also overwhelmingly positive, especially for those who are not actively advising or mentoring undergraduates in research. Faculty reported being able to adapt the co-mentoring principles in the classroom on team projects, during advising/mentoring meetings with other undergraduate students, and in their research labs. **Conclusions:** The multi-pronged approach was highly successful in achieving the project goals, the inclusion of co-mentoring played a significant role in increasing students' STEM identity, confidence and autonomy with additional benefits to the faculty co-mentors' professional development. Funding: NSF RII-BEC 2225771

PS 3409 Cannabis Safety Considerations and Informed Decision Making by Cancer Patients: Drug Interactions

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Background and Purpose: Cannabis use is becoming more common among cancer patients for managing symptoms. However, inconsistent regulation and limited education about toxicity create obstacles to safe use. Cannabis contains over 100 active phytochemicals, most notably Δ^9 -tetrahydrocannabinol (THC) and cannabidiol (CBD), which have the potential for adverse effects, drug interactions, and contamination. Despite legalization trends, many clinicians and patients are unaware of toxicological risks, highlighting the need for science-based education accessible to both professionals and the public. The goal of this work was to synthesize evidence on cannabis safety in cancer populations and to model how toxicologists can apply principles of exposure science, pharmacokinetics, and risk communication to enhance understanding among patients and providers. **Methods:** A narrative review was carried out using open-access and peer-reviewed literature, U.S. state and Canadian federal regulatory documents, and expert consultations with clinicians, dispensary staff, and cancer patients. Publications covering three safety areas—(1) adverse effects and misuse, (2) drug interactions, and (3) product safety—were included. The findings were adapted into a harm-reduction framework and combined with educational communication strategies for application in clinical and public settings. Case studies from oncology clinics were examined to illustrate real-world application of toxicological principles, including dose titration, pharmacokinetic monitoring, and contaminant risk assessment. **Results:** Three risk domains were identified. Adverse effects included cardiovascular (tachycardia, bradycardia), neurological (fatigue, dizziness, psychomotor impairment), psychiatric (psychosis, anxiety), and gastrointestinal events, including cannabis hyperemesis syndrome. Approximately 10-30% of users developed cannabis use disorder. Cannabinoid-drug interactions occurred through CYP450 enzymes (CYP2C9, CYP2C19, and CYP3A4), altering the metabolism of agents such as tamoxifen, warfarin, and chemotherapeutics. Product safety concerns included microbial, pesticide, and heavy metal contamination, particularly in unregulated markets, posing infection risk for immunocompromised patients. These findings informed educational modules emphasizing safe dose titration ("start low and go slow"), product verification, and non-inhalation routes of administration. **Conclusions:** This synthesis demonstrates how toxicological literacy can improve the safety and efficacy of cannabis use among vulnerable populations. Future toxicologists play a critical role in translating pharmacological and exposure data into accessible risk communication for clinicians and the public. Integrating toxicology education with real-world patient guidance strengthens the credibility of cannabis safety messaging and supports evidence-informed policy. Educating the next generation of toxicologists to communicate clearly about emerging toxicants, such as cannabinoids and their contaminants, will enhance public trust and advance the discipline's contribution to health literacy and harm reduction.

PS 3410 Interactive Learning Tools Improve Student Confidence and Learning in a Summer High School Program

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Background and Purpose: The Rutgers University Toxicology, Health and Environmental Disease (THED) Program is aimed at teaching high school students core principles of toxicology, pharmacology, experimental design and common laboratory techniques, as well as career opportunities in these fields. Tailored for students

interested in environmental and public health related fields, THED provides a platform for hands-on training in experimental methodology and career-based exploration. Herein we evaluated the impact of an interactive learning tool, Poll Everywhere, on student understanding, confidence, and satisfaction with topics presented in the Genetics Laboratory during the 2025 THED program. **Methods:** A total of 56 students participated in the two one-week programs. On the first (n=52) and the last (n=46) day each program, students completed pre- and post-program surveys. Pre-surveys assessed prior knowledge and confidence in the topics and techniques taught during THED. Post-program surveys assessed knowledge and confidence using the same set of questions as the pre-survey, with additional questions aimed at assessing satisfaction with THED activities. Confidence and satisfaction were assessed using qualitative 5-point Likert scales with one scale each for the Genetics Laboratory. Understanding was assessed by a series of true/false questions, three of which pertained to topics that were covered during the session. During the laboratory, students identified their genotype and phenotype for an inherited bitter taste receptor present on cells collected by a cheek swab, DNA extraction, amplification, and visualization. Lectures were also presented to students on key genetics principles including Mendelian genetics and single nucleotide polymorphisms, as well as methodology for DNA extraction, polymerase chain reaction (PCR), and gel electrophoresis. Students from Week 1 received a traditional lecture format, while students in Week 2 received lectures with 10 interactive questions imbedded using the Poll Everywhere software (Poll Everywhere, Inc., San Francisco CA.) Students responded to questions via mobile devices and received real time feedback from the instructor during the lecture. After the lectures, students applied these principles and techniques to a hands-on lab activity aimed at identifying their individual and population genotypes within their cohort. **Results:** Comparing student pre- (Week 1 n= 24; Week 2 n=27) and post-surveys (Week 1 n= 20; Week 2= 26), we observed a greater increase in confidence explaining the relationship between genotype and phenotype for Week 2 (49%) compared to Week 1 (28%). Satisfaction with the genetics lab was not appreciably different between students in Week 1 and students in Week 2. In contrast, greater understanding of the material was noted in Week 2 as indicated by an increase in the percentage of students with correct answers to Questions 1 (22%), 2 (18%), and 3 (17%), compared to Week 1 (14%, -13%, -15%, respectively). **Conclusions:** The use of the interactive learning tool, Poll Everywhere, increased student confidence and understanding, but did not affect student satisfaction with the laboratory. These findings provide insight into how to better engage students during lectures to ensure confidence in applying and understanding of the materials presented. Funding provided by Bristol-Myers Squibb, Johnson and Johnson, T32ES007148 and P30ES005022.

PS 3411 NURA: Free Toxicology Training on Sunscreens, Xeno-free Reagents, and More in 2026

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Background and Purpose: Animal-based methods have historically dominated the field of toxicology. Some of these methods, like the Draize test, have been used for as long as 80 years without questioning the reliability and reproducibility of the results. Today, most toxicology education is centered around these methods. Scientists are recognizing the ability of New Approach Methodologies (NAMs) to improve chemical assessments while providing a better understanding of the mechanisms of toxicology. Unfortunately, training is often cited as a key barrier to the use of NAMs. For this reason, easily-accessible training is needed to help overcome the barrier and enabling more widespread use and understanding of NAMs. **Methods:** The NAM Use for Regulatory Application (NURA) program coordinates with leading regulatory and policy experts and scientists across sectors to develop training programs that target the replacement of animal use with more effective nonanimal methods. To reduce financial and logistical barriers to NAMs training, NURA hosts free, live webinars that allow the audience to engage with experts via Q&A sessions, hands-on demonstrations, case studies, and more. Following the live sessions, all NURA webinars are added to our online learning platform where they are available on demand with manually verified English and Spanish subtitles. NURA also develops Continuing Education Courses and customized hands-on workshops to meet the individual needs of organizations or regulatory agencies. **Results:** Throughout 2025, NURA provided thousands of cumulative NAM training sessions. We coordinated eight virtual webinars. We hosted a timely training following the OECD's updated release of Guidance Document 497, Defined Approach to Skin Sensitisation, where our invited experts highlighted the SARA-ICE method. In our NAM Spotlight series, we featured ALIsens, ToxTracker, and NAMsNetwork as NAMs for use in regulatory applications. We developed the "NURA en Español" series with colleagues from the Institute for *In Vitro* Sciences (IIVS) and presented three webinars for our Spanish-speaking audience. In February, NURA presented a two-day customized training at the California Department of Pesticide Regulation with our IIVS partners that highlighted skin and eye irritation and sensitization, systemic toxicity, and the Integrated Chemical Environment. In October, NURA hosted an in-person continuing education course, "Demystifying QSARs," at the American Society for Cellular and Computational Toxicology Annual Meeting. **Conclusions:** NURA aims to remove barriers to NAM training by providing scientists and regulators with free, on-demand, accessible NAMs education to help modernize their toxicity assessments.

PS 3412 Research in action: An applied approach to understanding knowledge and preferred communication about chemical exposures in consumer and children's products.

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Background and Purpose: The Minnesota Department of Health (MDH), in partnership with a public health seminar course at Hamline University, conducted four focus group discussions with parents and caregivers to assess knowledge and preferred communication methods regarding chemical exposures in consumer and children's products. Students in this course had the opportunity to apply classroom learning on topics such as focus group facilitation, qualitative research coding, and health communication campaign design in a professional setting. **Methods:** From September to October 2025, eight students facilitated four focus group discussions with a total of 55 participants. The students conducted quality checks and coded the transcripts to identify key themes. Findings from the results will be used to develop a health communication campaign for parents and caregivers focused on chemical exposures in consumer and children's products. **Results:** Most participants (56%) identified as Black, African, or African American, 61% were male, and 45% were between the ages of 25 and 34. Many participants reported relying on a variety of sources for health information, including social media, parenting blogs, and government websites. In addition, healthcare providers were highlighted as trusted messengers whom parents and caregivers rely on to provide information about potential chemical exposures and related health effects. Some participants indicated that they checked product labels before making purchasing decisions; however, many others reported that they did not prioritize reviewing product information prior to buying. Economic constraints were also identified as a factor influencing purchasing decisions. Final results are being compiled and will be shared at the conference. **Conclusions:** Findings indicated that multiple factors influenced participants' purchasing decisions and that parents and caregivers used a range of sources to obtain health information. The insights generated from this project will inform MDH's future exposure reduction and health communication efforts to improve the public's understanding of the potential for harmful chemical exposures from consumer and children's products and their related health effects.

PS 3413 Interactive Lesson on Microplastics and Nanoplastics for Summer Undergraduate Interns

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Background and Purpose: Micro/nanoplastics are now recognized as ubiquitous in the environment. Exposure can occur via ingestion, inhalation, skin contact and potential health impacts include cytotoxicity, DNA damage that enhance absorption of other toxins and exacerbation of respiratory pathologies. To increase awareness of micro- and nanoplastic particle toxicology, we designed an interactive training lesson for college students participating in a 10-week summer research internship at Rutgers University. **Methods:** The session was comprised of a 1) 45-minute didactic lecture on polymer types, particle characteristics, and particle deposition, 2) two hands-on team-based activities, and 3) utilization of Applied Research Associates, Inc. Multiple-Path Particle Dosimetry Model (MPPD) Software. For the first team activity, students were provided with recyclable plastic items and asked to estimate the decomposition time for each product. For the second team activity, students were provided toys and balls as surrogate micro- and nanoparticles. Students were asked to first identify the physical and chemical properties of the particles, then compare and contrast the properties with a neighboring team and theorize how these differences may impact biological behaviors or cellular interactions. For the Dosimetry activity, students were provided 4-case studies consisting of a model system and particles differing in density and size distributions; they were then asked to compare human and rodent pulmonary deposition using the MPPD software. Thirteen knowledge questions were tested before the interactive session and one-week after the training. The activity was rated using a 5-point Likert scale (1-poor to 5-excellent). **Results:** Twenty-one summer interns participated in the micro- and nanoplastics training and response rates on pre-/post-test questions ranged between 91-100%. The scores on the assessment questions increased 24% between the pre- and post-test. Across the 13 questions, the mean normalized gain of knowledge was 23%, with large variation ranging between -42% and +95% depending upon the question asked. At the end of the internship, the micro- and nanoplastics lesson was one of the most favorably rated career development activities (mean: 4.5, SD: 0.7, N=17). **Conclusions:** An interactive approach that combines didactic instruction, case studies on a modeling platform, and a fun team-based activity can be used to teach undergraduate students about micro- and nanoplastic characteristics and their biological disposition after pulmonary exposure. Supported by R25ES020721, T32ES007148, R01ES036575, P30ES005022, and the SOT and ASPET SURF Intern Programs.

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Background and Purpose: Artificial intelligence (AI) is rapidly transforming drug development, safety assessment, and toxicological research. However, a critical workforce gap exists while many toxicologists recognize AI's growing importance, they lack confidence applying AI terminology and concepts within scientific and regulatory contexts. This disconnect between awareness and application creates a barrier to responsible AI integration in toxicology practice. Building on a 2024 pilot study that introduced AI literacy, this 2025 study examined how learners progress toward fluency, defined as the ability to translate AI concepts into professional practice. This study aimed to strengthen "AI bilingualism," helping adult learners bridge computational and toxicological languages to prepare the next generation of translational scientists. **Methods:** Graduate students (n=87) enrolled in health sciences courses completed pre- and post-intervention surveys assessing progression from AI literacy toward fluency with seven key terms: Generative AI, Large Language Models (LLMs), Ethical AI Use, AI Bias, TRACI (Task, Role, Action, Context, Input) Prompting Framework, Predictive vs Reasoning AI, and AI Audit Trail. Students rated their proficiency using a five-point Likert scale (1 = not familiar to 5 = can apply this term in context). Between surveys, students completed structured assignments requiring application of AI concepts to toxicology and drug development scenarios. This study was approved by The Ohio State University Institutional Review Board (Protocol 2024E0184, Amendment 1). **Results:** Proficiency scores increased across all seven terms (preliminary n=87 pre-intervention, n=57 post-intervention to date; final data collection ongoing). Foundational concepts such as Generative AI and Large Language Models showed moderate gains (mean scores increased from 3.02 to 3.95 and 2.15 to 3.16, respectively), while applied and ethical topics including Ethical AI Use and AI Bias showed strong improvements (2.92 to 3.88 and 2.71 to 3.89, respectively). The largest gains occurred in advanced reasoning and prompting frameworks: TRACI Prompting Framework showed the greatest improvement (1.60 to 3.65, $\Delta=+2.05$), followed by Predictive vs Reasoning AI (1.98 to 3.18, $\Delta=+1.20$) and AI Audit Trail (1.99 to 3.65, $\Delta=+1.66$). These findings demonstrate that structured learning substantially enhances AI fluency among graduate students. **Conclusions:** Embedding AI fluency into toxicology education helps learners progress from terminology recognition to practical application. As AI continues to evolve within toxicology, from modeling to automated literature reviews and regulatory submissions, graduates need true bilingual fluency. Building confidence with AI terminology prepares toxicologists to critically evaluate AI outputs, recognize algorithmic bias, and navigate ethical considerations essential for responsible integration and regulatory decision-making.

PS 3415 **Efficacy of StressCURE: A Course-Based Undergraduate Research Experience Focused On Biological Stress In Various Laboratory Courses at Four Institutions.**

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Background and Purpose: Research has shown for undergraduates that earlier exposure to research leads to increased student retention in STEM, self-identification as a scientist, and progression towards graduate school. Course-based Undergraduate Research Experiences (CUREs) are an effective way to expose undergraduates to research in the regular course of instruction throughout their educational program. StressCURE is an NSF-funded program supporting six faculty members to develop a modular approach to CURE design which focuses on the concept of biological stress in the *C. elegans* model system. Biological stress is defined as anything that disrupts the homeostasis of an organism, which may include exposure to toxicants, radiation, temperature extremes, diets, and genetic alterations. **Methods:** Using the pathway model approach to program assessment, we linked key activities of the project with short-, middle-, and long-term outcomes and identified key assessment points needed to demonstrate programmatic success. With this trajectory in mind, StressCURE was implemented in two courses in the Fall of 2024: a biochemistry laboratory at the US Coast Guard Academy (N=12) and an organic chemistry laboratory at the University of Maine (N= 32); one course in the Spring of 2025: a toxicology laboratory at Washington College (N=11); and three courses in the Fall of 2025: a molecular genetics laboratory at Lincoln Memorial University (N=39), an organic chemistry laboratory at the University of Maine (N=35), and a biochemistry laboratory at the US Coast Guard Academy (N=11). Students were assessed using the Experimental Design Ability Test (EDAT) which is an open-ended response for students to design their own experiment to a prompt, before and after the course, assessed by the professor using a validated rubric. Students also took the laboratory course assessment survey (LCAS) to evaluate their perceptions of courses which contained the StressCURE. **Results:** Initial results of the EDAT (N=57) showed a statistically significant ($p < 0.05$) increase in all measured values in the ability of students to design experiments. As assessed by the LCAS survey (N=51), students perceived the courses employing StressCURE as having increased collaboration and iteration as shown by statistically significant ($p < 0.001$) increase in all measures.

Since retention in STEM and at institutions is important for growth in STEM disciplines, we assessed the sense of belonging in the course and institution. Results from this validated instrument, revealed there was an increase ($p < 0.05$) in students comfort, respect, and belonging in the course when results were compared in the pre- and post-survey. These data extended to the institution where students had an increase ($p < 0.05$) in feeling comfortable on campus and supported in the major and institution when comparing results in the pre-and post-survey. **Conclusions:** Taken together, these data show the importance of utilizing CURE approaches like StressCURE for increasing critical student skills needed for their development as scientists and sense of belonging in STEM and retention at institutions. This material is based upon work supported by the National Science Foundation under Grant Number 2416714 as part of the Improving Undergraduate STEM Education (IUSE EDU). StressCURE course materials are freely available at stresscure.org.

PS 3416 **Transforming Toxicology Education through Interactive Online Modules: The ToxMSDT Approach**

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Background and Purpose: A major component of the five-year Toxicology Mentoring and Skills Development Training (ToxMSDT) program was to develop engaging online curriculum resources that were accessible to learners and instructors worldwide free of cost. These resources introduce foundational toxicology concepts in ways that are relevant, thought-provoking, and accessible to students with introductory biology and chemistry backgrounds. Original pilot modules were updated and continued development has produced new modules on toxicology topics and case studies focused on particular toxicants. The ToxMSDT program was designed to inspire and prepare STEM undergraduates for graduate study and careers in toxicology through mentoring and professional skills development; 138 undergraduates were paired with mentors and participated in career development during a pilot project and the five-year grant. **Methods:** Undergraduate educators and subject experts were recruited to write updates for five modules that were originally released in 2018 and to create new materials. Changes for existing modules include updated content and increased interactivity. All modules and case studies were aligned with the SOT Undergraduate Toxicology Learning Framework and the SOT Eminent Toxicologist Lectures were integrated. In addition, new modules and case studies were developed by teams organized by SOT. Grant partners at Michigan State and Tuskegee University also created two modules. The SOT oversight team recruited authors, reviewed content, ensured interactivity and consistency, and coordinated programming and completion of the products. Module completion and competency were tracked through earned certificates, and website analytics provided complementary insights into broader global use and reach. **Results:** Teams organized by SOT, comprising 27 authors representing unique institutions, contributed to updating five modules, developing two new modules (Neurotoxicology and Developmental and Reproductive Toxicology), replacing the Regulatory Toxicology module with a case study, and creating seven additional case studies focused on specific toxicants. The SOT oversight team managed the final stages of production for two additional modules, one produced by an author at Michigan State, and one created by six authors at Tuskegee University. Data collected through June 2025 show steady engagement across the online learning modules. Among the most utilized are Genetics (175 certificates), Biochemistry (117), Regulatory Toxicology (150), Applied System Toxicology (116), Pathophysiology (164), Lead (150), and Vaping (79). Newer modules such as Aflatoxin (4) and Ethanol (46) continue to expand the program's topical reach, while a recent addition, Reproducibility and Rigor, has begun awarding certificates, with 3 issued to date. For those achieving certificates as a course assignment, learners represent a broad network of 23 institutions, including universities such as Rutgers, Saint Louis University, Michigan State University, and the University of Illinois. Certificate completers were from over a dozen countries and territories spanning the United States, Canada, Pakistan, Egypt, Puerto Rico, Uganda, and others, underscoring the program's growing international reach. The oldest module, ToxTutor, originally adopted from the National Library of Medicine, has provided 3,612 certificates and 1,148 quiz completions as of June 2025. **Conclusions:** The ToxMSDT modules and case studies developed and refined during the ToxMSDT project demonstrate the power of collaboration and innovation in advancing toxicology education. These engaging, evidence-based learning resources enrich undergraduate toxicology curricula, provide valuable training for ToxMSDT participants, and broaden the global accessibility of toxicology education. Ongoing use in a new project, MSOT-One Health, and in toxicology-related courses and programs, will amplify the impact of these materials, increasing understanding of toxicology. Funded by R25GM1139200 and 1R25GM113989.

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Background and Purpose: StressCURE is a Course-based Undergraduate Research Experience (CURE) focused on the theme of biological stress in the *C. elegans* model system. CUREs are designed to bring authentic research experiences to undergraduates, a practice which increases student retention in STEM, increases student identity as a scientist, and provides other benefits. StressCURE is designed to offer faculty flexibility in choice of scientific problems to investigate, as biological stress can be induced through exposure to toxicants, dietary changes, genetic modification, and a variety of other parameters. The *C. elegans* model system is readily adoptable, inexpensive, and includes a wealth of genetic models, making it ideal for undergraduate research. StressCURE is designed to be used in any undergraduate course, from basic biology through upper level laboratories. The goal of StressCURE is to expose more undergraduates to research by facilitating the adoption of the CURE methodology by faculty. **Methods:** The StressCURE module design was developed and tested by faculty members and students in their courses for three semesters. Each module is focused on a laboratory protocol and includes a brief overview, difficulty rating, list of materials, prerequisite knowledge, faculty goals, student learning objectives, faculty instructions, sample data, student handout, and additional information. A detailed timeline contains instructor preparation instructions and student responsibilities to be completed for the module. Protocols were tested at the primary author's institution and at least one other institution, refined, and then evaluated by the advisory board. Protocols were then published on the StressCURE website (www.stresscure.org) and communicated at scientific conferences to encourage adoption. The Reproduction, Chemotaxis, Aversion, and Basal Slowing Response modules were tested. Efficacy of the overall StressCURE model was also assessed using validated survey instruments according to a pathway model approach to program assessment. **Results:** StressCURE was successfully deployed at four institutions: the US Coast Guard Academy, University of Maine, Washington College, and Lincoln Memorial University. Faculty reported that the modules significantly reduced the workload required to adopt a new laboratory protocol for the teaching laboratory and that they would continue to teach in this way. Website traffic grew as more modules were published and surged around the time of conferences. Fourteen individuals signed up to receive notifications regarding the StressCURE project. While many individuals reported interest in using CUREs for Introductory Biology, several are interested in Microbiology, Biochemistry, Molecular Biology, Genetics, Genomics, and Cell Biology. Individuals reported hearing about StressCURE through conferences and their professional networks. In 2025, there were 713 visits to the site. **Conclusions:** StressCURE's first six modules have produced significant interest and the launch of more modules over the remaining two years of the project. The modules have been successfully adopted in the laboratories of the investigators and several faculty members plan to adopt them as well. Future assessment instruments will determine efficacy outside of the investigator group through surveys and ongoing communication as a community of practice is developed. In the third year, a regional conference will present all of the modules (approximately 30). This material is based upon work supported by the National Science Foundation under Grant Number 2416714 as part of the Improving Undergraduate STEM Education (IUSE EDU). StressCURE course materials are freely available at stresscure.org.

PS 3418 **An Innovative National Research Education, Mentoring, and Skills Development Training Program in One Health and Toxicology: MSDT-One Health**

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Background and Purpose: The NIH NIGMS has awarded a new 5-year IPERT mentoring and skills development training grant (R25) in One Health (OH) to a consortium of institutions led by the University of California, Davis. Collaborating institutions include the Society of Environmental Toxicology and Chemistry (SETAC), Meharry Medical College, and Iowa State University. One Health research is an integrated approach aimed at optimizing the health of people, animals, and ecosystems in their shared environment. Environmental toxicants are environmental stressors that negatively impact the health of people, animals, and the ecosystem in their common environment. Currently, OH research is facing a major challenge: the lack of engagement of toxicologists and/or people with toxicology knowledge in OH transdisciplinary research. This is partly due to inadequate training of toxicologists in skills essential for successful transdisciplinary OH research and/or a lack of awareness of opportunities in this field, as OH is not offered as a major at most undergraduate institutions. MSDT-One Health is a national program targeting early career scientists and undergraduate students in STEM fields. **Methods:** The MSDT-One Health training program has three specific aims (SA). SA1: Develop and deliver a year-long mentoring program for STEM undergraduate students. SA2: Develop and deliver a year-long professional development training and mentoring program for early career

scientists (Post-docs, Fellows, Project Scientists, Assistant Professors) to increase their awareness of the opportunities in OH research and to increase their knowledge and ability to obtain funding necessary to conduct OH research, including community-engaged toxicology research. SA3: Develop case studies and e-modules at the intersection of OH and toxicology and make them widely available. The program, which is mostly virtual, is delivered through a combination of assigned modules and case studies, in-person and virtual workshops, webinars, attendance at a national OH conference, and 1:1 mentoring with near peer and senior mentors. Training duration is 1 yr. **Results:** For the 1st cohort (November 2025 to April 2026) we recruited 20 undergraduate mentees, 9 early-career scientists, and 18 mentors, from across the United States, including Puerto Rico and Hawaii, to foster skills development in toxicology and transdisciplinary OH research. **Conclusions:** Expected outcomes include: a) a total of 100 undergraduate students mentored in OH with a toxicology emphasis; b) 5-10 early career scientists securing intramural seed funding and 2-3 early career scientists securing extramural funding to conduct OH research with community engagement components; c) development of highly interactive case studies in one environmental health toxicology (5) and learning modules on OH (3) to be widely available for training undergraduates and early career researchers. Ultimately, this research education program in OH and toxicology will strengthen the biomedical research enterprise by increasing the number of scientists with the necessary knowledge, skills and networks to engage in transdisciplinary OH research. Supported by NIGMS R25GM160671

PS 3419 **Advancing Neural Exposome Research at NINDS: Strategic Initiatives and Scientific Priorities**

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Background and Purpose: Most neurological diseases and disorders cannot be explained by genetic factors alone. The *neural exposome* encompasses the totality of physical, chemical, biological, and psychosocial exposures that influence brain health and disease across the lifespan. The Office of Neurological Exposome and Toxicology (ONETOX) at the National Institute of Neurological Disorders and Stroke (NINDS) was established to advance basic, translational, and clinical research on how these diverse exposures shape nervous system development, function, and pathology. This abstract describes ONETOX's strategic role in supporting neural exposome research and fostering multidisciplinary collaboration to accelerate discovery and improve neurological health. **Methods:** To guide near- and medium-term efforts to cultivate neural exposome research, NINDS launched a two-phase process to identify research priorities in neural exposome. Phase 1 was conducted in 2023-2024 by the Neural Exposome Top Priorities Working Group (NEXT WG) that brought together experts in neurobiology, neurological disorders, and exposomic science to define key needs for the field. Phase 2, led by ONETOX during 2025, involved a working group internal to NINDS that developed an implementation strategy to address these recommendations. **Results:** Phase 1 identified, six priority areas: 1) Identification of exposome factors that affect neurological health, 2) fundamental mechanistic research to determine how these factors influence neurological health, 3) Evaluation of exposome factors in human/population research studies, 4) Creation of a multidisciplinary research community, 5) Development of exposome assessment tools, and 6) fostering collaborations to accelerate progress. Phase 2 organized these recommendations into five implementation focus activities: (1) foster partnerships and amplifying research; (2) leverage resources and tools; (3) train investigators and build multidisciplinary teams; (4) convene workshops; and (5) support neural exposome research. Together, these efforts have strengthened the scientific foundation for neural exposome research by hosting scientific meetings, promoting team science, expanding access to exposure assessment tools and repositories, and funding neural exposome research. **Conclusions:** Neural exposome research offers significant potential to uncover disease mechanisms, identify modifiable risk and resilience factors, and improve the prevention and treatment of chronic neurological disorders. By coordinating resources, expertise, and collaborative networks, ONETOX drives innovation in exposomic science and strengthens the broader neuroscience research ecosystem. The Neural Exposome Program at NINDS underscores an enduring commitment to public health by advancing scientific understanding of how lifelong exposures shape brain and nervous system health.

PS 3420 **The Environmental Health Language Collaborative (EHL): Spotlight on Community-Driven Projects that Harmonize Toxicology Data**

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Background and Purpose: The availability of data is rapidly advancing opportunities to answer large-scale, complex questions in toxicology and environmental health sciences (EHS). However, a significant challenge remains around the development and use of harmonized language to allow for streamlined and customized workflows to find, share, and reuse this data. The Environmental Health Language Collaborative (EHL), <https://www.niehs.nih.gov/research/programs/ehlc> was created to facili-

tate a community-of-practice-driven effort to advance the development and adoption of harmonized language approaches within toxicology and environmental health science fields. Central to EHLC's work are three use case working groups. These use cases are distinct, but parallel, efforts that seek to address unique challenges related to promoting environmental health science data interoperability, discovery, and reuse. This poster will share information on recent products, publications, and next steps from these use case working groups and how SOT attendees can become involved in ongoing or future efforts. **Methods:** EHLC has grown into a robust community of practice made up of transdisciplinary subject matter experts led by a leadership committee. EHLC currently supports use case working groups focusing on Data Discovery, Data Harmonization, and Biomarkers and Biological Processes of Exposure. These groups explore how language-based solutions can 1) make it easier to find and use published toxicology and EHS data and resources, 2) support the combination and cleaning of data sets across independent toxicology and EHS studies, and 3) generate a conceptual model linking potential sources of particulate matter to human exposures and respiratory health outcomes, respectively. In addition, EHLC hosts community workshop and webinar events to fill identified needs within the community of practice, advance potential topics for focused activities, and promote outreach opportunities to toxicologists and environmental health scientists. The Adverse Outcome Pathway (AOP) Standards Workshop, a virtual webinar event held in June 2025, focused on ways to improve the computability of AOPs through a range of approaches that leverage language standardization, expanded AOP information structures, AI, and lessons learned from biomedical knowledge base development efforts. Subsequent products developed based on discussions and presentations at the AOP Standards Workshop include a summary report, a proposal entitled "AOP Reporting Standards for Biomedical Data", and a discussion article entitled "The FAIR AOP roadmap for 2025: Advancing findability, accessibility, interoperability, and re-usability of adverse outcome pathways" (Mortensen et al., 2025). **Results:** Five public webinars on EHS topics—such as data annotation, ontology curation, and large language models—expanded knowledge and skills among the community. The AOP Standards Workshop fostered new collaborations and led to tangible outputs, including a workshop summary and a peer-reviewed article. The EHLC Executive Committee, a chartered group of volunteer experts from across the EHS field, coordinated these efforts to align with community needs. Of the three EHLC use case working groups, two are developing manuscripts to share best practices, while the Data Harmonization Use Case published recommendations now informing the field. These initiatives have deepened the understanding of data management gaps and are shaping future directions as AI and machine learning become more prominent in environmental health sciences. **Conclusions:** This presentation shares updates, resources, information about participating agencies, and opportunities to get involved with EHLC use case working groups focused on harmonizing and sharing toxicology data relevant to SOT participants. Upcoming opportunities for SOT attendees to contribute to the development of a harmonized language to ensure that toxicological and EHS data can effectively be shared and best used to answer the fields' most pressing questions have also been collated. The goal of this presentation is to inspire collaborative engagement with EHLC and bring awareness of available resources to help advance environmental health sciences.

PS 3421 Contamination in Pharmaceuticals: Public Health, Medico-Legal Implications, and Recent Incidents

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Background and Purpose: Pharmaceutical contamination with toxic adulterants has emerged as a major toxicological and regulatory challenge, particularly in LMICs. Recent fatal outbreaks in India (2025), The Gambia (2022), and Uzbekistan (2023) were linked to diethylene glycol (DEG) and ethylene glycol (EG) contamination of pediatric cough syrups. These incidents highlight persistent vulnerabilities in drug manufacturing, excipient sourcing, and global supply-chain integrity. The objective of this study was to analyze recent contamination incidents, characterize associated toxicological outcomes, and evaluate the regulatory, medico-legal, and industrial implications. **Methods:** We conducted a multi-source review integrating toxicological data, regulatory reports (WHO, CDSCO, UNODC), and peer-reviewed literature. Cases were analyzed for contaminant type, exposure route, clinical presentation, mortality, and regulatory response. Legal and ethical dimensions were assessed through policy documents and published medico-legal analyses. Quantitative data on DEG levels and case-fatality rates were synthesized where available. **Results:** Across three major incidents, DEG and EG concentrations in contaminated syrups ranged from 1.5% to 48.6% (v/v), far exceeding pharmacopoeia limits (<0.1%). Reported mortality included more than 70 pediatric deaths in Gambia, 19 in Uzbekistan, and at least 20 in India (2025), primarily due to acute renal failure and metabolic acidosis. Root-cause investigations are either underway or those concluded have identified substitution of industrial-grade solvents, inadequate excipient testing, and documented GMP violations in affected facilities. Regulatory actions included WHO global alerts, product recalls, factory closures, and criminal proceedings against manufacturers and practitioners. **Conclusions:** Pharmaceutical contamination represents a convergence of toxicological hazard and governance failure. DEG toxicity remains preventable with robust quality control and supply-chain verification. These incidents underscore the need for toxicovigilance systems, internationally harmonized GMP enforcement,

and ethical accountability mechanisms. For toxicologists, such events highlight the societal role of the discipline beyond mechanistic toxicity toward prevention, policy engagement, and protection of global public health.

PS 3422 From Sensors to Solutions: Novel Co-Planning Frameworks to Guide Air Pollution Monitoring and Input into Environmental Public Health Policy in the Columbus, Ohio Metropolitan Statistical Area

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Background and Purpose: The College of Public Health of the Ohio State University led, in partnership with the City of Columbus via Sustainable Columbus, Mid-Ohio Regional Planning Commission, Power A Clean Future Ohio, and Impact Community Action, a low income and disadvantaged community (LIDAC) benefits analysis study as a component of Comprehensive Climate Action Plan (CCAP). The CCAP outlined a comprehensive strategy to coordinate pollution reduction and mitigation strategies for greenhouse gas emissions across 6-sectors while prioritizing LIDACs. In order to facilitate the collection and analysis of real time air contaminant data in LIDACs, SimpleAQ air monitoring system was utilized. The online platform retains the information gathered from the sensors with the goal of providing data on the concentrations of criteria air pollutants in LIDAC communities and a dashboard for environmental literacy. **Methods:** Our methodological approach utilized several frameworks: a) Public Health Exposome; b) Health Opportunity Index; c) Public Engagement to Re-imagine Community Co-Planning (PERCC); and d) an enhanced engagement model E6. Multiple (n=30) engagement sessions were held. The protocols focused on educating the public on strategies to strengthen environmental literacy and implement co-planning strategies between residents and environmental public health professionals for pollution reduction strategies. During these sessions, information, thoughts, and opinions were collected relevant to potential exposures to air contaminants and the burden of responsibility. These data were collated and analyzed. LIDAC residents expressed their preferences, stories, opinions, and beliefs onto a bullseye template. To determine whether the Simple AQ air monitors would be beneficial, informative, and contribute to the weight of evidence in LIDACs for PM2.5 potential exposures; we modeled the associations of PM2.5 with the Health Opportunity Index (HOI) contextualized by readiness-to-read test scores in a LIDAC preschool cohort (n=236 students). To assess the benefits and costs of the interventions, we combined techniques from environmental public health policy analysis, decision analysis, and engineering. **Results:** LIDAC engagement sessions identified 6-greenhouse gas and other harmful air pollutant sectors, 17-strategies were developed from which 65-measurable actions are being implemented that will reduce these emissions to promote equity, resilience, health, jobs, and affordability. The LIDAC benefits analysis identified 6 win-win outcomes. 1) Reductions in Criteria Air Pollutants by Tree Canopy Type; 2) Animal Husbandry; 3) Electric Vehicle Adoption; 4) Reduced Vehicle Dependence; 5) Electrified Building Systems; 6) Equipment and Process Emission Reductions; and 7) Expanded Waste Diversion; and 8) Improved Water Use Efficiency. The SimpleAQ air monitoring network was deployed, tested, calibrated and validated in Columbus, OH and now monitors pollutant exposures in LIDACs. Finally, our collective frameworks identified significant associations between PM2.5 pollutant exposures and readiness-to-read in an (n= 236) LIDAC preschool children's cohort. **Conclusions:** The conduct of a LIDAC community benefits analysis was grounded in the input of high-quality data integrated with CCAP measures, methodological rigor, that resulted in the integration of environmental, demographic, and health data to address latent disproportionate burdens on low-income, disadvantaged communities (LIDACs). The study demonstrates the important role of exposomics-informed data for use by local environmental public health policy-makers for the design of pollution adaptation, and mitigation strategies that support the MSA priorities and impact.

PS 3423 Effects of Asbestos Dosage on Kidney IgG Deposition

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Background and Purpose: Worldwide prevalence of autoimmune diseases is rising, which may be partially due to xenobiotic exposures. Numerous studies have linked amphibole asbestos exposures to autoimmune development, though only a couple of these studies have examined the impact of exposures at environmentally-relevant doses. Understanding the mode by which asbestos causes autoimmune conditions is essential to determining risk factors and treatments in exposed human populations. Immunoglobulin-G (IgG) is the most common serum antibody, making it a good indicator of immune activity. IgG deposits in the kidney have been associated with autoimmune disease, which has also been attributed to asbestos exposure.

Thus, in this study, we directly compare IgG markers of autoimmune development in mice exposed to high and low dose asbestos exposures. **Methods:** We exposed male and female wild-type C57/BL6 mice (n=48 total) to a high (100µg) or low (3µg) dose of Libby amphibole asbestos given in two treatments via oropharyngeal aspiration. These doses mimic occupational and environmental exposures in human populations. At four months post-exposure, kidneys and serum were assayed. Kidney structure and collagen deposition were assessed using Masson's Trichrome and Hemotoxylin & Eosin. Immunofluorescence staining techniques and image processing were used to determine the percent area of IgG deposition in mouse kidneys. IgG isotypes were measured, and all data was analyzed using two-way ANOVA to assess effect of fiber dose and sex. **Results:** Significant IgG deposition was found in the low-dose group (p adj<0.01) compared to control and high-dose groups. Although high-dose did have greater IgG deposition when compared to the control group, this difference was not significant. Sex was not a significant factor in IgG deposition. No apparent structural changes in kidneys were noted during preliminary examination, though follow-up analyses are underway. **Conclusions:** Our findings suggest that even small, environmentally relevant asbestos exposure may trigger immune dysfunction and autoimmune responses, and that these responses are likely similar in biological males and females. Environmental asbestos exposures pose an on-going human health risk due to the presence of legacy asbestos in older buildings and products as well as natural asbestos outcroppings found worldwide. Understanding the etiology of asbestos-induced autoimmunity can lead to improving diagnostic methods, treatments, and prevention techniques in exposed populations.

PS 3424 Humanized B-hIL4/hIL4RA mice model for efficacy and toxicity evaluation

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Background and Purpose: The emergence of alternative models for drug safety evaluation has become a critical need, particularly in light of the current shortage of non-human primates. One promising alternative is the humanized mice, where specific target genes in mice are substituted with their human counterparts. Recent trends indicate that many pharmaceutical companies are leveraging Biocytogen's humanized mice for toxicology studies, crucial for supporting Investigational New Drug (IND) applications to regulatory bodies such as the FDA in the United States and the NMPA in China. A noteworthy application of this technology is the development of the B-hIL-4/hIL-4RA mouse model, particularly relevant for studying the therapeutic monoclonal antibody Dupilumab, which targets IL4RA to inhibit IL-4 and IL-13 signaling pathways involved in asthma pathophysiology. **Methods:** To overcome the challenge that human IL-4 does not activate mouse IL4RA and vice versa, Double humanized B-hIL-4/hIL-4RA mice were generated for *in vivo* efficacy assessment. **Results:** B-hIL-4/hIL-4RA mice were characterized for physiological similarities to wild-type mice, including baseline leukocyte populations, body weight, and various blood biochemical indices, to ensure model validity and safety. Functional studies demonstrated that B-hIL-4/hIL-4RA mice could be successfully applied in asthma and atopic dermatitis (AD) models. Analysis revealed inflammation-related changes, such as elevated eosinophils, neutrophils, increased IgE levels, and distinct pathological changes in lung tissue. Notably, administration of a Dupilumab analog led to significant improvements in asthma-related phenotypes, confirming the model's suitability for evaluating therapeutic efficacy. **Conclusions:** The B-hIL-4/hIL-4RA mice represent a robust platform for both efficacy and safety evaluations of human-targeted therapies. These advancements could streamline human drug development and provide more ethically sound models for studying human disease mechanisms.

PS 3425 Woodsmoke as an Environmental Amplifier of Autoimmune Pathology in Experimental Autoimmune Encephalomyelitis (EAE) Mouse Model

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Background and Purpose: Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) that impacts nearly 2.9 million people worldwide and disproportionately affects women between the ages of 20-40. MS is driven by autoreactive CD4⁺ T cells, particularly proinflammatory subsets, Th1 and Th17. While disease is believed to initiate due to breakdown of peripheral tolerance, pathology is driven by autoreactive CD4⁺ cells that cross the blood-brain barrier and promote demyelination. Despite available disease-modifying therapies, there is no cure and no single known cause, underscoring the need to identify environmental factors that influence immune function and disease risk. One possible environmental risk factor for MS, is exposure to woodsmoke from wildfires. Woodsmoke (WS), a major source of fine particulate matter (PM_{2.5}) from biomass combustion, contains complex chemical mixtures that induce systemic and neuroinflammatory responses. Our laboratory has shown that WS exposure in C57BL/6 mice upregulates cerebrovascular adhesion molecules (VCAM-1, ICAM-1), activates microglia, and promotes infiltration of neutrophils and monocytes (Ly6G⁺, Ly6C⁺) into the brain. Because MS is associated with neuroinflammation and disruption of the blood-brain barrier, we hypothesize

that WS exposure will exacerbate disease severity in the Experimental Autoimmune Encephalomyelitis (EAE) mouse model of MS. This hypothesis is supported by our previous findings showing that WS exposure increases CD4⁺ T-cell infiltration into the brain and drives neuroinflammation lasting up to 28 days post-exposure. Additionally, WS exposure produced increases in splenic naïve CD4⁺ T cells and activated CD4⁺ T cells. This suggests that inhaled particulates can prime or expand peripheral CD4⁺ T-cell populations, potentially lowering the threshold for autoimmune activation.

Methods: To address this, the EAE model was used to test if WS exposure amplifies CD4⁺ T-cell activation, alters effector function, increases leukocyte trafficking into the CNS and worsens disease severity. Female C57BL/6 mice induced with EAE were exposed to WS or filtered air at 0.5 mg/m³ for 4 hours per session, twice weekly for two weeks. Clinical scoring and body weight quantified disease progression and flow cytometry characterized immune activation across CNS and peripheral tissues, using a brain panel to evaluate leukocyte infiltration, endothelial adhesion, and microglial activation, and a spleen panel to define T- and B-cell phenotypes associated with immune priming. To assess antigen specificity and effector function, whole splenocytes were cultured *ex vivo* with or without myelin oligodendrocyte glycoprotein (MOG₃₅₋₅₅) and stimulated with anti-CD3/CD28 as a positive control. After four days, proliferation was evaluated, supernatants analyzed by multiplex cytokine (MSD) assay and cell pellets processed for RT-qPCR to measure Th1, Th17, and Treg cytokines (IFN-γ, IL-17A, IL-10, TGF-β) and transcriptional markers (Tbx21, Rorc, Il17a, Foxp3), as well as trafficking and myelin-integrity genes (Icam1, Cxcr3, Mbp). **Results:** Findings elucidate the degree to which WS exposure enhanced peripheral CD4⁺ T-cell activation, increased CNS infiltration and microglial activation, and ultimately impacted EAE clinical outcomes. **Conclusions:** Together, these results shed light on a potential role for WS exposure in heightening immune activation and CNS infiltration, strengthening evidence that WS acts as an environmental driver of MS and potentially other autoimmune diseases.

PS 3426 Efficiency of Tdap in Pregnancy with PFAS Exposure and Altered Immune Cell Populations in Maternal Rabbit Model

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are widespread contaminants in water, air, and food, raising health concerns. PFAS can cross the placenta and breast milk, exposing the fetus during critical development. While linked to immunotoxicity and reduced vaccine efficacy in adults and children, the effects of PFAS mixtures and PFBS on maternal vaccination remain unclear. **Methods:** Using a rabbit model, we studied the immunotoxicity of the environmental levels of PFAS or PFBS exposure via drinking water during pregnancy. Female rabbits at six months of age were given either clean water or water contaminated with a mixture of PFAS compounds or PFBS alone to drink *ad libitum* for 30 days prior to pregnancy and throughout gestation. Rabbits were given two doses of a Tdap vaccine; the first dose was given seven days before pregnancy, and the booster was given during pregnancy at gestational day 11 (GD11). Dams and their fetuses were necropsied at GD25. To characterize body burden accumulation and validate exposure, maternal serum PFAS levels were quantified at three different time points (before exposure, day 23 of exposure, and day 55). Antibodies to the Tdap vaccine were measured using ELISA, and antibody titers were calculated by a 3-fold difference between GD-7 and GD25. Peripheral blood mononuclear cells (PBMCs) were analyzed by single-cell RNA sequencing (RNAseq), from which cells were computationally clustered and analyzed. **Results:** PFAS and PFBS Low exposure reduced maternal Tdap vaccine efficacy by decreasing diphtheria-specific binding antibodies and impairing their neutralizing capacity. Single-cell RNAseq of maternal PBMCs showed PFAS exposure alters immune cell composition, introducing myeloid lineage cells such as pDCs, while decreasing B cell proportions. The transcriptional profile revealed B-cell dysfunction, characterized by altered receptor signaling and antigen presentation, reduced ribosomal biogenesis, and impaired regenerative capacity, which explains the reduced vaccine efficacy. Monocytes showed a senescence-associated secretory phenotype that promotes chronic inflammation. However, maternal-fetal IgG transfer efficiency was preserved, suggesting that maternal immune dysfunction is the primary driver of reduced offspring immunity. **Conclusions:** This study demonstrates that exposure to a PFAS mixture or PFBS alone can alter the health of B cells in the mother and impact antibody production during gestation, potentially reducing the effectiveness of prenatal vaccination in protecting offspring from neonatal infectious diseases.

PS 3427 Cetylpyridinium chloride (CPC) disrupts phosphatidylinositol 4,5-bisphosphate (PIP₂) nanoscale clustering and protein interactions in immune cells

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Background and Purpose: People are exposed to the widely-used, *positively-charged*, antimicrobial cetylpyridinium chloride (CPC) in cleaning supplies, personal products, and food. Previously we showed that CPC inhibits mast cell (MC) function at exposure-relevant, non-cytotoxic doses. MCs are immune cells which release granules

containing chemical and protein mediators (degranulation) in response to antigen stimulation to modulate the immune system. Phosphatidylinositol 4,5-bisphosphate (PIP₂) is a *negatively-charged* plasma membrane (PM) lipid critical for MC signaling. While PIP₂ clustering and dynamics have not been previously investigated in immune cells, we hypothesized that CPC may *electrostatically interfere* with this key signaling lipid. **Methods:** Due to the tiny length scales at which PIP₂ clustering may occur, we utilized live-cell super-resolution microscopy (FPALM; resolution 10-20 nm), coupled with total internal reflection fluorescence (TIRF), to investigate PIP₂ clustering in the RBL-2H3 MC model. PIP₂ cluster characteristics (area, density, perimeter, eccentricity) and dynamics (turn angles and diffusion coefficients) were investigated under a range of conditions including in resting and stimulated MC with or without CPC. Airyscan confocal microscopy and PIP strips (PIP₂ lipids immobilized on a strip) were utilized to investigate if CPC disrupted binding between PIP₂ and two binding partners. **Results:** We discovered that PIP₂ clusters in this immune cell model, and at sizes inaccessible to conventional microscopy. PIP₂ clusters ~220 nm in size or smaller were identified. CPC decreased PIP₂ cluster size and density, while increasing circularity. Furthermore, PIP₂ diffusion increased by CPC, suggesting interference of this drug with PIP₂. Antigen stimulation resulted in significantly larger PIP₂ clusters, and CPC treatment along with antigen stimulation counteracts this effect. Binding of a PIP₂ specific protein, Grip, to PIP₂ was also found to be disrupted by CPC via experiments with PIP strips: CPC increased the K_d of Grip-PIP₂ binding. Confocal microscopy experiments revealed that CPC also disrupts binding of another PIP₂ binding protein, the PM-resident mRFP-MARCKS-ED SA4. CPC (5 μ M for 1 hr) reduces the amount of mRFP-MARCKS-ED SA4 at the PM by approximately half. **Conclusions:** These results indicate that PIP₂ clusters grow larger due to antigen stimulation, and that PIP₂ clusters may arise as needed for various processes throughout a cell's lifespan. Our data also suggest that CPC's disruption of nanoscale PIP₂ clustering and of PIP₂ partner binding are mechanisms underlying CPC MC inhibition.

PS 3428 Protein Corona-Aware Multi-Task modeling for Nanoparticle Immunotoxicity

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Background and Purpose: Nanoparticles (NPs) have become a vital part of biomedical innovation, with applications such as drug delivery, imaging or tumor therapy. Their size and surface chemistry give them unique advantages. However, once exposed to the bloodstream, nanoparticles are susceptible to plasma proteins coating their surface, forming a protein corona (PC). This PC alters the physicochemical identity of the NP, which not only can induce immune responses but also limits the therapeutic efficiency. Uncontrolled cytokine release and clearance by macrophages are the most significant consequences of protein corona formation. Traditional migration approaches have largely relied on a trial-and-error workflow that is slow, costly, and hard to generalize across NP design. As a result, there is a pressing need for predictive tools to forecast immune response before *in vivo* or *in vitro* trials. To address this, we built a multi-task neural network (MTNN) that learns nonlinear links between NP design and biological outcomes, leveraging shared patterns across endpoints while preserving task-specific signals. Our model ingests NP physicochemical properties and, when available, protein-corona features to jointly predict cell viability and cytokine responses as surrogate markers of immunotoxicity. We emphasize TNF- α and IL-10, two consistently reported, mechanistically informative cytokines, to deliver size- and design-aware predictions. **Methods:** We curated a compact dataset from 25 primary studies, harmonizing NP physicochemical descriptors (zeta potential, NP subtype, size, surface modification/charge), PC features (ApoA-I, fibronectin, complement factor H—selected for reporting consistency), and key experimental covariates (cell type, exposure time/ concentration, medium/serum). We train an MTNN model with a shared trunk and two task-specific heads to jointly predict continuous cytokine levels (TNF- α , IL-10) and their high/low immunotoxicity classes via calibrated thresholds. Inputs are z-scored; categorical fields are one-hot encoded; missingness is handled with simple imputation plus a missing-indicator. Data is split by study (to avoid leakage) into train/validation/test (~80/10/10%), with grouped k-fold cross-validation for robustness on the small N. We tune learning rate, L2 weight decay, hidden size/depth, dropout, and epochs with early stopping. Performance is reported for both regression (MAE, RMSE, R2) and classification (ROC-AUC, PR-AUC, balanced accuracy, MCC, precision, recall, F1); we plot ROC/PR curves for each cytokine and assess probability calibration (isotonic/Platt). Decision thresholds use literature-based or validation-optimized cutoffs, and uncertainty is estimated via bootstrap ensembling. **Results:** The proposed MTNN model demonstrated promising predictive performance for cytokine response classification. During cross-validation, the model achieved an ROC-AUC of 0.69, balanced accuracy of 0.80, Matthews correlation coefficient (MCC) of 0.68, overall accuracy of 0.83, precision of 1.00, recall of 0.60, and an F1 score of 0.75. These results indicate that the MTNN effectively distinguished between high and low cytokine release levels, with particularly strong precision suggesting a low false-positive rate. The model's ability to generalize across cytokine endpoints underscores its potential to capture the shared nonlinear relationships between NP properties, protein corona composition, and immune activation pathways. **Conclusions:** The study introduces a predictive framework that connects NP design, PC composition, and immune outcomes using an MTNN approach. Even with a limited dataset, the model demonstrates the feasibility of a computational approach to anticipate immunotoxicity and identify NP likely to induce strong cytokine responses. Future works will focus

on expanding the dataset, refining the model, and validating predictions against a controlled experimental study to strengthen its use as a computational tool for immune safety assessment.

PS 3429 Uncovering the Impact of Common Antimicrobial Cetylpyridinium Chloride on Mast Cell Degranulation-Associated Mitochondrial Dynamics

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Background and Purpose: Consumers are exposed regularly to antimicrobial agent cetylpyridinium chloride (CPC) in personal care, cleaning, and food products. Mounting evidence suggests CPC breaches the blood-brain barrier, is bioavailable, and is toxic to eukaryotes at low doses, such as nanomolar toxicity to developing oligodendrocytes. Our lab previously showed that low-dose exposure to CPC causes mitochondrial toxicity on par with canonical mitotoxicant carbonyl cyanide-3-chlorophenylhydrazone (CCCP) in primary human keratinocytes, mouse NIH-3T3 fibroblasts, and RBL-2H3 cells. In addition, CPC limits function of the nervous and immune system cell type, mast cells (MCs), by an unknown mechanism, yet the research to fully explore the toxicology of this drug is sparse. In response to various stimuli, MCs undergo a functional response, degranulation, in which bioactive substances like histamine and serotonin are exocytosed from pre-formed granules. Upon exposure to the stimulant calcium ionophore, which enables the calcium influx precondition for the MC degranulation pathway without any IgE receptor crosslinking or early tyrosine phosphorylation, we have found CPC actually enhances MC degranulation, with a U-shaped dose response. This effect lies in stark contrast to the potent CPC inhibition of degranulation and early tyrosine phosphorylation we have observed when the activation pathway is initiated by antigen stimulation. We have also found that CPC causes mitochondrial fission, which is, curiously, both a sign of mitotoxicity and a requirement to energize immune signaling by promoting mitochondrial translocation to the plasma membrane. Thus, we hypothesize that CPC-induced mitochondrial fission is actually supporting and stimulating degranulation when CPC's inhibition of early phosphorylation events is alleviated through use of ionophore. **Methods:** Translocation of mitochondria in MCs was assessed via confocal microscopy and image analysis. RBL-2H3 rat MCs were cultured and activated, either using 0.7 ng/mL antigen or by bypassing it through 3.0×10^{-7} M calcium ionophore as determined by preliminary dose-response testing to represent low antigen response (~7% degranulation). Cells were incubated with 2.0×10^{-8} M MitoTracker Red FM and 1.0×10^{-7} M Hoechst 33342 dyes and treated with CPC (0-10 μ M). Images were taken via the Zeiss LSM 980 Airyscan confocal microscope, at 37°C. The oil-immersion 63X objective was used. Images were analyzed using a Fiji ImageJ software mitochondria plug-in. **Results:** The data show that antigen-stimulated degranulation as a result of receptor crosslinking coincides with enhanced mitochondrial translocation to the plasma membrane. This effect is dampened by the addition of 10 μ M CPC, in concert with CPC's degranulation inhibition at this dose. Stimulation with calcium ionophore also leads to a significant increase in mitochondrial translocation, but in this case, translocation is *enhanced* by low concentrations (2.5 μ M) of CPC, a result also aligned with CPC's increase in degranulation with ionophore. **Conclusions:** This study contributes to basic knowledge of mitochondrial involvement in immune signaling and also provides information regarding the complex effects that CPC and related quaternary ammonium antibacterials have on the immune system.

PS 3430 Investigating an *in vitro* method for determining the dermal and respiratory allergenic potential of proteins

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Background and Purpose: Proteins can exhibit allergenic potential following dermal or respiratory exposure. However, no standardized *in vitro* test method currently exists to evaluate this potential. In contrast, the biopharmaceutical field has developed *in vitro* immunogenicity assays. This study investigated whether the enzyme-linked immunospot (ELISPOT) assay using cultured peripheral blood mononuclear cells (PBMCs), originally designed for immunogenicity assessment, could also be applied to determine the allergenic property of proteins. Furthermore, as the ELISPOT assay detects cytokine secretion at the single-cell level. The development of protein-induced allergic sensitization involves not only T-cell activation (IL-2) but also type 2 helper T cell (Th2) cytokines, we compared the predictive performance across different cytokine readouts (IL-2, IL-4, IL-5 and IL-13). **Methods:** Based on epidemiological data for allergy after dermal or respiratory exposure to naturally occurring proteins, we selected the test proteins as follows: those with several reported cases were classified as high-allergenic proteins, whereas those with limited evidence from clinical or experimental studies indicating a significant role in sensitization were classified as low-allergenic proteins. The high-allergenic proteins included papain (papaya-derived protease), Der p 1 (mite-derived protease), and Asp f 1 (fungal ribonuclease), whereas potato agglutinin (potato-derived lectin) was classified as a low-allergenic protein. Moreover, because certain enzymes had numerous occupational case reports and are considered potentially allergenic, we included two lipases

and two amylases as occupational exposure-related proteins. PBMCs collected from multiple healthy donors were exposed to each test protein and cultured for 2 weeks. The cells were then divided into an antigen restimulation group and a media control group, and the ELISPOT assay was used to quantify spot-forming cells that produced IL-2, IL-4, IL-5, and IL-13. The positivity rate was calculated using the stimulation index (SI), defined as the ratio of spot-forming cell counts in the restimulation group to those in the control group. **Results:** Among the naturally occurring proteins, high-allergenic proteins generally exhibited higher positivity rates than the low-allergenic protein not only across IL-2 but also across IL-4, IL-5, and IL-13. In contrast, Asp f 1 exhibited no detectable IL-2 response, and papain exhibited no detectable IL-5 response. Moreover, potato agglutinin demonstrated higher positivity rates for IL-4 and IL-13 than for IL-2 and IL-5, indicating protein- and cytokine-dependent differences in responsiveness. Among the occupational exposure-related proteins, allergenicity was detected in all enzymes. For some enzymes concordant responsiveness was detected across cytokines, with the same enzymes exhibiting the highest positivity rates across multiple cytokines. **Conclusions:** The ELISPOT assay may be useful for the *in vitro* evaluation of protein allergenicity. Nevertheless, the most appropriate cytokine readout may differ depending on the protein. Future research will expand the test set and further validate the mechanistic basis to establish an optimal *in vitro* method for determining protein allergenicity.

PS 3431 Involvement of damage-associated molecular patterns (DAMPs) in the immune checkpoint inhibitor-induced myocarditis in A/J mice.

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Background and Purpose: Myocarditis is one of the adverse events induced by immune checkpoint inhibitors (ICI). The damage-associated molecular patterns (DAMPs) are endogenous molecules that induce potent inflammatory responses by activation of signaling pathways related to pattern recognition receptors (PRR) including Receptor for Advanced Glycation End products (RAGE) and Toll-Like Receptor 4 (TLR4). Although DAMPs have been presumed as aggravating factor of ICI-induced myocarditis, the details are not fully understood. This study was conducted to clarify the variations of DAMPs and their related factors in plasma and heart in the mouse model of ICI-induced myocarditis. **Methods:** Anti-mouse Programmed cell death 1 (PD-1) and anti-mouse Cytotoxic T-lymphocyte antigen-4 (CTLA-4) antibodies (200 µg/body each) or isotype control antibodies were intraperitoneally administered to male A/J mice (n=5/group) twice weekly for 12 weeks. Plasma cytokines and calprotectin were measured 4, 8, and 12 weeks after the first dosing. The immunophenotyping, expression analysis of DAMPs and their related protein, and histopathological examination in the heart were performed 12 weeks after the first dosing. **Results:** The levels of Th1, Th2, and pro-inflammatory cytokines, calprotectin, and effector memory CD8⁺ T cells in plasma were increased in the ICIs-treated group. In the protein expression analysis, the RAGE and TLR4 were detected in the heart of both control and ICIs-treated groups, with no noticeable differences observed. In contrast, the phosphorylation levels of JUN and NF-κB, which are downstream signaling of RAGE and TLR4, were significantly increased in mice given ICIs. In histopathological examination, loss of cardiomyocytes with inflammatory cells infiltration was observed in 40% of mice treated with ICIs. Increased protein expressions of HMGB1 and Galectin-3 were noted in the hearts of animals exhibiting cardiomyocyte loss accompanied by immune cell infiltration, whereas these changes were not observed in animals lacking cardiomyocyte loss, despite the presence of immune cell infiltration. **Conclusions:** Increased plasma calprotectin levels, accompanied by activation of downstream signaling pathways including JUN and NF-κB were observed in A/J mice treated with ICI, suggesting their involvement in the induction of local inflammatory response in the heart. Furthermore, increased cardiac HMGB1 and Galectin-3 are involved, at least in part, in the exacerbation of myocarditis observed in A/J mice treated with ICIs.

PS 3432 Glycolytic metabolism of CD8⁺ T cells affects susceptibility to human leukocyte antigen-mediated abacavir-induced hypersensitivity

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Background and Purpose: Drug hypersensitivity reactions (DHRs) associated with a specific human leukocyte antigen (HLA) allotype do not manifest uniformly in all individuals within an HLA model population. This scenario highlights the complexity of predicting DHR risk without considering additional factors. Furthermore, conducting prospective clinical studies of DHRs in humans is unfeasible. Therefore, this study aimed to investigate additional risk factors beyond HLA genetics that determine susceptibility to HLA-mediated abacavir (ABC)-induced hypersensitivity reactions by using a unique transgenic mouse line harboring HLA-B*57:01 (B*57:01-Tg).

Methods: B*57:01-Tg mice or their littermates were orally administered either an ABC-containing or a vehicle-only diet. Subsequently, analyses were performed on isolated CD8⁺ T cells collected from the lymph nodes, ear biopsies, or plasma of these mice. **Results:** RNA sequencing and proteomic data analyses performed on the isolated CD8⁺ T cells demonstrated that the expression levels of several glycolytic enzymes, including hexokinase 2, were significantly upregulated in ABC-treated B*57:01-Tg mice. Moreover, the glycolytic rate and metabolite production significantly increased in these mice. Although treatment with the pyruvic acid carrier inhibitor UK5099 did not affect CD8⁺ T cell activation, inhibiting glycolysis using 2-deoxy-D-glucose or diet-based calorie restriction attenuated the increase in IFN-γ-secreting effector memory CD8⁺ T cells. Consequently, this attenuation prevented CD8⁺ T cell dermal infiltration in ABC-treated B*57:01-Tg mice. **Conclusions:** Overall, these results suggest the indispensable role of glycolytic CD8⁺ T cell metabolism in HLA-mediated ABC-induced hypersensitivity reactions, which may further determine susceptibility to HLA-mediated DHRs in humans.

PS 3433 Comparing macrophage differentiation from monocytes isolated via negative or positive magnetic bead selection and performance in an immunotoxicity assay

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Background and Purpose: Macrophages play a key role in immunomodulation, driving initial inflammatory responses to diseases, wounds, and toxins, while also inhibiting inflammation and promoting healing. The process of generating macrophages and then polarizing them into their distinct sub-forms while still retaining their desired functionality in immunological assays requires a delicate balance of many factors. Everything from monocyte isolation protocols to macrophage differentiation protocols can influence the sub-phenotype and behavior of the resultant macrophages. In this study, we compared the phenotypes of cryopreserved monocytes isolated from human peripheral blood mononuclear cells via negative or positive magnetic bead selection. We then compared the sub-phenotypes and phagocytic capacity of M1 and M2 macrophages differentiated from each type of monocyte with and without an immunotoxic inhibitor. **Methods:** Cryopreserved human monocytes were isolated from fresh peripheral blood mononuclear cells via either positive (selecting for CD14^{pos}) or negative (selecting against all other cell types; CD14^{neg}) magnetic bead purification (3 donors each). The resulting monocytes were seeded at 0.1 million cells/well in 96-well plates and differentiated into macrophages using a modified KuGM[®] medium supplemented with 35 ng/mL M-CSF for 6 days. On day 6, three different polarization media were used to differentiate monocytes into M0 (naïve), M1 and M2 macrophage sub-phenotypes. Cells were cultured in this media for 24 hours and harvested on day 7 for immunophenotyping. Additional wells were used on day 7 to assess phagocytic capacity of each macrophage subtype. The phagocytosis assay used live-cell imaging to quantify the rate at which naïve, M1 and M2 macrophages engulfed fluorescently labeled bioparticles loaded at 10 µg/well final concentration. As a negative control, 1 µM cytochalasin D, an immunotoxic inhibitor of phagocytosis, was included to confirm the assay's specificity by blocking actin polymerization. **Results:** Naïve, M1 and M2 macrophages derived from either CD14^{neg} or CD14^{pos} cryopreserved monocytes displayed cell morphology consistent with each of the respective macrophage subtypes. All three macrophage subtypes showed the same overall patterns of surface and intra-cellular marker expression as determined via FACS analysis, though CD14^{pos}-derived macrophages tended to have more distinct differences in CD80 (M1 macrophage marker) and CD206 (M2 macrophage marker) expression when comparing among naïve, M1 and M2 subtypes. In general, macrophage subtypes derived from either CD14^{neg} or CD14^{pos} cryopreserved monocytes behaved the same in the phagocytosis assay. The general pattern showed M1 macrophages having the lowest rates of phagocytosis, while M2 and naïve macrophages were tied for highest rates of phagocytosis across all but one donor. In the outlying CD14^{neg} donor, naïve macrophages had very low rates of phagocytosis, similar to M1 macrophages. Regardless of donor or macrophage subtype, cytochalasin D significantly inhibited phagocytosis. **Conclusions:** Isolating monocytes via positive or negative magnetic bead selection had no significant impact on the morphology, overall marker expression, or phagocytic activity of macrophages. M1 macrophages had the lowest rates of phagocytosis, likely due to their overall phenotype as well as the use of LPS as a differentiating compound, which can inhibit phagocytosis. The use of M-CSF to differentiate monocytes into macrophages and the use of magnetic beads to purify monocytes from peripheral blood can push monocytes and naïve macrophages towards anti-inflammatory M2-like phenotypes, which was generally observed in the phagocytosis assay. However, donor-to-donor variability can predisposition monocytes and naïve macrophages towards the inflammatory M1 phenotype, which was observed in one of the CD14^{neg} donors where naïve macrophages had very low rates of phagocytosis, similar to M1 macrophages. Despite this, there was still a very high degree of separation between M2 and M1 phenotype sub-populations across all donors, indicating that the protocol used for macrophage differentiation is effective in generating distinct macrophage sub-phenotypes, regardless of whether the parent monocytes are isolated via positive or negative magnetic bead selection.

PS 3434 tBHQ inhibits dendritic cell-mediated CD4⁺ T cell activation in a Nrf2-dependent manner

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Background and Purpose: Dendritic cells (DCs) are professional antigen-presenting cells that bridge the innate and adaptive immune systems by activating naive T cells through antigen presentation on MHC class II molecules. Previous work from our lab demonstrated that tert-butylhydroquinone (tBHQ)—a widely used food preservative and potent activator of nuclear factor erythroid 2-related factor 2 (Nrf2)—reduces DC expression of key functional markers, including MHC II, CD80, and CD86. We also found that tBHQ suppresses CD25 and CD69 expression and decreases IL-2 secretion in CD4⁺ T cells, suggesting impaired T cell activation. However, it remains unclear whether the effects of tBHQ on T cell activation are a direct consequence of its impact on dendritic cells, given the essential communication between these two cell types. Since DCs are critical for T cell priming and activation, we hypothesized that tBHQ impairs DC-mediated CD4⁺ T cell activation in a Nrf2-dependent manner. **Methods:** To test this, splenic DCs were isolated from female wild-type or Nrf2-knockout mice, pulsed with OVA, and treated with vehicle (0.005% ethanol) or tBHQ (0.5, 1, or 5 μ M), then activated with LPS overnight. DCs were washed, counted, and co-cultured with splenic CD4⁺ T cells isolated from wild-type female mice for three days. T cell activation was assessed by measuring CD25 and CD69 expression, as well as CD44 and CD62L expression via flow cytometry and IL-2 secretion via ELISA. **Results:** tBHQ treatment significantly suppressed expression of T cell activation markers and reduced IL-2 production by T cells, but only when co-cultured with wild-type DCs. These effects were abolished in co-cultures using Nrf2-knockout DCs, indicating a Nrf2-dependent mechanism. **Conclusions:** Overall, our findings demonstrate that tBHQ impairs the ability of DCs to activate CD4⁺ T cells in a Nrf2-dependent manner, providing new insight into how this common dietary additive may alter adaptive immune responses.

PS 3435 Human cell-based miRNA investigation of occupational allergic asthma pathways

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Background and Purpose: Asthma is a chronic and heterogeneous disease of the lower airways, affecting approximately 300 million people worldwide. The most prevalent and extensively studied form is allergic asthma, which occurs in both children and adults. Its primary pathological hallmark arises from complex interactions between immune cells and immunological mediators. Recent evidence has highlighted alterations in microRNA (miRNA) expression across various lung diseases, including allergic asthma. Several miRNAs have been linked to asthma and airway inflammation; however, for most studies, the identification of their specific targets remains unresolved. This study, conducted entirely on human cells, aims to characterize miRNA expression patterns and identify potential targets involved in occupational allergic asthma. **Methods:** The *in vitro* approach is based on the human lung epithelial cells, namely Calu-3, exposed to different chemicals classified as respiratory sensitizers, skin sensitizers and irritants through a liquid aerosols system (Cloud Alpha System - VITROCELL® Systems). Additionally, human biological samples derived from healthy patients and patients affected by common and occupational asthma have been collected collected in collaboration with Dott. Liviero, Occupational Unit of Padova University and evaluated. **Results:** The miRNAs expression is evaluated through microRNA panels (mircury Panel Qiagen) for the *in vitro* part while a miRNA sequencing have been performed on serum human samples. Results obtained suggest an involvement of several miRNAs, such as miR-17-5p, miR-21-5p, miR-135b-5p, and let-7a-5p induced by respiratory sensitizers, in particular HClPt. **Conclusions:** The final purpose of the project is to compare data derived from the two systems, human and 3D *in vitro* assay and to identify possible target involved in the regulation of the miRNAs after respiratory sensitizers exposure. The translational potential of the project is due to the use of an *in vitro* cell culture model as well as human samples. Furthermore, implementation of miRNA patterns could be useful as prevention tool (biomarkers for diagnosis) of allergic asthma. *This study was supported by a grant from Ministero dell'Università e della Ricerca (PRIN 2022, project number PRIN202223VGLB_01).*

PS 3436 Prenatal exposure to inorganic arsenic and immune cell abundances in newborns

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Background and Purpose: Inorganic arsenic (iAs) is a toxic environmental contaminant that poses a risk to chronic disease, endocrine disruption, and immune dysfunction, particularly *in utero* exposure. T cells, essential to the immune system, have been hypothesized to mediate certain biological processes and pathogenic effects of prenatal iAs exposure. This study aimed to investigate the association between maternal exposure to iAs and the abundance of cell types at birth. **Methods:** We

included 215 infant-mother pairs from the TIMICS (Taiwan Maternal and Infant Cohort Study), TIMCS-P (Taiwan Maternal and Infant Cohort Study-Pilot), and TEC (The Taiwan Early-life Cohort Study) cohorts. We estimated cell type abundances based on RNA sequencing and DNA methylation array data from cord blood samples. We determined concentrations of arsenic species, including arsenite [As(III)], arsenate [As(V)], monomethylarsonate (MMA), dimethylarsinate (DMA), and arsenobetaine (AsB), using high-performance liquid chromatography in maternal urine samples at 28 to 36 weeks of gestation. **Results:** The abundance of CD8⁺ T cells in cord blood was inversely associated with maternal iAs exposure in the TIMICS cohort ($\rho = -0.257, p = 0.005$) and validated in the combined TIMICS-P and TEC cohort (partial $\rho = -0.224, p = 0.028$). Gene set variation analysis revealed that maternal iAs exposure was inversely associated with unfolded protein response and Myc targets, but positively associated with estrogen response and bile acid metabolism ($p < 0.05$). **Conclusions:** Prenatal iAs exposure may reduce CD8⁺ T cell abundance in cord blood, potentially via the estrogen response, highlighting CD8⁺ T cells as a marker of iAs-related immunotoxicity. Further experimental validations are warranted.

PS 3437 Investigating the Impact of Irregularly-shaped PET Microplastics (1-20 μ m) on Macrophage Function Using a Comparative Biology Approach with *Xenopus laevis* and a Human Cell Line

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Background and Purpose: Microplastics (MPs) (1 μ m-5mm) are ubiquitous in the environment and exhibit a variety of different shapes and compositions. Although the biological effects of pristine polystyrene microspheres at high concentrations (>1 mg/mL) have been extensively characterized, more realistic MPs with variable sizes (1-20 μ m) and irregular shapes (I-MPs) at lower concentrations have yet to be investigated. By employing a comparative biology approach with macrophages from the amphibian *Xenopus laevis* and a human monocytic cell line (THP-1), we hypothesize that artificially aging I-MP will increase the frequency of phagocytosis. In addition, we anticipate that internalization of I-MPs will induce macrophage dysfunction related to the expression and processing of major histocompatibility complex II (MHC-II). This macrophage dysfunction may ultimately increase susceptibility to pathogen infection. **Methods:** For this study, polyethylene terephthalate (PET) I-MPs were generated from cryomilled water bottles, sieved on silicone nanomembranes to 1-20 μ m, and subsequently stained using Nile red fluorescent dye. Primary macrophages from *Xenopus laevis* frogs were collected by peritoneal lavage and compared to macrophages differentiated from a human monocytic cell line (THP-1) using phorbol 12-myristate 13-acetate (PMA). Macrophages were exposed to PET I-MP concentrations of 0.1, 1, and 10 μ g/mL. The phagocytosis and the expression of MHC-II were assessed by confocal microscopy, ImageStream flow cytometry, and RT-qPCR. Ongoing studies are investigating techniques for artificially aging PET I-MP, which involve incubating PET I-MP in water samples from Lake Ontario or laboratory-generated freshwater under ultraviolet (UV) light for 7-14 days. **Results:** The early stages of this project have largely focused on method development. PET I-MP can be reliably stained using Nile red fluorescent dye and visualized by epifluorescence microscopy. Preliminary results from confocal microscopy and ImageStream flow cytometry indicate that PET I-MP are phagocytosed by macrophages even at low concentrations. However, the frequency of phagocytosis is low (7-10%) compared to polystyrene microspheres. Preliminary data also suggest that the phagocytosis of PET I-MP affects the relative MHC-II gene expression of both *Xenopus laevis* and human macrophages. **Conclusions:** The phagocytosis of PET I-MP by macrophages is observed at lower concentrations than currently found in the literature for pristine polystyrene microspheres. Our current studies are exploring the impact of PET I-MP or artificially aged PET I-MP internalization on MHC-II expression and processing in macrophages. Future studies will investigate the phagocytosis of I-MPs produced from other polymer types, the phagocytosis of I-MPs coupled to pathogen exposure, and the long-term effects of I-MP phagocytosis by following the macrophages in tadpoles over a longer period of time. Overall, this research will provide new insights across phylogeny into the potential of microplastic water pollutants to alter macrophage function.

PS 3438 Intracellular HLA-drug interaction in keratinocytes drives HLA-B*57:01-dependent abacavir skin toxicity

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Background and Purpose: Immune-mediated idiosyncratic drug toxicity often shows strong associations with specific human leukocyte antigen (HLA) alleles but remains difficult to model prospectively, particularly for skin-restricted reactions. Abacavir hypersensitivity (AHS) tightly linked to HLA-B*57:01 is a representative case. While HLA is classically viewed through surface antigen presentation, the determinants of skin tropism remain unclear. We set out to define cell-intrinsic mechanisms in keratinocytes that gate the onset of drug-provoked dermal immune responses. **Methods:** Primary keratinocytes from HLA-B*57:01-Tg, HLA-B*57:03-Tg, and their littermates were exposed to abacavir. ER-stress/UPR activation (e.g., Ca²⁺ flux), cytokine/chemokine induction, and T-cell chemotaxis were quantified. Mechanistic involve-

ment was tested with the chemical chaperone 4-phenylbutyrate (4-PB), and intracellular abacavir-HLA binding/aggregation were assessed by HLA immunocapture with LC-MS/MS and confocal microscopy. *In vivo*, HLA-B*57:01-Tg mice received abacavir $\pm$ 4-PB, and epidermal ER-stress markers (p-IRE1, XBP1), vascular permeability, serum CCL17, dermal CD8 infiltration, and CD8 effector-memory accumulation were measured. **Results:** Abacavir triggered rapid Ca^{2+} release and pro-inflammatory cytokine/chemokine induction in HLA-B*57:01 keratinocytes, but not in HLA-B*57:03 or their littermates, which drove T-cell chemotaxis toward keratinocyte supernatants. These responses were blunted by 4-PB. *In vivo*, HLA-B*57:01-Tg mice exhibited epidermal p-IRE1/XBP1 induction, increased vascular permeability, elevated serum CCL17, dermal CD8⁺ T-cell infiltration, and CD8⁺ effector-memory accumulation. 4-PB attenuated dermal readouts without reducing lymph-node effector-memory frequencies. Biochemically, abacavir co-purified with HLA-B*57:01 (but not HLA-B*57:03), coincident with higher-order HLA species and BiP engagement, consistent with intracellular HLA-drug binding and misfolding-driven ER stress. **Conclusions:** Our results clarify that HLA-mediated intracellular stress responses are pivotal to the onset of HLA-B*57:01-mediated AHS, suggesting that the intracellular behavior of HLA is a critical determinant of drug eruptions.

PS 3439 Single-cell analysis reveals AhR-dependent myeloid reprogramming and myeloid-derived suppressor cell migration following TCDD Exposure

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Background and Purpose: Under inflammatory conditions, the immune system generates suppressor cell populations that migrate to affected areas to limit excessive activation. Activation of the aryl hydrocarbon receptor (AhR) by high-affinity ligands has similarly been shown by our lab to expand highly immunosuppressive myeloid-derived suppressor cells (MDSCs). However, the mechanisms by which AhR signaling governs the induction, trafficking, and functional polarization of MDSCs remain to be established. **Methods:** C57BL/6 mice and mast cell-deficient mice were injected intraperitoneally with 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD, 10 $\mu\text{g/kg}$) or the vehicle, Dimethyl sulfoxide (DMSO). Bone marrow (BM) & peritoneal exudate cells (PEC) were isolated for downstream analyses using Single cell RNA-seq (scRNA-seq). **Results:** TCDD induced high levels of MDSCs (CD45⁺, CD11b⁺, Gr-1⁺) at the site of administration compared to vehicle. This induction was significantly downregulated in mast cell-deficient mice, however reconstitution of these mice with wildtype BM cells restored the MDSC accumulation in the peritoneum. scRNA-seq revealed a marked expansion of polymorphonuclear and monocytic myeloid-derived suppressor cell (PMN-MDSC and M-MDSC) clusters in the peritoneal cavity compared to bone marrow. In the bone marrow, RNA-velocity and CytoTRACE analyses showed stable hematopoietic trajectories and unchanged differentiation potential, indicating that AhR activation does not affect MDSC differentiation. In contrast, peritoneal velocity vectors were long and directional towards MDSC, with higher CytoTRACE scores denoting incoming, transcriptionally immature myeloid cells that acquire suppressive functions. Differential gene expression and pathway analyses demonstrated that TCDD up-regulated (S100a8, S100a9, Cxcr2, Mmp9, Csf3r, Il1b, Trem3, etc.) genes enriched for granulocyte migration, leukocyte chemotaxis, and cytokine-mediated signaling. In contrast, TCDD-treated bone marrow samples showed enrichment of mitotic and chromosomal-organization pathways without chemotactic or suppressive signatures. CellChat network analyses revealed that AhR activation increased intercellular communication, and MDSCs as dominant signaling hubs sending and receiving chemokine signaling molecules (CCL, FN1, GALECTIN, MIF, CD86, CD80, BST2, CEACAM & LAIR1, CD52, SELPLG, SIRP, SEMA4, CXCL, SELL), whereas bone-marrow networks remained largely unchanged between Vehicle and TCDD. **Conclusions:** These data demonstrate that AhR activation by TCDD mobilizes myeloid precursors from the bone marrow through chemokine signaling and transcriptional reprogramming into suppressive MDSCs within the peritoneal cavity, a process partially involving mast-cell signaling (This work was supported in part by NIH grants R01ES030144, P01AT003961, P20GM103641, and R01AI123947, R01AI160896).

PS 3440 Optimizing Antibody Selection and Gating Strategy for Cynomolgus Monkey Bone Marrow Immunophenotyping by Flow Cytometry

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Background and Purpose: Given the translational relevance to human immune system, accurate immuno-phenotyping of bone marrow immune cells in cynomolgus monkeys is essential for the immunotoxicological effects in toxicology studies. Repeat dose toxicology studies for the pharmaceutical drug development are generally conducted by the contract research organizations (CROs) who provide appropriate endpoint methods. Frontage-Suzhou developed and validated a flow cytometry-based method to optimize antibody selection and gating strategy for the quantification of key immune cell populations in the bone marrow of cynomolgus monkeys. **Methods:** Bone marrow from naïve cynomolgus monkeys was processed

by resuspending in PBS, filtering through a 70 μm mesh, and adjusting to 10^6 cells/ml. Cells were lysed with erythrocyte lysis buffer, and labeled with a high-specificity antibody panel (BD Biosciences and BioLegend). Staining was performed for 20 minutes, followed by washing and resuspension in staining buffer. Multi-dimensional gating strategies, including FSC/SSC gating to assess cell morphology and live/dead staining to exclude debris and non-viable cells, and dual-marker validation were implemented to identify T cells (CD3⁺), CTL cells (CD3⁺CD8⁺), Th cells (CD3⁺CD4⁺), monocytes (CD11b⁺CD66⁺CD14⁺), eosinophils (CD11b⁺CD66⁺Siglec-8⁺CCR3⁺), basophils (CD11b⁺CD117⁺CD123⁺FcεRIα⁺) and different stages of granulocytes. Data quality was assessed for reproducibility. **Results:** In a representative sample, live cells comprised 98.2% of events, confirming effective debris exclusion. Immunophenotyping revealed lymphocytes at 18.50%, monocytes at 5.63%, eosinophils at 0.03%, basophils at 0.10%, myeloblasts at 0.14%, promyelocytes at 0.26%, myelocytes at 17.28%, metamyelocytes at 25.82% and mature granulocytes at 39.27% of the parent population. The optimized gating strategy achieved high-resolution separation of cell subsets, with minimal non-specific binding. **Conclusions:** Flow cytometric immunophenotyping methodology with bone marrow in cynomolgus monkeys was established and validated using multiple fluorescently labelled antibodies and gating strategy was optimized to enable precise and robust assessment of immunotoxicological evaluations.

PS 3441 Development of human alveolar macrophage model cell lines for the assessment of immunotoxicity of nanoparticles

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Background and Purpose: The immunotoxicity of nanoparticles has recently attracted attention. Nanoparticles reach the deepest parts of the lungs through respiration, where they are phagocytosed and removed by alveolar macrophages (AMΦ). Some nanoparticles can cause severe allergic reactions, thus there are needs to develop alternative methods of predicting such reactions. However, primary cells are not reproducible and human AMΦ-like cell lines have not yet been established. These are obstacles to studying the immunotoxicity of nanoparticles in the respiratory system *in vitro*. Furthermore, the adverse outcome pathways (AOPs) of nanoparticles in the respiratory system remain unclear. Therefore, the purpose is to develop a method of assessing the immunotoxicity of nanoparticles by developing AMΦ model cell lines and examining their immune response to the nanoparticles. To create these cell lines, we adapted a recently reported method for differentiating human primary peripheral blood mononuclear cells into AMΦ like cells. **Methods:** We used two types of human monocytic cell lines, acute monocytic leukemia derived cells (THP-1 cells) and iPS-derived myeloid cells (iPS-ML). THP-1 cells were cultured in RPMI1640 containing FBS and iPS-ML cultured in MEM-α containing FBS, GM-CSF and M-CSF. These cells were differentiated by stimulation with GM-CSF, TGF-β, IL-10, and pulmonary surfactant (SURFACTEN), as previously reported. Differentiation into AMΦ was evaluated by examining the expression of surface markers (CD11b, CD14, CD45, HLA-DR, CD163, CD169, CD206, MARCO) using FACS. Also, the number of viable cells during differentiation was counted to evaluate cell proliferation. The function of AMΦ was assessed using ELISA to measure IL-1α production in response to stimulation with allergy-inducing aluminium hydroxide [Alum, Al (OH)₃], non-inducing aluminium oxide (Alumina, Al₂O₃). **Results:** As results of the differentiation method using surfactant cocktails, the expression of HLA-DR⁺, CD206⁺/CD169⁺ and CD206⁺/MARCO⁺ increased in differentiated THP-1 cells, compared with untreated THP-1 cells. Also, the expression of CD206⁺/CD169⁺ and CD206⁺/MARCO⁺ was higher in differentiated iPS-ML than in untreated iPS-ML. As AMΦ is known to express several scavenger receptors, these results suggest that the surfactant cocktail method has the potential to differentiate THP-1 cells and iPS-ML into AMΦ like cells. The number of viable THP-1 cells increased five-fold during differentiation, but the number of viable iPS-ML decreased. This may mean that M-CSF is an essential growth factor for iPS-ML proliferation. Primary mouse AMΦ have been reported to produce IL-1α in response to allergen-inducing nanoparticles; therefore, IL-1α was also used as an indicator in this study. IL-1α production increased in response to Alum stimulation compared to Alumina stimulation in untreated iPS-ML; however, untreated THP-1 cells did not produce IL-1α in response to both Alum and Alumina stimulation. Differentiated THP-1 cells produced IL-1α in response to Alum stimulation, but the amount remained low. Also, differentiated iPS-ML produced little IL-1α, and there was no difference between Alum and Alumina stimulation. These results suggest that IL-1α is a promising indicator when human AMΦ-like cell lines are exposed to allergic nanoparticles. We also investigated another method without using surfactant cocktails. Interestingly, continuous 7-day culture of iPS-ML in GM-CSF and M-CSF increased CD206⁺/CD169⁺ and CD206⁺/MARCO⁺ expression. 7-day cultured iPS-ML increased IL-1α production in response to Alum stimulation by twice compared to untreated iPS-ML. The differentiation mechanisms of iPS-ML during the 7-day culture with GM-CSF and M-CSF are currently under study. **Conclusions:** Our results suggest that iPS-ML and its differentiated cells could be used as AMΦ-like cell lines, with IL-1α serving as an indicator to evaluate the immunotoxicity of nanoparticles *in vitro*. However, the optimal differentiation method into AMΦ cell lines from iPS-ML is still under investigation.

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Background and Purpose: Xylene substances (xylene) are high production chemicals that are currently undergoing new data generation and dossier evaluation under European Regulation on the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH). This includes consideration of an additional immunotoxicity testing cohort to an extended one-generation reproduction toxicity study (EOGRTs). Recently there has been a significant expansion to the dataset, providing an opportunity to evaluate the immunosuppressant potential of a legacy industrial chemical with a significant pre-existing animal and human dataset, and evaluate if an additional animal cohort is warranted. **Methods:** A systematic literature review and weight of evidence approach evaluation was conducted that included consideration of all available immunotoxicity information on xylene derived from relevant *in vitro*, *in vivo*, and epidemiology approaches including previously unpublished new studies of xylene substances. The specific mechanisms/modes of action of xylene and xylene metabolites that would indicate immunosuppression were reviewed. **Results:** Multiple high-quality guideline *in vivo* studies have repeatedly interrogated xylene's immunosuppressive potential including systemic haematological and clinical chemistry markers as well as pathological evaluations of bone marrow, lymph nodes, spleen, and thymus. Despite some statistically spurious and inconsistent immune system reported findings, overall, the dataset in its totality demonstrated no signal of xylene-mediated immunosuppression. The previously unpublished repeated dose studies also align with this conclusion. The limited available epidemiology data showed no xylene exposure-related effects on white blood cell counts, and EOGRTs data relevant to hippuric acid, a structural analogue of the xylene metabolite methyl hippuric acid, showed no indication of developmental immunosuppression. Similar data on ethylbenzene, a xylene reaction mass constituent, showed no indication of developmental immunosuppression. **Conclusions:** The evidence collectively supports that xylene substances are not immunosuppressants and does not support the need for an immunotoxicity cohort evaluation in any subsequent EOGRTs study per Regulations Commission Delegated Regulation (EU) 2015/ 282 of 20 February 2015 amending Regulation (EC) No 1907/2006.

PS 3443 Sex-Specific Immunotoxic Effects of Pesticides on Hematopoiesis

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Background and Purpose: The Central Valley is known for its agricultural productivity, and populations that are highly exposed to pesticides are known to manifest chronic dysregulation of molecular and cellular pathways eventually leading to disease. Some toxicology studies have established that broad-spectrum fungicides such as pyraclostrobin (PYRA) can cause mitochondrial dysregulation and oxidative stress in aquatic life such as zebrafish (Lin and Yeh 2025). However, the effects of such pesticides on cellular mechanisms and immunotoxicity in mammalian vertebrates remain unclear. Our objective is to investigate the effects of PYRA induced changes on hematopoiesis - the process of generating white blood cells, red blood cells and platelets - and the extent of its toxicity. The cell populations were extracted from primary lymphoid organs of bone marrow (BM), and thymus as well as a secondary lymphoid organ, spleen, to reveal early cellular toxicity. **Methods:** Eight to twelve-week-old female and male C57BL/6 mice, were intraperitoneally injected for 30-34 days with PYRA at a 10 mg/kg body weight dose. The control group received corn oil (vehicle). BM, thymus, and spleen samples from individual mice in each group were processed for cell counting and flow cytometric analysis. **Results:** Our findings indicate that PYRA exposure alters immune cell composition in a sex-dependent manner. In males, PYRA increased the percentage of myeloid-biased MPP2 progenitors without affecting their absolute numbers, and no additional changes in hematopoietic progenitor populations were detected. PYRA-treated females similarly exhibited no differences in any hematopoietic progenitor subsets, including MPP2s. These results suggest that PYRA's effects occur downstream of hematopoietic progenitors, primarily influencing differentiated immune cell populations. PYRA treatment induced sex-dependent remodeling of lymphocyte populations across primary and secondary lymphoid tissues. In males, PYRA increased both the percent and absolute number of bone marrow CD4⁺CD8⁻ T cells, whereas females exhibited no significant bone marrow changes. Within the spleen, males showed an increase in CD4⁺CD8⁻ (double-negative) T-cell percent without a corresponding rise in absolute numbers, along with a reduction in total CD3⁺ T cells and no change in overall splenic cellularity. In contrast, females displayed a pronounced increase in splenic cellularity, accompanied by increases in CD3⁺ T cells in both percent and number, resulting in elevated absolute counts across all splenic T-cell subsets. Females also exhibited an increase in splenic B-cell numbers, whereas monocyte and granulocyte populations remained unchanged, indicating that PYRA's effects were predominantly lymphocyte

specific. Thymic cellularity was unchanged in both sexes; however, female CD8⁺CD4⁻ thymocytes were decreased in percent and number. **Conclusions:** Collectively, these findings demonstrate that PYRA selectively modulates T-cell (and to a lesser extent B-cell) populations in a strongly sex-dependent and subset-specific manner, with minimal impact on myeloid lineages. Our results highlight the importance of accounting for sex specific outcomes so there can be more targeted and effective mitigation strategies. Building upon our previous work, we are beginning to experiment with a widely known herbicide, glyphosate (GLY) to investigate its potential hemotoxic and carcinogenic effects. This study will elucidate the pathway mechanisms of how certain immune cell populations are being disrupted by GLY, while contributing to cancer risks among highly exposed communities. Future studies will investigate the combined effects of exposure to GLY and PYRA, reflecting real-world scenarios. By studying the biological impacts of pesticide exposures, the study maintains its broader commitment to environmental stewardship in protecting vulnerable communities and promoting safer pesticide use.

PS 3444 Imaging analysis of the effects of Zn deficiency during pregnancy on placental tissues

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Background and Purpose: Nutritional Zn deficiency induces immune dysfunction, including the development of inflammatory diseases. Zinc deficiency in pregnant women can restrict intrauterine fetal growth. However, the mechanism by which zinc deficiency causes intrauterine fetal growth restriction remains unclear. This study investigated the effects of zinc deficiency on placental tissue structure (basal and labyrinth layers) in zinc-deficient rats during pregnancy. **Methods:** Nine-week-old male SD rats and eight-week-old female SD rats were mated, and pregnant female rats were fed a standard diet (3 mg/day), a zinc-insufficient diet (0.3 mg/day), or a zinc-deficient diet (0.03 mg/day) for 19 days (n = 8 in each group). After dietary manipulation, implantation marks from uterus and amniotic fluid were collected, and the fetus and placenta were measured. Placental sections were stained with H&E, and the total areas of the placental tissue, basal layer, and labyrinth layer were measured using image analysis. **Results:** The final body weights of the zinc-deficient group were significantly lower than those of the other groups. The weight of the placenta and fetal rats in the zinc insufficiency or zinc deficiency groups was significantly lower than that in the standard group. Also, the miscarriage (%) of the zinc-deficient group was significantly higher than those of the other groups. For placental tissue imaging analysis using H&E staining, the total area, basal layer, and labyrinth layer of zinc insufficiency and deficiency groups were significantly lower than those in the standard group. **Conclusions:** It was suggested that zinc deficiency in rats during pregnancy leads to decreased serum zinc concentrations, resulting in atrophy of the placenta (basal and labyrinth layers). Consequently, it has been demonstrated that impaired supply of nutrients and oxygen to the fetus increases the risk of intrauterine growth restriction and miscarriage.

PS 3445 Recapitulating Immune-Driven Hepatotoxicity Using a Liver Microphysiological Platform

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Background and Purpose: With the April 2025 U.S. Food and Drug Administration (FDA) roadmap promoting New Approach Methodologies (NAMs) for regulatory testing of monoclonal antibodies (mAbs) and other drugs, there is a need for models with greater complexity and human relevance. For biologics such as mAbs, immune-mediated drug-induced liver injury (IDILI) remains a significant concern in drug development. Human-specific immune system interactions with new modalities, such as therapeutic antibodies, can lead to unpredictable hepatotoxicity, which often fail to be captured in traditional animal models and high-throughput cell line models, leading to poor translational outcomes and high attrition rates. The FDA-recognised PhysioMimix® DILI assay was modified to incorporate peripheral blood mononuclear cells (PBMCs) to address this with an immune-competent, human liver relevant MPS model. The model aims to enhance translational relevance and improve safety profiling in drug development pipelines. **Methods:** Primary human hepatocytes were cultured under dynamic perfusion in Liver-12 plates by PhysioMimix Core to form 3D liver microtissues. The liver microtissues were analysed for functionality (CYP activity, albumin, urea) and clinically relevant injury markers (ALT, AST). Following four days of hepatocyte culture, HLA-matched PBMCs were added to the circulating media with the liver microtissues for a further four days and functionality assessed (cytokine profiling). Ipilimumab (anti-CTLA-4) or infliximab (anti-TNF-alpha) was dosed into the media of hepatocyte only and co-cultures for 48 hours and samples were taken at 1-, 4-, 8-, 24- and 48-hours post-dosing. The liver microtissues were evaluated for hepatotoxic responses while immune activation and inflammation was assessed by cytokine release. **Results:** Immune cell interaction and recovery from the Liver-12 was first completed to determine the ability of the PBMC to transverse the microfluidic channels and scaffolds unhindered (60-70% recovery). Following initiation of co-culture, maintenance of liver microtissue functionality and stable immune

phenotypes was demonstrated over four days of co-culture. Upon dosing with the mAbs in the co-culture model, immune-mediated hepatotoxicity was detected with significant elevation in LDH and ALT/AST, and reduction in the functional biomarkers, albumin and urea. Interestingly, significant cytokine increases were only seen in ipilimumab-treated conditions, aligning with clinical data which demonstrated a pro-inflammatory phenotype via activation of T-cells. **Conclusions:** This proof-of-concept study using two clinically relevant monoclonal antibodies demonstrates the ability of the PhysioMimix DILI assay to predict immune-mediated hepatotoxicity. Future studies will look to further profile immune alterations and interactions with the liver microtissues. Together, this assay provides a human-specific solution to the assessment of hepatotoxicity of large molecules. The FDA's endorsement of NAMs for investigational new drug (IND) applications marks a paradigm shift toward human-relevant, non-animal testing strategies, positioning the PhysioMimix® Core System as a pivotal tool for the future of immunotoxicity assessment and safer drug development.

PS 3446 Comparative uptake of perfluorooctanoic acid (PFOA) from water or milk matrices in an *in vivo* mouse model

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Background and Purpose: Traditional oral gavage with water is an efficient and effective way to administer a dosing solution in an *in vivo* mouse model but may induce stress from inexperienced personnel. This study aims to investigate the efficacy of an alternative, potentially less stressful dosing approach using sweetened condensed milk (SCM) delivered with a micropipette as a way to administer per- and polyfluoroalkyl substances (PFAS). **Methods:** 64 C57BL/6 mice (32 males, 32 females) underwent 15-day exposures, with SCM compared to water gavage, using perfluorooctanoic acid (PFOA) as a model PFAS. The mice were divided into 4 groups (n=8 per group): water vehicle control, SCM control, water + 7.5 mg/kg PFOA, SCM + 7.5 mg/kg PFOA. Urine was collected and pooled per cage for both sexes and collected at 3 timepoints: pre-dosing, and after 24 hours and 5 days. Six days prior to study terminus, mice were injected with sheep red blood cells to elicit T cell-dependent antibody responses (TDAR). Terminal blood was collected and organ samples (liver, spleen, thymus, kidney) were collected, weighed, and archived for later analyses. **Results:** It appeared as if mice receiving SCM did not consistently consume the full dosing volume from the micropipette, leading to incomplete dosing in some individuals. Preliminary organ weight data illustrated this as liver weights in the PFOA-gavaged group was approximately double its control groups. Whereas liver weights in the SCM-PFOA group were increased by 110% on average. Observations suggested similar stress behaviors during handling for both techniques, although gavage was faster to administer. Quantitative analysis of PFOA in urine and blood samples is ongoing and the TDAR will be evaluated with an enzyme-linked immunosorbent assay. **Conclusions:** SCM as a dosing matrix has the potential to serve as an alternative to water gavage; however, incomplete consumption may reduce dosing accuracy. Differences in liver weights and in liver peroxisomal enzyme activity between PFOA-treated and control mice indicate treatment-related effects, but the data also indicate that SCM alone induces liver peroxisomal enzyme activity. These findings suggest that SCM may not be a viable alternative dosing matrix for PFAS with the potential to induce peroxisomal enzyme activity.

PS 3447 PET Imaging of Myeloid Cell Modulation in a Mouse Tumor Model

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Background and Purpose: Immunosuppressive myeloid cells play a critical role in tumor progression, metastasis, and therapy resistance. In solid tumors, myeloid cells can constitute up to 50% of the tumor mass, making them the most abundant immune population in the tumor microenvironment (TME). These cells promote tumor cell proliferation, metastasis, chemoresistance, and angiogenesis, and are emerging as key targets for therapeutic strategies such as depletion, repolarization, and recruitment inhibition. Quantifying myeloid cells is essential for preclinical drug development, providing insights into therapeutic efficacy and potential drug resistance mechanisms. Additionally, visualizing myeloid cells can support patient selection for myeloid-specific treatments and serve as a prognostic tool. A positron emission tomography (PET) imaging tracer targeting a myeloid biomarker, such as CD11b, enables quantification of these cells, facilitating preclinical immunosuppression studies and non-invasive prediction and monitoring of patient response. **Methods:** The human/mouse cross-reactive anti-CD11b antibody, M1/70, was selected for quantifying CD11b+ myeloid cells. In a proof-of-feasibility experiment, C57BL/6 mice were injected with fluorophore-conjugated M1/70 or an isotype control. Optical imaging (IVIS) and flow cytometry were used to quantify specific binding *in vivo*. Next, the antibody was conjugated with deuterium (DFO), and radiolabeled with ⁸⁹Zr, achieving high radiochemical purity (>99%) and immunoreactivity (>98%). To test the CD11b PET probe *in vivo*, mice were implanted with MC38R tumors and treated with Talazoparib or vehicle for 7 days to induce systemic myeloid depletion. Flow cytometry, PET imaging, and *ex vivo* γ-counting were performed across tissues to quantify changes in the myeloid cell population. **Results:** IVIS imaging revealed significant signal in myeloid-rich femur (9.7 % injected dose ID/g)

and spleen (6.4% ID/g) in fluorophore-labeled anti-CD11b injected mice compared to isotype (4.7 and 3.1 %ID/g respectively), demonstrating the imaging capability of this antibody. Flow cytometry confirmed these results with isotype group showing negligible binding in the blood and spleen. Talazoparib treatment resulted in significant depletion of CD11b+ myeloid cells in the tumor (1.6-fold), bone marrow (femur) (2.5-fold), and the spleen (3.3-fold) as quantified by flow cytometry. PET imaging and *ex vivo* γ-counting successfully validated this depletion showing reduced tracer uptake in the tumor (1.29-fold), femur (2-fold), and spleen (2-fold). No significant differences were observed in other organs such as the liver, kidneys, lungs, and heart indicating low non-specific uptake of the tracer. **Conclusions:** In conclusion, the specificity of anti-CD11b to quantify CD11b+ myeloid cells was demonstrated. The antibody was successfully radiolabeled to generate a PET tracer and was evaluated in a mouse tumor model. This technique provides a sensitive tool for assessing immunotoxicity and therapeutic interventions targeting the myeloid compartment. The approach supports preclinical evaluation of immunomodulatory strategies and has potential translational relevance for patient stratification and monitoring in oncology. All procedures performed on animals were in accordance with regulations and established guidelines and were reviewed and approved by an Institutional Animal Care and Use Committee or through an ethical review process.

PS 3448 Plant-derived compound sulforaphane suppresses activation and functional marker expression in primary murine NK cells

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Background and Purpose: Sulforaphane (SFN), a bioactive compound found in cruciferous vegetables, has gained considerable interest for its ability to modulate multiple cellular pathways, including those involved in antioxidant defense and inflammation. Through these pathways, SFN plays an important role in modulating immune responses. However, current studies have predominantly focused on T cells and dendritic cells, while research on its effects in natural killer (NK) cells remains limited. Previous work in our lab demonstrated that tert-butylhydroquinone (tBHQ), a well-characterized Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2) activator, suppresses the upregulation of CD25 and CD69 in NK cells following activation with phorbol 12-myristate 13-acetate (PMA) and ionomycin. This suppression led to a more mature NK cell phenotype accompanied by significantly reduced NK cell function. Building on these findings, the present study investigates the effects of sulforaphane, another potent Nrf2 activator, on the activity of primary murine NK cells. **Methods:** Murine splenocytes were treated with 1, 3, 5, 10, 15, and 20 μM of L-SFN, followed by activation with either PMA/ionomycin or IL-12/IL-18 for 24 hours. Key NK cell activation events were assessed via flow cytometry by measuring the induction of surface markers CD25, CD69, CD107a, CD44, and NKG2D. Functional activity was evaluated through intracellular staining for interferon gamma (IFN-γ), granzyme B, and perforin. In addition to effector functions, NK cell maturation was also analyzed. Data were compared between cells activated by PMA/ionomycin, a robust yet non-physiological method that bypasses surface receptors and directly triggers intracellular signaling, and IL-12/IL-18, which provides a more physiologically relevant cytokine-driven activation. **Results:** Results indicate that treatment with SFN impacts NK cell biology by suppressing their expansion following activation in a dose-dependent manner. In addition to limiting proliferation, SFN decreases NK cell activation across both PMA/ionomycin and IL-12/18 stimulations, as evidenced by reduced CD25 and CD69 expression. Under IL-12/18 stimulation, SFN promotes an immature NK cell phenotype and a shift away from terminal maturation, whereas PMA/ionomycin stimulation does not show this effect. **Conclusions:** Correspondingly, SFN reduces NK cell effector functions, including cytokine production of interferon gamma, granzyme B, and perforin, in response to both PMA/ionomycin and IL-12/18 stimulation. Together, these findings suggest that SFN broadly dampens NK cell responses, affecting proliferation, activation, maturation, and effector function.

PS 3449 Δ⁹-tetrahydrocannabinol impairs enhanced IFNγ secretion associated with chronic HIV infection in primary human monocyte-CD8⁺T cell co-cultures.

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Background and Purpose: The widespread implementation of combined antiretroviral therapy has shifted human immunodeficiency virus (HIV) infection to a manageable yet chronic disease. However, these individuals are at risk of developing HIV associated neurocognitive disorders (HAND), which is characterized by a persistent state of neuroinflammation which can result in impaired cognitive function. Monocytes and CD8⁺ T cells are known to enter the central nervous system (CNS) during chronic HIV infection, and IFNγ producing CD8⁺ T cells correlate with worse cognitive outcomes. The HIV+ population self-report *Cannabis sativa* use to mitigate symptoms of chronic infection, and the side effects from combined antiretroviral therapy. Δ⁹-tetrahydrocannabinol (THC) treatment suppresses monocyte interleukin

(IL)-1 β and CD8 $^{+}$ T cell IFN γ production. The objective of this study is to characterize the effects of THC on monocyte and CD8 $^{+}$ T cell interactions in the context of neuroinflammation. **Methods:** Peripheral mononuclear blood cells were isolated by Ficoll density gradient centrifugation from human donor. Subsequently, two independent secondary magnetic negative selection isolations were performed to isolate the CD8 $^{+}$ T cell and monocyte populations. CD8 $^{+}$ T cell and monocyte monocultures were established at 1x10 ^{6} cells/mL and monocytes were seeded at 2.5x10 ^{5} cell/mL, respectively. CD8 $^{+}$ T cell and monocyte cocultures were established at a 4:1 ratio. All cultures underwent a 30-minute pretreatment with either vehicle (0.03% DMSO) or THC (1, 5, or 10 μ M) at 37°C. The cultures were subsequently activated with 10 μ g/mL R848, 25 μ L/mL ImmunoCult, and supplemented with 5ng/mL IL-2. Supernatants and cells were collected up to 3-days post-activation and cytokine responses were quantified by ELISA and flow cytometry. **Results:** IFN γ secretion was observed to be upregulated in CD8 $^{+}$ T cell monoculture and coculture condition with a seven-fold increase in supernatant associated IFN γ from coculture samples. Furthermore, THC treatment suppressed coculture IFN γ secretion in a concentration dependent manner. Analysis by flow cytometry determined an approximately a two-fold increase in the geometric mean fluorescent intensity associated with IFN γ production in the CD8 $^{+}$ and CD8 $^{+}$ CD14 $^{+}$ populations. Utilization of trans-well inserts, recombinant cytokine treatment, in addition to neutralizing antibodies demonstrate that enhanced IFN γ secretion is associated with IL-12p70 (IL-12p70), which is also susceptible to suppression with THC treatment. Monocytes from HIV+ donors produce more IL-12p70 than those from HIV- donors. While there is no significant difference in IFN γ secretion by CD8 $^{+}$ T cells from HIV+ and HIV- donors, IFN γ secretion by all populations is suppressed with THC treatment. **Conclusions:** Monocytes and CD8 $^{+}$ T cells are both known to enter the CNS of HIV+ individuals, and IFN γ production by the CD8 $^{+}$ T cell population is associated with worse cognitive outcomes. This work demonstrates that monocyte-derived IL-12p70 enhances CD8 $^{+}$ T cell secretion of IFN γ . While IFN γ secretion is not directly enhanced during chronic HIV infection, there is an increase in monocyte production of IL-12p70. Furthermore, both IFN γ and IL-12p70 are susceptible to suppression with THC treatment. This demonstrates that enhanced inflammatory endpoints from monocyte and CD8 $^{+}$ T cell interactions are susceptible to suppression with THC treatment. This work is potentially generalizable to other disease states where IFN γ has been directly implicated.

PS 3450 Cholesterol esterification in macrophages: The effects of ACAT1 on macrophage activity

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Background and Purpose: Lipid laden macrophages (LLMs) are characterized by the formation of lipid droplets. Alveolar macrophages (AMs) are poised to form LLMs being bathed in lipid rich surfactant. LLMs have been observed in many inflammatory pulmonary pathologies and are largely associated with exacerbating injury: EVALI, sulfur mustard, silica dust, etc. LLMs are traditionally characterized by accumulation of oxidized low-density lipoprotein (oxLDL). To reduce lipid droplet formation in cells, we targeted acyl-coenzyme A: cholesterol acyltransferase 1 (ACAT1), the protein responsible for esterification of cholesterol for lipid droplet synthesis. Previously, we found intratracheal instillation of a pharmacological inhibitor reduces macrophage cholesterol accumulation and blunted lung injury. To further explore ACAT1's role in macrophage lipid handling, we generated a myeloid specific ACAT1 knock out (*Acat1*^{-M/M}). We hypothesize that *Acat1*^{-M/M} will reduce LLM formation and reduce lung injury. **Methods:** Male and female WT and *Acat1*^{-M/M} mice given intratracheal bleomycin (ITB) (3U/kg) to induce acute lung injury and necropsied after 7d. Daily body weight was measured to assess weight loss. Bronchoalveolar lavage (BAL) cells were cytospun to assess cell differentials, counted for enlarged foamy appearance, and stained for Oil Red O (ORO) to assess lipid content. A bone marrow derived macrophage (BMDM) model was established to assess *Acat1*^{-M/M} macrophages when treated directly with lipids. As a preliminary study, we treated BMDMs with oxLDL to induce a LLM phenotype. BMDMs of WT and *Acat1*^{-M/M} mice were isolated and differentiated for 7d and treated with oxidized LDL (oxLDL) on d5 or LPS and IL-4 on d6. BMDMs. On d7 macrophages were stained for ORO, Seahorse glycolytic and mitochondrial metabolism, and qPCR for lipid related genes: *Scarb1*, *Msr1*, *Cd36*, *Abca1*, *Cpt1a*, *Nr1h3*. **Results:** Within *Acat1*^{-M/M} mice ITB led to increased weight loss (5.77 $\pm$ 1.94% vs 10.10 $\pm$ 1.32%), worse histological lung injury, and increased neutrophils in bronchoalveolar lavage (BAL) fluid (3.99 $\pm$ 0.65% vs 7.82 $\pm$ 1.66%). *Acat1*^{-M/M} mice treated with ITB had significantly higher numbers of enlarged cells than WT ITB (21.14 $\pm$ 4.43% vs 39.52 $\pm$ 5.74%). When stained for ORO, *Acat1*^{-M/M} BAL cells, both PBS (vehicle) and ITB, did not stain positively for neutral lipids, while WT BAL cells, both PBS and ITB, both stained positively. This is consistent with a lack of ACAT function leading to increased phospholipid accumulation. Both WT and *Acat1*^{-M/M} derived BMDMs stained positively for ORO when treated with oxLDL. *Acat1*^{-M/M} BMDMs show a trend of increased glycolytic metabolism (404.6 $\pm$ 51.2% relative to WT), glycolytic capacity (514.1 $\pm$ 222.6% relative to WT) mitochondrial basal respiration (156.6 $\pm$ 38.5% relative to WT) and mitochondrial maximal respiration (147.8 $\pm$ 13.8% relative to WT). But when treated with oxLDL there is a trend of an increase in glycolytic metabolism in WT, which is not seen in *Acat1*^{-M/M}. The qPCR results showed a general increase in lipid handling genes in WT with oxLDL treatment, consistent with an LLM phenotype. But *Acat1*^{-M/M} oxLDL BMDMs showed a decrease of these genes, showing oxLDL did not induce a similar LLM phenotype in *Acat1*^{-M/M}. **Conclusions:** These results show, unlike a whole lung targeted pharmacological inhibition, a myeloid specific loss of

ACAT1 function results in exacerbated lung injury and increased number of LLMs. LLMs from WT stained positively for ORO while *Acat1*^{-M/M} were negative, indicating either an accumulation of free cholesterol or non-neutral lipid within *Acat1*^{-M/M} macrophages. When trying to induce a foam cell phenotype in BMDMs by treating with oxLDL, both WT and *Acat1*^{-M/M} showed positive ORO staining, contrasting with LLMs seen *in vivo*. This indicates the lipid species accumulating *in vivo* *Acat1*^{-M/M} ITB macrophages is not cholesterol but rather a non-neutral lipid, such as phospholipid from lung surfactant. *Acat1*^{-M/M} BMDMs treated with oxLDL did not show the shifts in metabolism and transcriptional activity of lipid handling genes found in WT oxLDL BMDMs. Indicating that cholesterol is not driving *Acat1*^{-M/M} macrophages into an LLM phenotype. These results contrast the current paradigm which assume LLMs can only be formed from cholesterol and triglycerides. Future work will assess the lipid species found in LLMs of injured *Acat1*^{-M/M} by treating BMDMs directly with BAL lipids of injured animals.

PS 3451 A Novel Method for Enhanced Bone Marrow Collection in Non-human Primates: Improving Yield and Cell Viability for Downstream Analysis

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Background and Purpose: Bone marrow tissues are valuable and essential samples to diagnose, monitor and research a wide range of hematologic and systemic diseases. As the primary site of blood cell production, bone marrow tissues are valuable samples for studies on hematopoiesis and immune cell status, and to detect malignant or abnormal cells. In both preclinical and clinical settings, bone marrow collection is an important method to obtain samples to further scientific understanding of therapeutic responses in hematopoietic and immune systems and advance patient care. Therefore, proper collection techniques to ensure adequate volumes of high-quality samples are critical to ensure cell viability and assay accuracy. **Methods:** The centrifugation of whole bones is an effective and widely used technique for post-mortem bone marrow collection in animal research models, usually in mice and rats. After harvesting long bones such as the femur or humerus, the distal epiphyses are removed and the bone is placed vertically in a collection tube for centrifugation, with the proximal epiphysis at the top of the conical tube. This process uses centrifugal force to rapidly expel marrow from the medullary cavity, providing a high-yield, minimally diluted sample. Biomere has expanded this technique to non-human primates to further collection methods and optimize the terminal bone marrow yield. **Results:** A comparison was performed to further validate the centrifugation method as the superior collection method in comparison to manually scraping. Terminal humerus samples were collected from N=2 cynomolgus macaques. Comparing collection yield through total live cell counts and cell viability (%) showed that centrifuging method yielded a significantly increased count compared to scraping. The left humerus for both animals was scraped and yielded 332.5 million live cells at 89% viability for the first animal and 289.3 million live cells at 90% viability for the second animal. The right humerus for both animals was centrifuged and yielded 737.5 million live cells at 89% viability for the first animal and 602 million live cells at 90% viability. **Conclusions:** Both methods showed comparable viability while a clearly significant increase in total live cell counts were detected in samples prepared using the centrifugation method.

PS 3452 Comparison of Immunological Function and Ability to Produce ADA to Drug Products for Neo-Thy versus CD34 Immune-Humanized Mouse Models

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Background and Purpose: Immune-humanized mouse models are increasingly used to evaluate biological therapeutics. However, differences in immune reconstitution and function between model types could impact interpretation of results. Here, we compare the immune reconstitution, function and ability to produce anti-drug antibodies between Neo-Thy and CD34 immune-humanized mice using two products, infliximab and salmon calcitonin (sCT), both known to cause immunogenicity in patients. Neo-Thy immune humanized mice are produced through implantation of human thymic tissue under the kidney capsule, followed by CD34 $^{+}$ hematopoietic stem cell (HSC) transplantation, supporting human thymopoiesis. CD34 immune-humanized mice only have HSC transplantation and T cells are trained by the vestigial murine thymus. **Methods:** Immune humanized mice were produced in paired groups, with half receiving a neonatal human thymus and all receiving HSC from the same donor, using a total of four unique HSC donors. Following humanization, they were administered infliximab, sCT, a combination of these treatments, positive or negative control on treatment schedules appropriate to each therapeutic over 9-weeks. Longitudinal blood/serum sampling (day 0, 21, 42) and collection of blood/serum, bone marrow, spleen, and lymph nodes were completed at necropsy. Immune

function and antibody responses were evaluated through quantification of total IgM and IgG (by isotype), anti-drug antibody (ADA) assays, flow-cytometric phenotyping, intracellular cytokine production and proliferation. **Results:** Neo-Thy mice had higher frequencies of activated/mature T cells and B cells in PBMC, spleen and lymph nodes; and greater IFN γ and IL-2 cytokine responses, compared to CD34 mice. IgM and IgG Isotype antibody production were measured. In general, all humanized mice produced IgM, with some CD34-humanized mice having levels greater than the upper limit of quantitation (ULOQ) at Day 0. Many CD34-humanized mice produced IgG1, including saline treated, with some having high levels at Day 0. In contrast, Neo-Thy humanized mice had significantly fewer mice producing IgG1, however, when produced, showing a treatment responsive pattern. For both IgG2 and IgG4, CD34 mice produced more of both isotypes than Neo-Thy mice, but depending on the donor, levels were high at Day 0. Despite increased IgG1 production by CD34 mice, only Neo-Thy mice produced neutralizing antibodies to infliximab. With respect to sCT, both produced donor dependent binding antibodies; however, Neo-Thy tended to show a dose response, whereas CD34 mice generally did not. Binding antibodies to KLH for both mouse types showed responsiveness, however, both KLH-treated and saline mice produced responses, when saline treated mice had never encountered the antigen. **Conclusions:** Overall, Neo-Thy mice produced from the same donor as CD34 mice exhibited more functionally relevant and specific anti-drug immune responses. This study shows that presence of human thymus is critical to development of neutralizing ADAs and suggests they may support immunogenicity assessment in the drug-development/nonclinical space. Our results further highlight critical model-dependent differences that can influence interpretation of results and inform selection of appropriate humanized mouse models for regulatory-oriented immunosafety studies. **Disclaimer:** This abstract reflects the views of the author and should not be construed to represent FDA's views or policies.

PS 3453 Neo Thy immune humanized mice can produce anti-drug antibodies to support immunogenicity assessment for biological drug products

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Background and Purpose: Immunogenicity can be a critical determinant of safety and efficacy for biological drug products, yet conventional animal models typically fail to recapitulate human immune responses, as human drug products are recognized as foreign by their immune systems. Neo-Thy immune humanized mice develop a functional human immune system and may provide a translational platform to assess anti-drug antibody (ADA) formation, and immune cell activation. In this study, we evaluated the immunogenicity of two therapeutics known to induce ADAs in patients, infliximab and interferon- β (IFN- β), administered individually and in combination, in Neo-Thy immune humanized mice derived from multiple human donors with distinct HLA profiles. **Methods:** Neo-Thy immune humanized mice were generated using neonatal thymus and CD34⁺ hematopoietic stem cells (HSC) at two different locations, creating six donor-defined experimental groups. Mice (4-6 per treatment per group) received infliximab, IFN- β , a combination of infliximab and IFN- β , positive or negative control with treatment schedule appropriate to the therapeutic. Peripheral blood/serum was collected on day 0, 21, 42, and 63; tissues (BM, spleen, lymph nodes) were harvested at necropsy (day 63). Immune reconstitution and treatment effects were assessed by multiparameter flow cytometry, intracellular cytokine (ICC) assays, proliferation, multiplex total IgM/IgG isotyping, ADA ELISAs for infliximab and IFN- β , and histopathology. **Results:** Neo-Thy immune-humanized mice showed robust human immune reconstitution with donor-dependent differences in B, T, and NK cell subsets. Treatment with infliximab, IFN- β , and KLH induced marked modulation of B-cell populations shown through changes in CD27⁺IgD⁺ and CD27⁺IgD⁺; and shifts from naïve (CD45RA⁺) to memory (CD45RO⁺) T cells. ICC and proliferation assays demonstrated antigen-specific IgM/IgG production and enhanced lymphocyte proliferation, respectively, upon drug re-stimulation *ex vivo*. In several cases, humanized mouse serum immunoglobulin levels approached or exceeded those from normal human serum, which served as a control. As expected, most immune humanized mice produced significant amounts of IgM, with some individual mice produced levels comparable to human control serum. IgG levels were measurable for all IgG isotypes for each donor group, regardless of treatment. Some mice in Groups 2 and 4, that were infliximab or combination-treated *in vivo*, produced IgG1, IgG2 and/or IgG4 at levels that approached the human control. Binding ADAs to IFN- β and neutralizing ADAs to infliximab were detected in multiple mice in a donor- and treatment-dependent manner. **Conclusions:** Neo-Thy humanized mice mount human-like, donor-dependent immune responses to biologics, including class switching, antigen-specific proliferation, and ADA formation. These findings support Neo-Thy humanized mice as a translational preclinical model for immunogenicity risk assessment and evaluation of immune modulation by biological drug products. **Disclaimer:** This abstract reflects the views of the author and should not be construed to represent FDA's views or policies.

PS 3454 Associations Between Tobacco Smoke Exposure and Cadmium Exposure in the U.S. Population: Findings from NHANES 2015-2020

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Background and Purpose: Cigarette smoke contains toxic chemicals, such as cadmium, which accumulate in the body and lead to serious health problems. Overall, smoking rates in the U.S. have decreased significantly due to prevention and cessation efforts. However, it is still uncertain how much tobacco smoke exposure currently contributes to cadmium burden at the population level. This study examines the current effect of tobacco smoke exposure on cadmium levels using biomonitoring data from the U.S. population. **Methods:** We analyzed data from the National Health and Nutrition Examination Survey (2015-2020) for children, adolescents, and adults in the U.S. The association between serum cotinine levels and blood and creatinine-adjusted urine cadmium levels was investigated. Smoke exposure status was categorized by serum cotinine levels into no exposure, light secondhand exposure, heavy secondhand exposure, and active smoking. Survey multivariable linear regression was conducted to estimate geometric means (GMs) and the ratios of GMs, adjusting for all covariates. **Results:** Among the adult population, compared to non-exposure, high secondhand smoke exposure and active smoking were associated with 1.55 times and 3.20 times higher blood cadmium levels, respectively, with evidence of a dose-response relationship (p -trend<0.001) after adjusting for all covariates. However, for urinary cadmium, active smokers showed significantly elevated levels (1.58 times higher), while no significant elevation was observed for secondhand smoke exposure. Among children and adolescents, cadmium levels did not differ across smoke exposure statuses. Additionally, older age, female sex, lower poverty-income ratio, lower education, and racial minority status were associated with higher cadmium exposure. **Conclusions:** This study demonstrates that tobacco smoke exposure and key socioeconomic and demographic factors remain important drivers of cadmium burden in the U.S. population. It emphasizes the importance of addressing these factors in public health interventions and environmental risk reduction efforts.

PS 3455 Cytotoxicity of Freebase Nicotine and Its Analogs Using Cellular and Statistical Methods

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Background and Purpose: (S)-nicotine has been used for decades in traditional cigarettes and, more recently, in modern e-cigarettes. However, (S)-nicotine induces adverse health effects, particularly in the lungs. Analogs of (S)-nicotine, such as (R,S)-nicotine, 6-methyl nicotine, nicotinamide, nicotine lactate, and nicotine benzoate, are growing in popularity as (S)-nicotine replacements. These analogs are marketed as 'safer alternatives,' yet little information is available on their induced health effects or toxicological properties. **Methods:** In this study, we systematically investigated methods for predicting the lethal concentration (LC) values of (S)-nicotine and five of its analogs using cell cultures and statistics. Human bronchiolar epithelial lung cells (BEAS-2B) were exposed to five nicotine analogs over a 24-hour exposure period. Cellular metabolism and cellular membrane integrity were measured and subsequently modeled using a four-parameter dose-response function in RStudio. Both models (culture- and statistics-based) were used to rank the chemicals in order of potency. **Results:** Out of the 5 analogs of interest, 6-methyl nicotine had the most adverse effects on cell health according to the overall ranking. However, all the analogs showed differential responses depending on which endpoint was being analyzed. **Conclusions:** The methods used in this study establish a framework for comparing toxicological evaluations and provide important insight into the lethality of emerging nicotine analogs.

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Background and Purpose: Extrapolating *in vitro* toxicology results to humans would be more meaningful if clinically relevant exposure doses were known. For both Electronic Nicotine Delivery Systems (ENDS) and Heated Tobacco Products (HTP) clinical studies reporting plasma nicotine values were used to calculate clinically relevant *in vitro* exposure doses for the extrathoracic, tracheobronchial and alveolar regions of the human respiratory tract on a mass of nicotine/cm² - min basis. **Methods:** A recently published PubMed search was duplicated and identified 95 papers as of November 2024, which were compared with previous summaries of pharmacokinetic studies of ENDS product use. Elimination of duplicates resulted in 65 and 15 unique clinical studies reporting nicotine plasma levels after ENDS and HTP use, respectively. To calculate the total amount of absorbed nicotine from each clinical study reporting *ad libitum* ENDS use, reverse dosimetry was performed using 3 recently published nicotine specific physiologically based pharmacokinetic (PBPK) models and one commercial PBPK model. To calculate nicotine vapor and particle deposition within regions of the human respiratory tract (ET, TB, AL regions), 3 published aerosol dosimetry models specifically designed for semi-volatile ENDS aerosols were used. Finally, the surface of each respiratory tract region was used to determine the clinically relevant *in vitro* exposure doses on a nicotine mass/cm² - min basis. Forty of the 65 clinical trials conducted around the world reported plasma nicotine values from *ad libitum* ENDS product use by 1323 participants. Participants used a variety of ENDS products with nicotine concentrations ranging from 0-59 mg/ml (0 - 5% nicotine) and product use from 4.5 minutes to 24 hours. Nine of the 15 clinical trials reporting *ad libitum* use of HTPs also reported plasma nicotine values (C_{max}) that were similar to nicotine C_{max} values from *ad libitum* use of ENDS products. **Results:** The range of total absorbed nicotine predicted by the four PBPK models using reverse dosimetry from these 40 clinical trials was 0.0018 - 2.04 mg nicotine/min. The three semi-volatile aerosol deposition models predicted absorbed nicotine that ranged from 0.016 - 775 µg in the ET region, 0.022 - 483 µg nicotine/min in the TB region, and 1.01 - 1,977 µg nicotine/min in the AL region. When these absorbed nicotine values are divided by the surface area of these respiratory tract regions, the clinically relevant *in vitro* exposure dose range for the ET region is 0.00009 - 5.0 µg nicotine/cm² - min. The clinically relevant *in vitro* exposure dose range for the TB region is 0.008 - 102 ng nicotine/cm² - min, and for the AL regions is 0.0014 - 4.6 ng nicotine/cm² - min. **Conclusions:** Based on the similarity of nicotine C_{max} values between *ad libitum* use of HTP and ENDS products, the same clinically relevant *in vitro* exposure dose ranges should be used for *in vitro* studies using HTP. Additionally, nicotine reverse dosimetry should allow extrapolation to clinically relevant doses of other ENDS and HTP constituents (i.e., flavors and HPHC) from characterized aerosols. These clinically relevant *in vitro* exposure dose ranges for nicotine are up to 370 times lower than levels of ENDS flavors tested in some *in vitro* studies with tracheobronchial cells. The magnitude of this difference is conservative since nicotine is typically used in ENDS products at a higher concentration than individual flavors. Here we show the utility of using the PBPK reverse dosimetry models to determine relevant exposure dose ranges for *in vitro* testing.

PS 3457 Impact of Benzaldehyde and Veratrylaldehyde on Acrolein-Induced Cytotoxicity

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Background and Purpose: Artificial flavors have become widespread in e-cigarettes (e-cigs), with over 14,000 classified e-liquid products currently available on the market. These flavoring agents significantly contribute to the appeal and uptake of e-cigs, particularly among the youth, raising concerns about their potential pulmonary toxicity. Since many flavor compounds are aldehydes, and potentially reactive with proteins, there is a need to assess the impact of the flavoring agents with known e-cig toxicants, such as acrolein. Therefore, the objective of this study is to assess the potential synergistic interactions between benzaldehyde (BA) and veratrylaldehyde (VA) in enhancing acrolein-induced cytotoxicity. **Methods:** BEAS-2B human bronchial epithelial cells and A549 human lung carcinoma cells were seeded into 96 mm cell culture plate inserts and treated with increasing concentrations of acrolein, either alone or in combination with BA or VA, for 24 and 48 h. Cell viability was evaluated through a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and colony-forming assay. Oxidative stress was assessed using the dichlorodihydrofluorescein diacetate (DCFDA) assay. **Results:** A549 cells exhibited significant

cytotoxicity to acrolein (IC₅₀: 60 µM at 24h and 58 µM at 48h), while the aldehydes were less cytotoxic, BA (IC₅₀: 2.5 mM at 24h and 2.0 mM at 48h) and VA (IC₅₀: 1.2 mM at 24h and 3.8 mM at 48h). Similarly, acrolein was toxic to BEAS-2B cells (IC₅₀: 34 µM at 24h and 30 µM at 48h), while the aldehydes were much less toxic, BA (IC₅₀: 2.0 mM at 24h and 1.1 mM at 48h) and VA (IC₅₀: 2.10 mM at 24h and 2.18 mM at 48h). The combination of BA or VA with acrolein, produced an additive cytotoxic effect. Our colony-forming assay supported the results obtained from the MTT assay. We also found that the combination of acrolein with BA or VA induced oxidative stress in A549 and BEAS-2B cells in a dose-dependent manner. In a similar manner, acrolein produced a dose-dependent increase in oxidative stress in both A549 and BEAS-2B cell lines. The IC₅₀ values for the aldehydes were in the millimolar range. The addition of BA or VA to the acrolein-treated cells did not synergistically increase the levels of oxidative stress. **Conclusions:** Altogether, our findings indicate that neither BA or VA impact the toxicity nor oxidative stress caused by acrolein in cell culture. However, further studies are warranted to elucidate the combined effects of acrolein with BA or VA in inducing pulmonary oxidative damage following e-cig exposure in humans.

PS 3458 High Levels of Toxic Aldehydes in High-Puff Number Disposable E-cigarettes

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Background and Purpose: Despite concerns regarding the potential health risks associated with e-cigarette use, their design, obtainable puff count, and e-liquid formulations have continued to evolve. Aldehydes are a key class of thermal degradation by-products of e-liquid constituents generated during the vaporization process. **Methods:** This study investigated the concentrations and toxic effects of chemicals and aldehyde reaction products in previously vaped and discarded disposable, higher puff number e-cigarettes. E-fluids were extracted from vaped devices obtained from users or collected from the field, and analyzed using gas chromatography-mass spectrometry to quantify a range of chemicals, including solvents, nicotine, coolants, flavor chemicals, and aldehydes. Pulmonary cells were exposed to aerosols and analyzed for toxic effects in multiple assays. **Results:** The percentage of e-fluid consumption averaged 46% - 77% across multiple brands. Levels of chemicals in e-fluids varied significantly by brand and mode of collection. Total Flavor chemicals varied across brands (means = 34 mg/mL ± 21.6 for Elf Bar and 9.7 mg/mL ± 7.9 for FLUM) and significantly decreased in vaped fluids. Compared to unvaped fluids, methylglyoxal, glyoxal, and formaldehyde significantly increased in vaped fluids, acetaldehyde and acrolein both decreased in vaped fluids, and glyceraldehyde and dihydroxyacetone were present only in vaped fluids. Increased concentrations were particularly pronounced in devices collected in the field, in contrast to those donated by users. Aldehyde levels were generally higher in Elfbar than in FLUM products. Methylglyoxal and acetaldehyde significantly disrupted BEAS-2B cell morphology and increased mitochondrial ROS in a concentration-dependent manner. **Conclusions:** Several factors influence the formation of aldehydes, which are subsequently inhaled by users, potentially leading to adverse health effects. These findings heighten important concerns regarding the safety of e-cigarette products, particularly with the long-term health implications of aldehyde exposure as a result of extended use. Public health policies and regulations can address these findings by considering the implications of puff count, device design, and e-liquid formulation on the formation of toxic by-products. Further research is needed to explore the cumulative health effects of aldehyde exposure from e-cigarettes, particularly among users with high consumption rates.

PS 3459 Thirdhand Vape Exposures Induce Immune Dysregulation and Epigenetic Alterations in a Mouse Model

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Background and Purpose: The widespread adoption of Electronic Nicotine Delivery Systems (ENDS), including e-cigarettes, has been driven in part by perceptions of reduced harm relative to combustible tobacco products. Despite reductions in conventional smoking, the rise in ENDS use has introduced new challenges for toxicology and public health. A growing body of evidence suggests that ENDS emissions can persist on surfaces long after active use. These residues contribute to a phenomenon known as thirdhand smoke (THS). When derived from ENDS product use, this is referred to as thirdhand vape (THV), and the toxicological and molecular consequences of THV exposures remain poorly understood. **Methods:** In this study, we sought to investigate the toxicological impacts of THV exposure *in vivo* using a mouse model. We hypothesized that chronic exposure to ENDS residues deposited on surfaces would drive measurable alterations in immune responses, transcriptomic regulation, and epigenetic stability. To test this, we conducted controlled exposure experiments, integrating cytokine profiling, RNA sequencing, DNA methylation analysis, and environmental residue confirmation through towel assays for nicotine and heavy metals. Nine-week-old C57BL/6J mice were indirectly exposed to ENDS residues via cotton towels pre-exposed for 1 hour to ENDS aerosol (6 mg/mL nicotine in 50/50 propylene glycol/vegetable glycerin) and placed in

cages as bedding for 1 hour daily/5 days a week, over 3 or 4 weeks. Control mice received unexposed towels. Nicotine and metal residues on towels were quantified via LC-MS/MS and ICP-MS. Bronchoalveolar lavage fluid (BALF), & serum were collected for cytokine profiling, lung and liver for RNA sequencing, and DNA methylation analyses. Cytokine/chemokine levels were measured using MILLIPLEX® MAP Mouse Cytokine/Chemokine Magnetic Bead Panels (MilliporeSigma). Group differences were assessed using ANOVA ($p < 0.05$). **Results:** A significant deposition of nicotine was observed on exposed towels compared to control towels, with nickel and chromium levels consistent with previous reports of ENDS aerosol constituents. Compared with control mice, thirdhand-exposed mice at a 3-week exposure time point had significantly elevated IL-13 levels in BALF ($p = 0.016$), with particularly strong increases in females ($p = 0.026$). Female BALF IL-1 α was reduced ($p = 0.039$), suggesting suppression of local pro-inflammatory signaling, while male BALF IFN- α was elevated ($p = 0.035$), indicating sex-specific antiviral responses. Systemically, serum IL-7 was decreased ($p = 0.003$), implicating disruptions in lymphocyte homeostasis. Serum KC (CXCL1) was elevated ($p = 0.045$), pointing to enhanced neutrophil recruitment, while serum IFN- α was decreased in females ($p = 0.038$). At the 4-week exposure time point, female BALF IL-7 was decreased ($p = 0.037$), while male BALF LIX (CXCL5) was reduced ($p = 0.009$). Systemically, serum LIX was also decreased ($p = 0.042$). These findings indicate a temporal shift from early Th2-skewed inflammation toward broader immune suppression with prolonged exposure. Compared with controls, exposed mice also showed overexpression of liver Socs2 (adjusted $p = 0.002$) and lung Gm22068 (adjusted $p = 0.012$) and reduced CpG methylation in liver (83.3%) and lung (99.5%) tissue. Pathway analyses highlighted enrichment of AP-1 signaling and dysregulation of metabolic networks. Differentially methylated regions overlapped with immune-related and cancer-related genes. **Conclusions:** This study provides evidence that exposure to THV from ENDS residues may be harmful. Environmental analyses confirmed that ENDS aerosols deposit nicotine and metals on surfaces, creating reservoirs of exposure that can lead to subsequent immune dysregulation and epigenetic alterations. Given the vulnerability of children and other sensitive populations to thirdhand exposures, our findings underscore the need for translational studies and public health policies that regulate indoor ENDS use.

PS 3460 E-Cigarette Modulation of T Helper Cell Subsets: Impaired IL-17 Production in Th-17 Cells

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Background and Purpose: Electronic cigarette (e-cigarette) use is rising, representing a growing public health concern among youth and young adults. E-cigarettes are electronic nicotine delivery systems designed to aerosolize liquid formulations for inhalation using a metal coil, a process termed vaping. Despite widespread use, e-cigarette effects on the developing immune system remain poorly understood. CD4+ T helper (Th) lymphocytes play an important role in orchestrating the antigen-dependent immune responses. Different Th subsets can direct either pro- or anti-inflammatory responses: Th1 produce interferon- γ (IFN γ), Th2 cells produce interleukin-4 (IL-4), IL-5, and IL-13; Th17 produce IL-17; and T regulatory cells (Tregs) act as immune suppressors to limit the immune response. We hypothesize that e-cigarette exposure will modulate T cell populations. **Methods:** We combined *in vivo* and *in vitro* murine models to assess the effects of e-cigarette aerosols and their components on helper T cell populations. Male and female mice were exposed for 2 weeks to air, propylene glycol and vegetable glycerin (PG/VG), nicotine salts, or tobacco-flavoured e-cigarettes. Splenocytes were analyzed by spectral flow cytometry for T cell subpopulations. Separately, lymph nodes from unexposed mice were processed into single cell suspensions, with or without CD4+ cell isolation, and cultured under Th17-polarizing conditions (anti-CD3/CD28, TGF- β 1, IL-6) in the presence of tobacco-flavoured e-cigarette extracts. Components of total extract including nicotine salts, PG/VG, tobacco flavouring (without nicotine) were also individually assessed *in vitro*. Cells were then profiled for T cell subtypes. **Results:** Two-week *in vivo* exposures induced sex-specific changes in splenic T cell subsets. Despite an increase in total CD4+ T cells, tobacco-flavoured e-cigarette aerosol exposure decreased Th1 and Th17 populations in female mice. In males, nicotine salt aerosol exposure decreased Th2, Th17, and Tregs populations. Exposure to PG/VG did not significantly alter any T cell populations. We further studied the effects on Th17 cells as they were affected in both males and females. To understand whether exposure to e-cigarette aerosols inhibited differentiation of Th17, differentiation of Th17 cells from lymph nodes was performed *in vitro* in the presence or absence of e-cigarette extracts. Th17 differentiation was not impaired, but IL-17A production was significantly suppressed. Furthermore, this was a direct effect on CD4+ T cells and did not require other lymph node-resident cells. *In vitro*, inhibition of IL17A was observed with nicotine salts and tobacco flavouring alone, suggesting that both components may impair Th17 responses. **Conclusions:** Our findings suggest that e-cigarette exposure may compromise T cell populations, particularly Th17 responses; these responses are particularly important in autoimmune diseases, recruitment of neutrophils, and defence against fungi. Future experiments will characterize gene expression changes in Th17 cells upon exposure and use a Th17-dependent murine model of psoriasis to provide insight on how vaping alters these responses.

PS 3461 Oral-Immune Co-Culture Model Reveals Significantly Lower Toxicity of Tobacco-free Nicotine Pouches Compared to Cigarettes

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Background and Purpose: Tobacco-free Nicotine Pouches (TFNP) are an emerging category of nicotine products gaining popularity with adult smokers. As TFNP don't contain or burn tobacco, research demonstrates they contain significantly fewer and substantially lower levels of harmful chemicals compared to cigarette smoke, therefore offering adult smokers a potentially less harmful way to consume nicotine. Initial *in vitro* investigations of these products reveal marked reductions in cytotoxicity when compared to cigarette smoke extracts (>200 fold less cytotoxic) and no genotoxicity (under the conditions of Ames and *In Vitro* Micronucleus tests). Building on this data, we sought to better reflect the complex cellular interactions occurring at the oral mucosal interface, by employing a novel oral-immune co-culture model to assess the potential impact of TFNP and cigarette smoke extracts. **Methods:** The coculture system, utilised TR146 buccal mucosa cells (supplied by SkinEthic™) on Transwell® inserts combined with wells containing ImmuPHAGE™ (ImmuONE Ltd) macrophages. The TFNP product (obtained from the European market) was extracted using ISO-10993-12:2021 methodology, in PBS. Cigarette smoke (CS) extracts were generated via bubbling through PBS (CS-PBS) or via DMSO extraction of Cambridge filter pads (e.g. CS-Total Particulate Matter (TPM)). Using nicotine as a dosimetry marker, cigarette samples were tested at concentrations between 0.7 - 23 μ g/ml nicotine, while the TFNP extracts were assessed at 90 and 180 μ g/ml nicotine. These concentrations were chosen based on experience of the biological impact of cigarette smoke extracts on similar co-culture systems. Macrophage morphological and stress assessment were performed using high content image analysis at 48 hours post exposure to test articles. Images were acquired using an InCell Analyser 6000 system (GE Healthcare). Images were analysed and quantified for cell morphology (cell area, number of vacuoles and total area of vacuolation) and cell health (mitochondrial and caspase 3/7 activity). Cytotoxicity of was determined at the same timepoint using PrestoBlue™ measurement. **Results:** Both CS extracts elicited dose-dependent toxicological effects. CS-TPM increased caspase 3/7 activity, displayed reduced PrestoBlue metabolism and induced a foamy macrophage phenotype (enlarged cells with increased numbers of vacuoles). CS-PBS caused cell death at the highest concentration, marked by reduced cell count, low PrestoBlue metabolic activity and smaller cells without vacuoles. For the TFNP extract, the highest test concentration increased caspase 3/7 activation, however, no significant changes in macrophage morphology were observed at any concentration. The responses to TFNP extracts were observed at concentrations approximately 7 to 250-fold higher than those elicited by CS extracts. **Conclusions:** The results presented here add to the growing body of scientific evidence supporting a promising role for TFNPs in THR strategies, however, further chemical characterisation, pre-clinical, clinical, and behavioural studies are proposed for further scientific substantiation.

PS 3462 Progressive Immunologic Dysregulation with Exposure to Tobacco Smoke in a Model of Chronic Obstructive Pulmonary Disease (COPD)

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Background and Purpose: Our research objective is to examine the role of dysregulated immune responses in disease progression and changes in lung physiology in a rat model of COPD. **Methods:** Spontaneously hypertensive rats were exposed to tobacco smoke (TS) (90 mg/m³) or filtered air (FA) for up to 12 weeks. Necropsy timepoints of 4, 6, 8, 10 and 12 weeks were chosen to define the immunological and pathophysiological changes in the lung. Rats exposed to TS showed significant increases in macrophage, neutrophil and lymphocyte number in bronchoalveolar lavage compared to FA at all weeks examined. **Results:** The influx of inflammatory cells detected in the bronchoalveolar lavage was significantly elevated at all timepoints measured (4, 6, 8, 10, and 12 weeks). Increased inflammatory cells included macrophages, neutrophils, and lymphocytes. Proinflammatory cytokines IL-1 β , IL-6 and IFN- γ in whole lung homogenates were significantly increased following exposure to TS compared to FA controls. However, exposure to TS over time dampened IFN and TNF levels in whole lung homogenates. Physiological airway obstruction due to TS exposure was noted by significant elevations in central airway resistance and tissue damping. Central airway resistance was significantly increased at 4 weeks, decreased at 6 weeks, but returned to significantly elevated levels at 10 and 12 weeks following TS exposure. Lung tissue damping showed a peak at 4 weeks, then a decreasing trend, although remaining significantly elevated through 12 weeks of TS exposure. Strong positive correlations were noted between proinflammatory cytokines IL-1 β , IL-6, IFN- γ , TNF- α and the degree of lung tissue damping. These changes were associated with increases in central airway squamous epithelial metaplasia and distal airway mucous metaplasia. **Conclusions:** These findings suggest progressive airway obstruction with an abnormal inflammatory response to TS exposure.

in this rodent model of COPD. We conclude a dysregulated immunologic response, along with airway inflammation are strongly associated with the impairment of physiological lung function in TS-exposed rats.

PS 3463 Low dose, sub-chronic exposure to Dihydroxyacetone (DHA) causes increased heart strain, alters cardiac mitochondrial metabolism, and induces DNA damage

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Background and Purpose: Electronic cigarettes (e-cigarettes) have become increasingly popular in the last decade and evolved with various devices and e-liquid components. E-liquids are heated to create the aerosol the user inhales, and dihydroxyacetone (DHA) is produced by the oxidation of glycerol and propylene glycol in the e-liquids. DHA is also an active ingredient in sunless tanning products. While DHA has been considered safe for external exposures, the risks from vaping and spray tanning have raised concerns about inhalation exposures. Exposures to DHA vary between e-cigarette devices with micromolar to low millimolar doses potentially inhaled. Once inhaled and absorbed by cells, DHA is incorporated into several metabolic pathways through its conversion to dihydroxyacetone phosphate (DHAP). Given the metabolic incorporation, DHA exposure affects highly energetic tissues like the heart are needed. **Methods:** Using human cardiomyocytes (AC16) dosed with 0.2mM DHA, we analyzed DNA lesion content and DNA repair protein changes. We examined mitochondrial and metabolic changes utilizing immunoblotting, qPCR, and microscopy. We then used cardiac tissues from A/J mice sub-acutely exposed to 5 µg of DHA for 14 days. We looked at mitochondrial changes, using qPCR for mitochondrial dynamics and mitochondrial DNA copy number, TEM for structure and counts, and echocardiograms for heart function. Additionally, we looked for oxidative DNA damage, mitochondria content, structural changes, and pro inflammatory markers. **Results:** Overall, we found an increase in mitochondrial copy number after 2 weeks of exposure to the exposed cells. Additionally, we found that there is an increase in oxygen consumption, increased mitochondrial proteins and mitochondria number as well. We also see an increase in DNA oxidative and alkylating lesions. However, we see a change in the DNA lesion profile across 4 and 8 weeks with altered levels of DNA crosslink-type lesions and uracil content. We also see altered mitochondria copy number and mitochondrial protein measurement changes, where we see a decrease at 4 weeks and a return at 8 weeks compared to time-matched control cells. In the mice 2 weeks after exposure to 5 µg DHA, we saw a significant increase in mitochondria DNA copy number and mitochondria number in male and female mice. Additionally, we saw an increase in proinflammatory markers, most notably IL-6, in the female mice. We also saw an increase in collagen levels within the males only, with no change in the females. Conversely, we looked at oxidative DNA damage and found an increase in oxidative damage in female mice. Subsequently, when we performed echocardiograms, we saw an increase in ejection fraction and fractional shortening in the males, but a decrease in the females. There was also an overall increase in global stain, suggesting that the males are undergoing cardiac hypertrophy and the females are undergoing cardiotoxicity. **Conclusions:** This work is the first to examine genotoxicity of DHA in cardiomyocytes and characterize physiologically relevant exposures to understand potential long-term effects on cardiovascular health. Additionally, this is the first time sex specific responses to DHA exposure were observed.

PS 3464 Effects of long-term electronic-cigarette (E-cig) aerosol exposure on kidney health in diabetic mice

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Background and Purpose: Diabetes is one of the most important risk factors for chronic kidney disease (CKD), and end stage kidney disease. Tobacco smoking accelerates the progression of CKD of different etiologies, including diabetes. However, whether e-cig use also has a deleterious effect on CKD progression, is unknown. The aim of this study was to examine whether inhalation exposure of diabetic adult male mice to e-cig aerosols, with and without nicotine, alters gene expression and histology associated with kidney injury. **Methods:** For studying the effect of e-cig on progression of kidney disease, 7-8-wk-old BTBR obese male mice (a model of type II diabetes) with pre-existing kidney injury were exposed to either: 1) filtered air (control); 2) Propylene glycol/Vegetable glycerin (PG/VG; 1:1); or 3) PG/VG (1:1) + Nicotine (36 mg/ml) for 10-wk (3hr/d; 5d/wk). Kidneys were collected, flash frozen and subsequently stored at -80°C until needed. RNA was extracted from the kidney and after extraction, bulk RNAseq was performed, followed by RNAseq libraries preparation with TruSeq Stranded Total RNA kit (Illumina). Libraries were sequenced and aligned against the mouse genome. Differential gene expression analysis was performed with DESeq2 R/Bioconductor package. Ingenuity Pathway Analyses (IPA) was then used to comprehensively analyze the differentially expressed genes (DEGs) in order to infer the underlying causes of the observed fold-changes and predict downstream health events. One kidney from each mouse was fixed in formalin,

followed by transfer to ethanol after 24-hr and then used to assess kidney injury in PAS-, trichrome stained slides, and fibronectin (a kidney injury marker) by immunohistochemistry (IHC). **Results:** Inhalation exposure of diabetic mice to PG/VG + Nicotine resulted in gene expression changes in 724 genes, while only 3 genes were differently expressed in the PG/VG group, compared to controls. Kidney injury-related (i.e., Rorc, Col4a3, EGFR, LRRK2, VDR), cancer-related (e.g., Adamts1, Ccn1, Akr3) and immune-regulatory genes (e.g., Ace2, Cxcl6, Ccn1), were up-regulated in the PG/VG + Nicotine exposure group compared to controls. In the PG/VG only group, pro-inflammatory (Rorc) was up-regulated, while inflammation-protective Nr1d and kidney injury-associated Mt1 genes were down-regulated. IPA analyses of the DEGs from the PG/VG + Nicotine vs the Filtered Air group revealed cancer, organismal injury and tumor growth as the top three diseases/disorders. IPA further demonstrated about 50 significant canonical pathways which were affected in PG/VG + Nicotine group. These pathways included collagen biosynthesis, IL-10 signaling, mitochondrial dysfunction, Elf2 signaling, and oxidative phosphorylation. Pathways specific to the diabetic kidney injury i.e., transcriptional regulators (Hippo signaling pathway, YAP/TAZ), and transforming growth factor (TGF-β)/Smad signaling pathway, were activated. However, metabolic processes i.e., IL-10 (anti-inflammatory and renal production), synaptopodin (podocyte integrity and maintenance), STK11 (regulator energy homeostasis), SIRT6 (a sirtuin involved in mitochondrial protection and anti-fibrotic activity), MLXIP1 (a regulator of glucose and lipid metabolism) were downregulated. While IHC analyses revealed significantly higher glomerular fibronectin expression in the PG/VG-only and PG/VG + Nicotine groups, compared to air controls; the highest levels of fibronectin expression were observed in mice exposed to e-cig with nicotine. However, PAS-stained kidneys revealed mesangial expansion consistent with diabetic nephropathy, there were no significant differences between the 3 exposure groups. **Conclusions:** This study indicates that long-term, repeated exposure of adult diabetic male mice to e-cig aerosols alters gene expression and physiological markers related to kidney injury. Exposure of diabetic mice to e-cig aerosols containing nicotine produces significant changes in genes involved in CKD, diabetic nephropathy, cancer, and inflammation. Enhanced fibronectin expression in e-cig exposed mice also demonstrates the increased vulnerability to e-cig aerosols in diabetic mice. This groundbreaking study reveals the kidney as a target of e-cig use and opens the door for further studies concerning the impact of e-cig use on kidney health.

PS 3465 Multi-Omics Profiling Reveals Sex-Specific Oral Health Disruptions Associated with Vaping

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Background and Purpose: Electronic nicotine delivery systems (ENDS), or e-cigarettes, are marketed as safer alternatives to combustible tobacco, yet accumulating evidence links their use to gingivitis, periodontitis, and other oral diseases. However, sex-specific molecular and microbial responses to ENDS in the oral environment remain largely unexplored. This knowledge gap is particularly concerning given that e-cigarette aerosols may result in greater nicotine retention in the upper respiratory tract than combustible tobacco smoke, potentially amplifying localized oral effects. **Methods:** We conducted an integrated multi-omics analysis of 73 adults (47 females, 26 males; 67% vapers, 33% non-vapers) to investigate sex-specific biological responses to vaping. Subgingival plaque and saliva samples were analyzed using 16S rRNA sequencing, untargeted proteomics, and metabolomics, while exhaled emissions were characterized for volatile organic compounds (VOCs) and aldehydes. The study protocol was reviewed and approved by the Advarra Institutional Review Board (Protocol No. H22081). **Results:** Although vaping exposure was comparable between sexes, males exhibited higher systemic nicotine and cotinine levels, consistent with slower nicotine metabolism. Male vapers showed pronounced alterations in amino acid, lipid, glycan, and bile acid pathways, elevated aldehydes in exhaled breath, and upregulated aldehyde dehydrogenase 3A1 (ALDH3A1), indicating enhanced detoxification and oxidative-stress responses. Female vapers exhibited broader metabolic shifts, elevated dopamine, and upregulation of fibrinogen beta chain (FGB), reflecting elevated inflammatory and immune activation. Arginine depletion was common to both sexes, representing a shared metabolic signature of ENDS use associated with oral-health decline. Microbiome analysis identified sex-specific enrichment of pathogenic taxa, *Saccharibacteria* (TM7) and *Granulicatella* in females; *Serratia*, *Achromobacter*, and *Absoconditabacteria* (SR1) in males. These molecular and microbial perturbations correlated with higher Community Periodontal Index of Treatment Needs (CPITN) scores and elevated salivary IL-1β. **Conclusions:** ENDS use induces metabolic reprogramming that amplifies inflammatory signaling and weakens mucosal-barrier integrity, thereby increasing periodontal-disease risk. While both sexes are susceptible, males and females exhibit distinct biological trajectories, underscoring the need for sex-specific frameworks in oral-health and exposomics research.

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Background and Purpose: E-cigarettes are often marketed as safer alternatives to traditional cigarettes, yet their effects on metabolism remain unclear. Recent studies suggest vaping may disrupt lipid metabolism and contribute to fatty liver disease. This study examines how e-cigarette exposure influences hepatic metabolism and fibrosis and whether these effects occur independently of metabolic co-morbidities such as hyperlipidemia. We hypothesize that exposure to e-cigarette aerosols disrupts hepatic proteins involved in lipid metabolism and fibrosis. Contributing factors such as a high-fat diet and prolonged exposure duration may exacerbate these effects but are not required for their initiation. **Methods:** C57BL/6 mice were injected with AAV-PCSK9 and fed a high-fat diet to induce hyperlipidemia. Mice were then exposed to air, STLTH, or Vuse aerosols twice daily for 16 weeks. Livers were collected and protein was extracted for proteomic analysis. Validation studies used IHC, IF, and qPCR for major hits. Additional proteomic analysis was performed in wild-type mice exposed to STLTH or Vuse aerosols twice daily for two weeks, in the absence of AAV-PCSK9 and a high-fat diet. **Results:** Proteomics revealed distinct, device-specific hepatic alterations. Proteomic analysis revealed hepatic alterations following 16 weeks of e-cigarette exposure, characterized by changes in mitochondrial, oxidative phosphorylation, and lipid metabolism pathways, indicative of early metabolic dysfunction. Similarly, in short-term exposures, wild-type mice exhibited comparable disruptions in hepatic lipid metabolism despite the absence of diet-induced hyperlipidemia or prolonged exposure, suggesting that vaping alone is sufficient to perturb liver metabolism. **Conclusions:** E-cigarette use induces early, device-specific hepatic metabolic changes before visible fibrosis occurs. Importantly, comparable proteomic changes in wild-type mice indicate that e-cigarette exposure alone can initiate hepatic alterations, while co-morbidities such as high-fat diet and hyperlipidemia likely exacerbate, but are not required for, this phenotype. These findings underscore vaping as an independent risk factor capable of altering liver metabolism even in the absence of underlying metabolic disease.

PS 3467 Pancreatic Ductal Adenocarcinoma: Enduring Transcriptomic Changes Resulting from Smoking History

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Background and Purpose: Pancreatic Ductal Adenocarcinoma (PDAC) is a deadly disease. Like several other cancers, tobacco smoking is a notable risk factor for the disease. Among other things, smoking affects detoxification processes and the response to oxidative stress. It remains unclear the extent to which PDAC patients with a history of smoking are impacted after diagnosis. The purpose of this study is to characterize enduring changes in gene expression patterns in PDAC and their impact on processes associated with disease progression. **Methods:** We leveraged data associated with PDAC tumor samples from the Cancer Genome Atlas (TCGA-PAAD). Comparing different groups of PDAC patients on the basis of their smoking histories, (lifelong non-smokers, n=68; former smokers, n=60; current smokers, n=20), RNA-seq data were analyzed using the DESeq2 package. Differentially expressed genes (DEGs) were subjected to over-representation analyses using *enrichR* and network characteristics were further examined in Cytoscape. In parallel, Gene Set Enrichment Analysis (GSEA) was performed on ranked gene expression data to identify coordinated pathway-level changes across smoking groups. **Results:** A total of 127 DEGs were identified between non-smokers and former smokers, 28 between non-smokers and current smokers, and 18 between current and former smokers, all at an adjusted p value < 0.1. In non-smokers vs former, DEGs were enriched for detoxification and nuclear receptor signaling, and CYP2E1 and CYP2C8 appeared as hub genes with high connectivity in the STRING protein-protein interaction (PPI) network. In non-smokers vs current smokers, enrichment included inflammatory and lipid metabolic pathways, with WNT11 enriched in Reactome and a PLIN1-centered PPI cluster by MCODE and annotated by GO Biological Process (GOBP) terms related to lipid metabolism. GSEA of the non-smoker vs former-smoker comparison further revealed significant enrichment of the MUNSHI_MULTIPLE_MYELOMA_UP gene set in non-smokers (Normalized Enrichment Score = 1.70, p = 0.004), suggesting that immune-metabolic programs related to plasma-cell differentiation remain incompletely recovered after smoking cessation. **Conclusions:** Our study suggests that any smoking exposure leaves incompletely recovered transcriptomic marks in PDAC, especially in detoxification and immune-metabolic pathways. Even former smokers show persistent changes, which may influence pancreatic cancer biology after diagnosis.

PS 3468 Evaluating the direct impact of heated tobacco and cigarette smoke exposure on lung adenocarcinoma cell biology

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Background and Purpose: Smoking remains a significant public health concern due to the harmful effects of the wide range of toxic compounds generated during tobacco combustion. Many of these substances can induce DNA damage and increase the risk of carcinogenesis. The lungs are particularly vulnerable to such detrimental effects of smoking, which promote chronic inflammation and tumor development. As part of harm-reduction strategies, non-combustible tobacco products have emerged and gained popularity as alternatives to conventional cigarettes (CC), including e-cigarettes and heated tobacco products (HTPs). Notably, HTPs and CC differ in their mechanisms of aerosol generation: CC rely on combustion, whereas HTPs use controlled heating, resulting in lower levels of toxicants. Despite this, comprehensive toxicological data on these products remain limited, and their potential health risks remain unclear. In this context, the present study aimed to evaluate the effects of CC and HTP exposure on the biology of lung adenocarcinoma cells (A549), using an alternative *in vitro* model, providing insights into their potential role in cancer progression. **Methods:** The air-liquid interface (ALI) exposure system used simulates the physiological conditions of smokers by allowing direct interaction between smoke aerosols and the apical cell surface. The model was established and coupled with a puff-based delivery protocol to assess the direct effects of these exposures. Cells were exposed for 30 minutes to HTP (IQOS device), CC (Marlboro Red Selection), or airflow (control). To isolate the effect of nicotine, cells were treated with increasing concentrations of nicotine (0.2-20 μ M) for 30 minutes. Following the exposure or treatment, cells were collected and plated for functional assays, including migration assay, spheroid formation, proliferation, and colony formation. **Results:** Even relatively low nicotine concentrations significantly affected spheroid development and colony formation. Compared with the control group, treatments with 0.2 μ M and 2.0 μ M nicotine led to consistent reductions in spheroid area and diameter. Although cells exposed to 20 μ M nicotine also formed smaller spheroids than the control group, the decrease did not follow the same reduction trend observed at lower doses, suggesting a plateau effect at higher concentrations. A similar pattern was observed in the colony formation assay, indicating that increasing nicotine concentrations beyond a certain threshold does not further intensify the inhibitory response. Exposure to both tobacco products also influenced spheroid growth dynamics. CC smoke exerted a more pronounced inhibitory impact than HTP aerosol. In the spheroid formation assay, all groups initially presented comparable areas and diameters, indicating similar baseline growth. Over time, however, cells exposed to CC maintained significantly smaller spheroid dimensions than those in the air control, while HTP exposure caused a milder but noticeable suppression. Consistently, the proliferation assay showed decreased proliferation rates following both HTP and CC exposure. Notably, neither treatment induced cell death, suggesting that both tobacco products are able to modulate tumor cell behavior without causing cytotoxicity. **Conclusions:** Nicotine modulates tumor cell behavior, particularly proliferation and three-dimensional growth. The observed plateau suggests receptor saturation, indicating that maximal activity occurs at a specific nicotine concentration. These findings highlight the need to evaluate the risks associated with nicotine-containing products, regardless of their delivery method. Exposure to HTP and CC alone did not significantly promote tumor growth, suggesting that additional factors, such as the tumor microenvironment and immune modulation, are required for cancer progression. However, even short exposures altered cell behavior, reinforcing that these products are not biologically inert. Although HTPs showed milder effects than CC, both modified essential cellular features. Overall, these results reveal that non-combustible tobacco products still pose biological risks and underscore the importance of further studies to clarify their mechanisms of toxicity and aid in the formulation of effective public policies as well as therapeutic interventions regarding tobacco products.

PS 3469 An integrated Lung-on-Chip platform investigating molecular responses to whole cigarette smoke exposure

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Background and Purpose: COPD is a major global health concern and the third leading cause of death worldwide, placing a significant burden on both patients and healthcare systems. The inhalation of tobacco products not only contributes significantly to the progression of COPD but is also implicated in conditions such as E-cigarette or Vaping-product Use-Associated Lung Injury (EVALI) and lung cancer, due to the presence of highly reactive chemicals that disrupt and inflame alveoli. However, current research *in vitro* models often study only single cell types, which fail to represent the complex microenvironment of the distal lung, including its dynamic stretching. Additionally, cigarette smoke exposure in these models is commonly performed using cigarette smoke extracts in submerged cultures, resulting in experimental conditions that are far from reflective of true *in vivo* environments. **Methods:** In this study, we present a novel inhalation setup utilizing a continuous flow smoke exposure platform (CFAX12), coupled with the ^{AX}Lung-on-chip technology (AlveoliX

AG) [Stucki et al., 2018; Sengupta et al., 2023], to mimic realistic inhalation exposure to whole cigarette smoke (CS). The AX12 lung-on-chip system enables physiological exposure by integrating a co-culture of human alveolar epithelial cells (A⁴IECs) [Sengupta et al., 2022], human lung microvascular endothelial cells, and macrophages together mimicking the air-blood barrier of the distal lung. These cells were cultured within the chip under air-liquid interface (ALI) conditions with and without breathing-like dynamic stretch. This advanced co-culture system was directly exposed to CS, thereby more accurately reflecting the conditions of human inhalation. Moreover, porcine-derived native surfactant (Curosurf) was added onto the cells on-chip to study the protective effect of surfactant lining in cigarette smoke studies. **Results:** Our results demonstrated significant destruction in the air-blood barrier and cytotoxicity after cigarette smoke exposure, which was sighted in a significant decrease in Transepithelial/trans endothelial electrical resistance (~ 80% drop in TEER) and visible cell barrier disruptions. At 48 hours post-exposure, a notable elevation in LDH levels (~3 folds higher than control samples) was detected. This significant increase indicates acute cytotoxicity because of cigarette smoke exposure. Gene expression analysis additionally indicated increased inflammatory gene expression (like IL6, IL8, TNF- α). Moreover, enhanced secretion of IL8 cytokine (~3 folds higher than control samples) was measured in cigarette smoke-exposed cells on-chip. Furthermore, increased reactive oxygen species (ROS) generation (~50% higher than control samples) was measured post-smoke exposure. Interestingly, the addition of surfactant on the cells on-chip indicated strong protective effects post-smoke exposure when compared to the control samples without surfactant inclusion (Sengupta et al., 2025). **Conclusions:** This integrated CFAX12 platform significantly advances our understanding of the molecular mechanisms underlying tobacco-related diseases. It plays an essential role in toxicological testing of emerging new tobacco products, including e-cigarettes and heated tobacco devices, offering a reliable means for assessing their safety and health impacts. Ultimately, this research also supports the identification of novel biomarkers and therapeutic targets, contributing to more effective and personalized treatments for tobacco-induced lung diseases, while bridging critical gaps in preclinical testing to improve both public health and regulatory standards.

PS 3470 PDGF-BB as a Mediator of Pulmonary Microvascular Injury Following E-Cigarette Exposure

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Background and Purpose: E-cigarettes are frequently perceived as a less risky alternative to traditional cigarette smoking. However, there is a growing body of evidence that this perception is flawed, as e-cigarette aerosol (EC) exposure leads to altered immune function and injury to the cardiovascular and pulmonary systems. Currently, the cellular and molecular mechanism of EC-mediated lung vascular damage is poorly defined. Recent studies have shown that platelet-derived growth factor-BB (PDGF-BB) was elevated in the plasma of e-cigarette users compared to non-smokers. Here, we investigate the behavior of immune cells in the lung microcirculation following nicotine-free EC exposure. The goal of this study is to define the role of PDGF in recruiting neutrophils and enhancing neutrophil-platelet aggregates (NPA). Neutrophils are the most abundant type of white blood cells, and when stimulated, release neutrophil extracellular traps (NETs). These webs of extruded DNA fibers provoke an inflammatory response and vasculature damage. We hypothesize that EC exposure triggers PDGF-mediated neutrophil-platelet aggregates and NET release in the lung microcirculation, leading to lung injury. **Methods:** Male and female BALB/c mice underwent 150 puffs/day of nicotine-free EC exposure for 3, 6, or 9 days. Control mice received room air (RA). Other groups of mice received either PDGF-BB to activate PDGFR or imatinib mesylate to inhibit PDGFR. The last experimental group received both EC exposure and imatinib mesylate for 9 days. The following day, lung fluorescence intravital microscopy (IVM) was performed to assess inflammation by observing fluorescently labeled neutrophils and platelets within the pulmonary microvasculature. Collected lungs were assessed with multiplex immunofluorescence staining and protein mass spectrometry. A mouse cytokine array evaluated harvested whole blood from the different exposure groups to analyze the change in cytokine expression. **Results:** Lung IVM revealed a significant increase of neutrophils and neutrophil-platelet aggregates in the pulmonary microvasculature for both male mice (650 μm^2 at day 6) and female mice (700 μm^2 at day 9) compared to the RA groups (250 μm^2). The PDGF-BB groups produced similar inflammation as the 9-day EC groups. Imatinib mesylate treatment blocked neutrophil-platelet aggregate formation in EC exposed mice. The cytokine array showed dysregulation of 9 pro-inflammatory cytokines in the male and female EC exposed mice compared to RA. Mass spectrometry showed a host of upregulated and downregulated proteins post EC exposure, with the most interesting being a downregulation of surfactant protein A post 9 day EC exposure. Surfactant protein A is responsible for lowering surface tension, which helps to prevent alveoli from collapsing. Lung immunofluorescence staining for the presence of PDGF-BB, PDGFR, and ROS is ongoing. **Conclusions:** In conclusion, the immune response is heightened in mice treated with EC. PDGF-BB recapitulated the inflammatory responses in EC exposed mice, while imatinib mesylate prevented aggregate formation. This research explores the pathway in which PDGF contributes to lung inflammation post EC exposure to educate the public about the dangers of EC.

PS 3471 E-Cigarette Aerosols Induce Oxidative Stress and Inflammation in Corneal Epithelial Cells: Potential Risk of Vaping to Ocular Surface

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Background and Purpose: Electronic cigarette (E-cigarette; EC hereafter) is a nicotine delivery device that generates aerosols by evaporating e-liquid containing humectants, nicotine, and flavorings. EC has become a commonly used tobacco product, among adolescents, with 1.63 million middle and high school students reporting current use of EC. Compared to conventional cigarette smoke generated by combustion, EC produces aerosols by heating which results in less tar, carbon monoxide, and tobacco-specific nitrosamines (TSNAs) emission. However, the thermal degradation of e-liquid emits potentially harmful compounds, such as volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons (PAHs), and metals. Recently, studies found that EC users reported moderate-to-severe eye dryness and tear film instability after vaping. Shorter tear breakup time, altered tear production, and loss of meibomian glands were observed in vapers, suggesting a possible correlation between EC exposure and dry eye symptoms. Our goal is to investigate the adverse effects of e-cigarette aerosols on eyes at the molecular levels, focusing on biomarkers related to eye dryness. **Methods:** Human corneal epithelial cells (HCE-2) were exposed to aerosols generated by electronic cigarettes with different device wattages (W) and e-liquid that contains varying nicotine levels and flavorings. Exposure program was controlled by Arduino-uno microcontroller board with servo motor to simulate human vaping behavior. Following exposure, cellular responses were evaluated using biochemical and molecular assays. Cytotoxicity was assessed by MTT cell viability assay. Oxidative stress was determined by measuring intracellular reactive oxygen species (ROS). Potential inflammatory response was examined by pro-inflammatory cytokine expression level using both quantitative real-time PCR (qPCR) and enzyme-linked immunosorbent assay (ELISA). **Results:** Cell viability was reduced by 10% at 10 W, 28% at 25 W, and 50% at 35 W after cells were exposed to 60 puffs of aerosols generated by e-liquid containing 12 mg of nicotine. ROS level exhibited a wattage-dependent increase of 1.5- to 2-folds at 4- and 6-hours post-exposure, indicating elevated intracellular oxidative stress. Further, we detected surged cytokine expression, including IL6, IL1 α , IL1 β at the RNA level, with IL1 β also upregulated at the protein level, suggesting that aerosol exposure contributed to activation of pro-inflammatory response in HCE-2 cells. Together, these results indicate that e-cigarette aerosol exposure induces cytotoxicity, oxidative stress, and inflammatory activation in HCE-2 cells in a wattage- and time-dependent manner. **Conclusions:** Oxidative stress can damage cellular components such as DNA, lipid, and protein, leading to cellular dysfunction and even cell death. The damage contributes to numerous eye diseases, such as cataracts, glaucoma, dry eye, and age-related macular degeneration. Moreover, inflammation triggered by oxidative stress can worsen existing conditions and promote disease progression. Our study shows that e-cigarette aerosols lead to elevated oxidative stress and pro-inflammatory response in human corneal epithelial cells. Together, these findings demonstrate that e-cigarette aerosols pose a potential hazard to eye surface health and highlight the need for further investigation into vaping-associated ocular damage.

PS 3472 Polycyclic aromatic hydrocarbons measured following electronic dabbing of Cannabis and Cannabis-derived products

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Background and Purpose: Electronic dabbing is the consumption of concentrated Cannabis and Cannabis-derived products vaporized at temperatures as high as 1000° F. Terpenes found naturally in Cannabis and added in excess are responsible for the flavor and aroma profiles characteristic of dabbing. At high temperatures, terpenes such as α -cedrene and β -caryophyllene are known to undergo pyrolysis, forming polycyclic aromatic hydrocarbons (PAHs), but the production of PAHs from dabbing Cannabis is largely unknown. PAHs pose a risk to human health; 16 are U.S.E.P.A priority pollutants and many are known or suspected carcinogens. Our hypothesis is that electronic dabbing of Cannabis products, especially those with high terpene concentrations, will lead to production of PAHs and subsequent toxic exposure. **Methods:** We developed a method for analyzing PAHs produced during electronic dabbing by collecting vapor produced at 1000° F from an electronic dabbing rig. Vapor was captured using C18 SPE cartridges, followed by measurement of the 16 EPA priority PAHs using gas chromatography/mass spectrometry (GC/MS). Control testing with multiple terpene mixes was performed at CU Anschutz, and various Cannabis concentrates with or without added terpenes were dabbed and prepared at Kaycha Labs in Denver, Colorado, for analysis at CU Anschutz. The Cannabis products, control oils, and terpene mixes that we tested are common in the Colorado market. Our protocol was validated by measuring samples spiked with PAHs before and after dabbing. **Results:** Our findings suggest the presence of multiple priority PAHs, particularly those of lower molecular weight, such as naphthalene and its methylated derivatives, in vapors produced from the dabbing of both control and Cannabis-derived samples, and PAH concentrations increase upon addition of terpenes. However, some PAHs were present at low concentrations in undabbed samples. Notably, the addition of terpenes to multiple Cannabis products resulted in significantly increased concentrations of four PAHs, including anthra-

cene. **Conclusions:** These results support the successful development of a method for the collection and analysis of PAHs from dab vapors. Additionally, our findings suggest that the addition of terpenes to electronic dabbing results in significantly increased production of PAHs. Further testing is underway to compare the effects of various added terpene mixes on PAH production, and current results will be analyzed for unknown metabolites using the NIST17 GC/MS database. Because current U.S. *Cannabis* testing facilities do not test for PAHs, our overall goal is to understand potential user exposures to prevent and protect the public health of Coloradans by providing evidence to improve regulation of device temperatures and product formulations. This research was supported by the Institute for *Cannabis* Research.

PS 3473 Characterization of Flavored E-cigarettes via Untargeted Mass Spectrometry to Identify Flavor Specific E-cigarette Markers of Exposure

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Background and Purpose: The use of flavored e-cigarettes is a public health concern due to previous exposure studies (both *in-vitro* and *in-vivo*) that have uncovered e-cigarette flavor-specific pathologies including gut inflammation, and immune dysfunction. Prior research studies on flavored e-cigarettes identified over 200 constituents, but the complete exposome has yet to be characterized due to the chemical diversity of e-liquids between flavor ingredients and brands. Determining associations between human health outcomes and flavored e-cigarette exposure has been hindered by the lack of flavor-specific biomarkers and comprehensive molecular characterizations. To overcome these challenges, we characterized the constituents of flavored e-cigarette street products within the New York City area. **Methods:** Street-circulating e-cigarettes were donated in the Fall of 2024 by the "We Are Atlas" organization, which collects used e-cigarettes in the Brooklyn area for recycling. Brand and flavor content of the donated used e-cigarettes (n=341) were recorded. The e-liquid was harvested from the sponge of each e-cigarette, then diluted with methanol to a final concentration of 80% methanol and centrifuged. The supernatant was analyzed via Reverse-Phase Untargeted Liquid Chromatography-High-Resolution Tandem Mass Spectrometry (LC-MS/MS) analysis. Molecular constituents were analyzed with standard in-house informatics methods, and two complementary approaches were applied and merged with novel scripts (Python, R) to annotate and cluster features by MS2 library matching and empirical feature annotation. Sample features were compiled into a large-scale feature library for consistent annotation across all samples. **Results:** The donated used e-cigarettes included 25 brands and 130 uniquely named e-cigarette flavors, with "clear" (n=65, 19.5%) and watermelon ice (n=36, 10.8%) being the most abundant flavors. The 130 uniquely named e-cigarette flavors were organized into 34 unique "flavor notes" so that nominal flavors could be associated with specific molecular constituents. For example, the flavor name "strawberry kiwi" would be annotated with two flavor notes; strawberry and kiwi. The most abundant flavor notes included; menthol/mint (n=206, 38.7%), berry (n=92, 16.9%), and melon (n=69, 12.7%). Twenty-six e-cigarettes from various brands that spanned the 34 flavor notes were selected for LC-MS/MS analysis. In total 19,480 putatively named and unnamed molecular features were detected. A subset (n=6,746) of high abundance features (>100X SNR) was used in a Principal Component Analysis Biplot Graph to identify shared molecular features within e-liquids associated with the same flavor note. We putatively identified such features that were unique to a flavor note, including but not limited to; berry (n=276), menthol/mint (n=189), watermelon (n=116), kiwi (n=16), and pineapple (n=13). Identification of flavor features present in multiple samples from assorted brands indicates that while flavor brand variation may exist, there is significant overlap in the constituents used to create a specific flavor. **Conclusions:** The donated e-cigarettes gave insight on e-cigarette flavor and brand usage within the Brooklyn area. From this analysis, we putatively characterized the chemical flavor landscape of over 26 flavored e-cigarettes highlighting the molecular composition of human exposures to e-liquids. The e-liquid flavor-specific features will be used in a novel prediction algorithm to blindly assign e-cigarette flavor exposure within an independent Human Vape Cohort (urine) with known flavor usage to test the hypothesis that flavor exposure can be determined by the urine exposome profile. Metadata from the positive control cohort, self-reported e-cigarette consumption with flavor preference, will be used to validate our assignment methodology. Our continuing work will build a molecular flavor library which can be used to putatively determine e-cigarette flavor exposure within human cohorts.

PS 3474 Disposable E-Cigarettes, Hidden Toxic Metals: Implications for Human Exposure

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Background and Purpose: Disposable pod e-cigarettes (dPODs) are currently the most popular type of e-cigarette on the market, particularly among adolescents and young adults. The majority of these products are not authorized for sale in the U.S. and come in a wide variety of designs and flavors, enhancing their appeal to youth. Despite their immense popularity, scientific research has only recently begun to

investigate the chemical compositions of dPOD aerosols and e-liquids, as well as the potential health risks associated with their use. A recent study by our group published in *ACS Central Science* (2025) (DOI: 10.1021/acscentsci.5c00641) identified nickel (Ni), antimony (Sb), and lead (Pb) in aerosols from three popular dPOD brands at concentrations exceeding established cancer and non-cancer risk thresholds, raising health concerns. This study revealed two major concerns with dPODs. First, the use of lead-based alloys for metallic internal components was identified in one brand, resulting in exceptionally high Pb concentrations in its aerosols, exceeding those found in a single pack of cigarette smoke by as much as 19-fold based on one day's worth of puffs (100 puffs). Second, the accumulation of toxic elements such as Pb, Ni, and Sb was observed with increased device use, possibly due to the closed system design of dPODs. While these findings are specific to the brands tested, they underscore a broader concern regarding the unknown toxicological profiles of the many dPOD brands on the black market. To provide an initial assessment of the human health implications of dPOD use, we conducted a controlled whole-body exposure study using C57BL/6 mice. Mice were exposed to an aerosol derived from a commercial dPOD e-liquid containing high concentrations of Pb, Ni, Sb, copper (Cu), and zinc (Zn). The objective was to determine whether toxic metals and metalloids from dPOD aerosols can enter systemic circulation, accumulate in vital organs, and induce immune responses. **Methods:** C57BL/6 mice (n = 12 per group; 6 males and 6 females, 12 weeks old) were exposed to aerosols generated from a commercial dPOD e-liquid previously found to contain high concentrations of Pb, Ni, Sb, Cu, and Zn. Exposures were conducted for 3 hours per day over either short-term (3 days) or prolonged exposure (4 weeks, 5 days per week). Control mice were exposed to HEPA-filtered laboratory air. Exposure chamber aerosols were collected and analyzed for gravimetric aerosol mass (mg/m³), total elements, and carbonyls. Following exposure, the lungs, olfactory bulbs, brain, liver, kidneys, blood, and femur were collected from all animals. Tissues were digested using microwave-assisted acid digestion, and elemental concentrations of Pb, Ni, Sb, Cu, and Zn were quantified by inductively coupled plasma mass spectrometry (ICP-MS). Immunohistochemistry (IHC) was conducted on lung tissues to identify changes in Club Cell Secretory Protein (CCSP) immunomodulatory function. **Results:** No significant differences in element concentrations were observed in mice following 3-day dPOD aerosol exposures for any element relative to controls. Conversely, significant increases in Pb concentrations were quantified in the bones and kidneys of mice exposed for 4 weeks (p = 0.03 to 1.3 x 10⁻⁶), in both males and females, compared to control mice. No significant increases in Pb or any other measured elements (Ni, Sb, Cu, Zn) were observed in the remaining tissues (p>0.05). Qualitative tissue staining identified subtle changes in inflammation, but with marked changes in airway integrity. **Conclusions:** Our *in vivo* exposure study demonstrated that sub-chronic (4-week) exposure to a commercial dPOD aerosol led to significant Pb accumulation in the bones and kidneys of exposed mice and alterations in airway epithelium. These findings indicate that e-cigarettes containing elevated Pb levels can promote systemic metal deposition and possibly disrupt airway immunomodulatory function, posing potential long-term health risks. Given the lack of toxicological characterization for most dPOD brands, consumers may be at risk of exposure to toxic metals from products that have not undergone proper safety evaluations.

PS 3475 Contribution of Metals, Carbonyls and TSNA's to the Excess Lifetime Cancer Risk of Illicit Flavored Disposable E-cigarette Products Available on the US Market

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Background and Purpose: To date, the U.S. Food and Drug Administration (FDA) has authorized only tobacco and menthol flavored electronic nicotine delivery systems (ENDS). Despite this, illicit flavored disposable ENDS have become widely accessible in the United States (US). Recent data show that illicit flavored disposables represent approximately 80% of the US ENDS market. While some studies have analyzed flavored disposable ENDS, to date, no comprehensive studies have examined the Excess Lifetime Cancer Risk (ELCR) of the e-liquids in these illicit products. The FDA's Center for Tobacco Products (FDA CTP) released two memoranda dated June 3, 2024, outlining recommendations for identifying genotoxicity hazards and classifying ENDS constituents according to carcinogenicity tiers and detailing methodologies for calculating ELCR in ENDS products to support Premarket Tobacco Product Applications (PMTA). Our objective is to apply the approach outlined in the FDA memos to calculate the contribution of the metals, carbonyls and tobacco specific nitrosamines (TSNAs) to the ELCR values of the selected 73 illicit ENDS products available in the U.S. market. **Methods:** Following the FDA CTP approach and incorporating inhalation unit risk (IUR) values from supplementary sources, we calculated ELCR attributable to metals, carbonyls and TSNAs measured in the e-liquids of the 73 illicit flavored disposable ENDS, assuming 100% transfer to the aerosol. We also included an IUR for antimony (Sb III) proposed by the California Office of Environmental Health Hazard Assessment. ELCR values were first calculated per gram of an e-liquid, then normalized per milligram (mg) of nicotine (for nicotine-containing e-liquids only) and subsequently adjusted by 40 mg daily nicotine consumption rate, cited by FDA CTP. For non-nicotine containing ENDS, the ELCRs were compared after normalization per weight of e-liquid. **Results:** High concentrations of chromium, nickel, antimony, and lead were observed in some illicit e-liquids, significantly contributing to their ELCR values. The Cr(VI) Inhalation Unit Risk (IUR) was applied to the total measured chromium concentration in ELCR calculations. Neither

cadmium nor arsenic were detected above the limits of quantification. The highest metal concentrations, and thus the highest ELCR values, were observed in non-nicotine e-liquid normalized per e-liquid weight. Some e-liquids contained TSNAs, which also contributed to the ELCR values. Finally, a moderate negative correlation was observed between concentrations of metals and e-liquid pH. **Conclusions:** Our work demonstrates that metals, carbonyls and TSNAs detected in the e-liquids of the illicit ENDS available on the US market significantly contribute (with maximum over 700) to the e-liquid ELCR values and some are higher than the median ELCR value of 31 calculated from measured Tier 1-3 HPHCs levels of authorized ENDS. However, as noted in the U.S. CTP memos, other factors, including e-liquid ingredients, and potential leachable compounds from ENDS aerosol components, may also contribute to the overall ELCR. Thus, the calculated ELCR values for the illicit ENDS are likely underestimated because these comprehensive data are largely unavailable for the illicit products.

PS 3476 Exposure assessment of toxicants in slim cigarette consumers

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Background and Purpose: Slim cigarettes often being chosen by consumers due to a perceived reduction in health risks. However, whether the consumption of these products genuinely lowers systemic toxicant exposure remains a critical unanswered question. To address this, a large-scale population-based pilot study was conducted across several cities in China. This research utilized urinary biomarker analysis to accurately assess and compare the exposure levels of various harmful constituents among slim cigarette smokers, regular cigarette smokers, and non-smokers, thereby providing essential real-world data for risk assessment. **Methods:** A robust cohort study was executed across six major Chinese cities, enrolling 1,141 volunteers (regular smokers, slim smokers, and non-smokers). Following a five-day smoking phase using designated products, 24-hour urine samples were collected. Utilizing a biomarker-based exposure assessment methodology, the study compared exposure levels to various toxicants, including nicotine, tobacco-specific nitrosamines (TSNAs, NNK), polycyclic aromatic hydrocarbons (PAHs, pyrene and naphthalene), volatile carbonyl compounds (acrolein and crotonaldehyde), and volatile organic compounds (VOCs, 1,3-butadiene, acrylonitrile, benzene, ethylene oxide) among slim cigarette, regular cigarette smokers and nonsmokers. **Results:** Compared to non-smokers, the consumption of slim cigarettes was found to significantly elevate exposure levels for nearly all measured toxicant biomarkers ($p < 0.05$). No statistical difference was observed in the average number of cigarettes consumed per day between the slim cigarette group (16.7 sticks/day) and the regular cigarette group (17.0 sticks/day, $p = 0.35$). Crucially, consumption of slim cigarettes was associated with significantly lower exposure to nicotine ($p = 0.001$), total NNK ($p = 0.02$) and naphthalene ($p = 0.02$) compared to regular cigarettes. Conversely, no statistically significant differences were detected for the biomarkers of VOCs. A strong positive correlation ($R^2 > 0.9$) was established between the number of slim cigarettes smoked and the measured toxicant exposure levels, confirming that daily cigarette consumption is a primary determinant of toxicant exposure. **Conclusions:** 1) Compared to non-smokers, slim cigarette smoking significantly increased exposure to all measured toxicants; 2) Exposure levels were positively correlated with the number of cigarettes smoked; 3) Compared with regular cigarettes, smoking slim cigarettes can significantly reduce user exposure to nicotine, TSNAs and naphthalene.

PS 3477 Accounting for Population Variability in Simulation Modeling of Tobacco Product Constituents

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Background and Purpose: Electronic nicotine delivery systems (ENDS) and combusted cigarettes contain chemical constituents that may be hazardous to human health when inhaled. Human health risk assessment of individual exposures provides critical information associated with the use of different inhaled tobacco products; but does not account for variability in the population or provide insights regarding risk to specific demographics. Capturing this variability illustrates how risks may differ across demographics, use patterns, and product types, offering valuable information for regulatory decision making. **Methods:** We simulated synthetic populations through statistical sampling from representative demographic, physiological, and behavioral distributions to assess tobacco-related health risks. U.S. Census data, the National Health and Nutrition Examination Survey (NHANES), and regression equations were used to generate demographic and physiological characteristics, while the Population Assessment of Tobacco and Health (PATH) and studies in the literature were used to represent behavioral patterns in tobacco product use. As a case study, we generated 1,000 individuals for a baseline population (Population A) using an 18 mg/mL nicotine fixed power ENDS and 1,000 individuals for a population using reduced-nicotine ENDS with compensatory puffing (6 mg/mL nicotine fixed power ENDS) (Population B). These synthetic individuals were then used as inputs to previously developed respiratory dosimetry and nicotine physiologically-based pharmacokinetic (PBPK) models to assess the effects of compensatory behavior for reduced nicotine devices on daily dose of nicotine and its metabolite cotinine, and the potential byproducts acetaldehyde, acrolein, and formaldehyde.

Results: Uptake and deposition of tobacco product constituents depends on properties such as flow rate, physico-chemical properties, clearance rates, and deposition mechanisms. Because these underlying parameters are not substantially altered between the two populations, the similarity in uptake/deposition fractions between the two populations can be expected. Compensatory puffing with the lower nicotine concentration ENDS led to 2-4 times higher median daily doses of formaldehyde, acetaldehyde, and acrolein across all regions of the respiratory tract. In addition, the standard deviation of aldehyde exposure was also higher in Population B, specifically in the mouth/throat. For example, the standard deviation for acrolein exposure is over 4 times higher in Population B ($0.036 \mu\text{g}/\text{cm}^2/\text{day}$) than in Population A ($0.0081 \mu\text{g}/\text{cm}^2/\text{day}$). In contrast, daily maximum nicotine and cotinine concentrations in venous blood and brain tissues were approximately twice as large in Population A due to the higher nicotine concentration in the e-liquid, despite reduced puffing intensity. For example, median concentrations in the venous blood were $2.69 \mu\text{g}/\text{L}$ for Population A compared with $1.17 \mu\text{g}/\text{L}$ for Population B. **Conclusions:** The modeling framework incorporates behavioral and physiological variability and allows for the comparison of risks between two populations that may have different tobacco use behaviors and device formulations. The approach enhances the ability to characterize variability in both dose and risk estimates by producing full distributions to estimate the full range of exposures. For example, such a framework provides estimates of the extremes within the population, which may be particularly important in identifying sensitive individuals and estimating the likelihood of exceeding the relevant health thresholds. Our findings from this case study suggest that compensatory puffing in response to reduced nicotine formulations can offset intended harm reduction by increasing exposures to byproducts.

PS 3478 Cancer Reference Estimates for Non-Mutagenic Flavoring Substances of Potential Concern

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Background and Purpose: Flavoring substances are used in many consumer products including e-liquids for electronic nicotine delivery systems (ENDS). Health hazard characterization, including the potential for cancer effects, is critical in the risk assessment of any ingredient. The cancer risk assessment can follow a nonlinear dose response model based on a threshold, below which there is no effect, or a no-threshold linear dose response model. The nonlinear model is applicable for non-carcinogenic effects; however, it is often used for cancer effects attributed to non-mutagenic mode of action (MoA), resulting in the estimate of the reference dose (RfD). The no-threshold linear model has been adopted as the default approach because it is considered health-protective. The linearity of the model allows the calculation of the cancer slope factor (CSF). CSFs are estimated using the benchmark dose level (BMDL) associated with a 10% response (BMDL₁₀) (i.e., 10% extra risk of carcinogenic effect in exposed test animals) to serve as the point-of-departure (PoD). The excess lifetime cancer risk (ELCR) characterization is used by the U.S. Food and Drug Administration's Center for Tobacco Products (USFDA CTP) to support Premarket Tobacco Product Applications (PMTA) for ENDS. ELCR characterization relies on cancer potency factors (CPF) for each ingredient which are inhalation unit risk (IUR) values, adjusted to daily exposure levels corresponding with a cancer risk of 1 in 100,000, over 52-year exposures. The CPF is similar to the non-significant-risk-level (NSRL), established by California's Office of Environmental Health Hazard Assessment (OEHHA), for cancer-causing chemicals listed under Proposition 65. The NSRL is a threshold of daily intake that is considered to pose no more than a 1 in 100,000 lifetime cancer risk. The USFDA CTP Memorandum states that additional information for specific constituents should be considered to increase the accuracy of the CPF, and therefore the ELCR analysis. This procedure was used to determine the potential contribution, if any, of five flavoring substances to the overall cancer risk assessment. The CPF estimates were compared to NSRL estimates to demonstrate validity in this approach. Additionally, for non-mutagenic substances the CPF was compared to the RfD to determine the presence of a buffer for potential non-mutagenic health hazards. **Methods:** The methodology described in the 2024 USFDA CTP Memorandum for calculating ELCR in ENDS products was used to estimate CPFs for the synthetic flavoring substances Benzophenone, Furfural, Methyl Eugenol, β -Myrcene, Pulegone, and Pyridine. The NSRL was estimated according to CalEPA OEHHA guidelines. Data used for the analysis primarily included the 2018 USFDA review that concluded these flavoring substances induce tumors by a non-genotoxic mechanism of action. First, if not available, oral CSFs were estimated using the BMDL₁₀ adjusted to a human equivalent dose (BMDL_{10(HED)}). Then, the IUR was calculated according to US EPA methodology and finally adjusted to CPF according to the USFDA CTP Memorandum. For comparison purposes, the RfD was also estimated following USEPA guidelines. **Results:** The adjusted IUR (CPF) estimates corresponding with a cancer risk of 1 in 100,000, for all flavoring substances using the linear model from a health-protective approach, were approximately 6-100x lower than the adopted default value of $1.5 \mu\text{g}/\text{day}$ in the USFDA CTP Memorandum. CPFs were found similar to NSRL estimates. In general, CPFs were approximately 250X lower (more conservative) than RfDs (considered protective for non-mutagenic cancer effects), when estimated using the nonlinear model appropriate for non-mutagenic substances. **Conclusions:** A method was employed to calculate IURs for flavoring substances in ENDS potentially carcinogenic via non-genotoxic MoA. The CPF estimate is aligned with NSRL indicating validity in the procedure. CPF estimates were higher than the proposed default CPF of $1.5 \mu\text{g}/\text{day}$ for single ingredients suggesting

a potential to overestimate the cumulative ELCR. The overestimate is even larger for those substances with a non-mutagenic MoA. The RfD estimate suggest a buffer may exist above the CPF for non-mutagenic carcinogens.

PS 3479 Tobacco and Nicotine Product Assessments: Quantitative Derivation of Excess Lifetime Cancer Risk for Oral Route of Exposure

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Background and Purpose: In 2024 the United States Food and Drug Administration (FDA) Center for Tobacco Products (CTP) published two toxicology memoranda focused on the hazard identification and carcinogenicity tiering of analytical constituents in Electronic Nicotine Delivery System (ENDS) products and the calculation of Excess Lifetime Cancer Risk (ELCR) as part of Premarket Tobacco Product Application (PMTA) evaluations. ELCR estimates the likelihood of cancer development over a lifetime of exposure to one or more substances. Globally there are over 300 million consumers of smokeless tobacco products such as snus, moist snuff, and nicotine pouches. Unlike combustible and ENDS products, smokeless tobacco products are generally placed in the mouth in direct and prolonged contact with the oral mucosa, resulting in a different absorption route for nicotine and Harmful and Potentially Harmful Constituents (HPHCs) - oral versus inhalation. This quantitative risk assessment approach centers on calculating the cumulative ELCR for smokeless tobacco products via the oral route of exposure, based on FDA CTP's toxicology memoranda for ENDS products published in 2024. **Methods:** To apply FDA's approach to calculating ELCR for smokeless tobacco products, oral cancer slope factors and average human body weight were utilized in lieu of inhalation unit risk factors and inhalation rate. HPHC data from CORESTA Smokeless Tobacco Reference Products, along with product usage assumptions under "average" and "heavy" consumption scenarios were applied to the model to estimate ELCR and demonstrate the feasibility of this approach which could be applied to smokeless tobacco product PMTA applications and evaluations. Additionally, non-cancer hazard quotients and indices were calculated based on EPA guidance. **Results:** Cumulative ELCR analysis of smokeless tobacco consumption under both the "average" and "heavy" use scenarios resulted in overall lower cancer risk profiles compared to that of the combustible reference cigarette 1R6F, which FDA CTP estimates to be 10,000 excess incidences of cancer in a population of 100,000 consumers. Non-cancer hazard indices were also below the regulatory level of unity (1) indicating that exposure to the constituents evaluated over the period of a lifetime are unlikely to result in the occurrence of deleterious non-cancer effects. **Conclusions:** This method demonstrates that FDA CTP's approach to calculating cumulative ELCR estimates for ENDS products can be modified for use via the oral route of exposure and applied to evaluate smokeless tobacco products.

PS 3480 G Protein Coupled Estrogen Receptor Modulation of Pulmonary and Neuroinflammatory Responses to Acute Inhaled Wood Smoke

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Background and Purpose: Wood smoke (WS) exposure is an emerging environmental health concern linked to systemic and neurological inflammation. Estrogen signaling, particularly through the G protein-coupled estrogen receptor (GPER), has been implicated in modulating immune responses, yet its role in pulmonary and neuroinflammation following WS exposure remains unclear. To address this, we investigated how loss of GPER signaling influences inflammatory outcomes following acute WS exposure. **Methods:** 10-12wk old GPER knockout (KO) and wild-type (WT) mice were exposed to either filtered air (FA) or WS (4h/d for 2d, ~0.575mg/m³) (n=8 per group/exposure). Brain tissue and bronchoalveolar lavage fluid (BALF) were collected to evaluate immune responses. In a separate cohort (n=5 per group/exposure), cerebral blood flow dynamics were assessed within 24 h of the last exposure using laser speckle imaging. **Results:** Laser Speckle Imaging revealed that WS significantly increased cerebral blood flow in WT mice but not in GPER KO mice (20.99 ± 9.553 SEM, $p=0.0335$, Welch's t test). Flow cytometry analysis of BALF revealed decreased resident alveolar macrophages in WT mice after WS exposure ($p=0.0309$, Nonparametric T test), accompanied by increased monocyte-derived macrophages in GPER KO/WS versus WT/FA (mean diff. = -0.388 ± 0.137 , $p=0.0243$, two-way ANOVA). We also observed elevated monocyte-derived macrophages in GPER KO/WS versus WT/FA ($p=0.0128$, Nonparametric T test). Additionally, T cell abundance was reduced in GPER KO mice compared to WT FA controls ($p=0.0070$, Nonparametric T test). **Conclusions:** Together, preliminary results demonstrate that GPER signaling influences immune responses and cerebrovascular adaptation following acute WS exposure. Future work will include evaluating mRNA cytokine gene expression in brain regions such as the hippocampus, cortex, and cerebellum, as well as to assess glial activation through IBA-1 and GFAP staining in the hippocampus and cortex.

PS 3481 HIF-1 α Regulates Acrolein-Induced Oxidative Stress and Antioxidant Signaling in Differentiated Human Bronchial Epithelial Cells under Hypoxic Conditions

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Background and Purpose: Exposure to acrolein, a hazardous environmental pollutant commonly found in industrial emissions and e-cigarette vapor, induces significant oxidative stress and cellular damage, particularly in hypoxic conditions. This oxidative stress and cellular toxicity is especially concerning for respiratory health. Hypoxia-inducible factor 1 alpha (HIF-1 α) is a critical regulator of cellular responses to low oxygen levels and plays a protective role by activating pathways involved in cell survival, antioxidant defenses, and stress response. However, the exact mechanisms by which HIF-1 α mitigates the toxic effects of acrolein on respiratory cells remain unclear, especially when comparing normoxic and hypoxic conditions. To address this, we differentiated primary normal human bronchial epithelial (NHBE) cells into pseudostratified epithelium at an air-liquid interface. After differentiation, we silenced the HIF-1 α gene in NHBE cells using RNA interference and exposed the cells to acrolein under both normoxic and hypoxic conditions. This study aimed to elucidate how HIF-1 α modulates oxidative stress and apoptosis through regulation of MAPK, PI3K/Akt, and Nrf2 signaling pathways and their downstream antioxidant and survival gene networks under normoxic and hypoxic conditions. This work will deepen our understanding of cellular resilience to environmental toxins and contribute insights toward developing public health strategies for mitigating pollutant-related respiratory damage. **Methods:** Differentiated normal human bronchial epithelial (NHBE) cells were cultured under air-liquid interface conditions and exposed to 40 or 80 μ M acrolein under normoxia (21% O₂) or hypoxia (1% O₂). HIF-1 α expression was silenced using siRNA transfection prior to exposure. Total RNA and protein were isolated to evaluate the expression of oxidative stress-related and apoptotic markers. Quantitative RT-PCR was performed to assess transcriptional regulation of MAPK, PI3K/Akt, and Nrf2 pathway genes. Western blot analysis was conducted to evaluate protein activation, including phosphorylated and total forms of JNK, p38, PI3K, and Caspase-3, along with Nrf2 protein expression and band intensities were quantified using densitometric analysis. Data were obtained from three independent experiments and expressed as mean $\pm$ SEM. **Results:** Western blot analysis revealed that acrolein exposure increased phosphorylation of JNK and p38 in a dose-dependent manner under hypoxic conditions, indicating activation of the MAPK stress pathway. However, HIF-1 α silencing altered this pattern: while phosphorylation of p38 increased at 40 μ M acrolein, both p-JNK and p-p38 levels were markedly reduced at 80 μ M, suggesting dysregulated stress-kinase signaling. Phosphorylated PI3K was also diminished in HIF-1 α -deficient cells, consistent with impaired pro-survival signaling. In parallel, Nrf2 protein expression was reduced following HIF-1 α knock-down, indicating attenuation of antioxidant pathway activation. RT-qPCR results also supported the protein findings. HIF-1 α -silenced cells exhibited lower mRNA expression of Nrf2-regulated antioxidant genes (NQO1, HMOX1, GCLM) and the survival-related gene AKT1 compared with control cells. The combined data demonstrate that HIF-1 α is essential for maintaining MAPK, PI3K/Akt, and Nrf2 pathway integrity under acrolein challenge, and its loss compromises both antioxidant and survival responses, particularly under hypoxia. **Conclusions:** This study highlights the critical protective role of HIF-1 α in mitigating acrolein-induced oxidative stress and apoptosis, particularly under hypoxic conditions and at lower acrolein doses. Silencing HIF-1 α exacerbated oxidative damage, disrupted antioxidant signaling, and heightened cellular susceptibility to acrolein toxicity. These findings underscore the importance of HIF-1 α in maintaining redox homeostasis and cellular resilience to environmental pollutants. In the broader context of toxicology, this work advances understanding of how airway epithelial cells adapt to oxidative stress and supports future strategies aimed at strengthening antioxidant defenses to reduce respiratory injury in vulnerable populations.

PS 3482 Vinyl chloride potentiates liver injury caused by chronic alcohol consumption in mice

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Background and Purpose: Vinyl chloride (VC), an industrial chemical, causes liver injury at high concentrations, but low-level exposure (<1 ppm) is currently considered safe. The consequences of sub-OSHA exposure on human health are not well understood, especially in the context of VC as a risk-modifying agent. Previous work from this group confirms the role of VC in exacerbating experimental dysfunction-associated steatotic liver disease (MASLD) caused by Western diet. Both MASLD and alcohol-associated liver disease (ALD) have similar liver pathologies, and the impact of VC exposure in ALD has not been investigated. Moreover, the prevalence of ALD worldwide raises concerns about how VC exposure can enhance disease progression to more severe stages of injury. The purpose of this study was to determine if VC exposure worsens liver damage caused by chronic alcohol (EtOH) consumption. **Methods:** C57Bl/6J mice, pair-fed or fed a Lieber-DeCarli EtOH diet, were exposed to VC (<1 ppm), or room air for 6 hrs/d, 5 d/wk for 6 weeks. Plasma and liver samples were collected for determination of injury. **Results:** VC exposure exacerbated EtOH-induced liver damage and oxidative stress, and moderately increased inflammation. Although the increase in lipids caused by ethanol was not impacted by VC exposure, the pattern of steatosis shifted to more micro-vesicular fat, which is often indicative

of mitochondrial dysfunction. In line with that VC also changed mRNA expression of genes regulating metabolism. **Conclusions:** Various environmental toxicants are known to modify or enhance liver injury in MASLD models. Findings from the present study suggest that VC interacts also with other lifestyle factors such as alcohol consumption resulting in exacerbated alcohol-associated liver damage. This emphasizes that environmental chemical exposure, such as to VC, potentially drives inter-individual risk for developing or enhancing disease.

PS 3483 Aryl hydrocarbon receptor signaling in club cells promotes Th17-driven lung injury following inhalation exposure to environmentally persistent free radicals

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Background and Purpose: Environmentally persistent free radicals (EPFRs) are redox-active combustion by-products generated during thermal combustion processes, including hazardous wastes remediation, and have been implicated in respiratory disease. We previously demonstrated that EPFR inhalation induces neutrophilic asthma associated with marked lung injury. This phenotype is driven by T helper 17 (Th17) cells, which secrete IL-17. We further identified the aryl hydrocarbon receptor (AHR) as a central regulator of EPFR-induced pulmonary Th17 responses and found that airway club cells are a primary site of AHR activation following EPFR exposure. The present study aimed to investigate the role of club cell-AHR signaling in mediating EPFR-induced pulmonary responses. **Methods:** Conditional knockout mice lacking AHR in club cells (*Ahr* Δ CC) were generated by crossing *Ahr*^{flx} mice [*Ahr*^{tm3.1Bra/J} strain] with *Scgb1a1*-Cre^{ERTM} mice [B6N.129S6(Cg)-*Scgb1a1*^{1tm1cre/ERT1Blh/J} strain]. Littermate (LM) control and *Ahr* Δ CC mice were exposed to air or EPFRs (30 min/day for seven consecutive days; average concentration = 200 μ g/m³) via inhalation. Pulmonary immunopathophysiological responses were assessed following the final day of exposure. **Results:** Targeted deletion of AHR in club cells was confirmed by flow cytometry and immunofluorescence. Following EPFR inhalation, LM controls exhibited a robust Th17-biased pulmonary immune response, increased neutrophil recruitment in bronchoalveolar lavage fluid (BALF), and elevated cytokines associated with IL-17 signaling (KC/GRO, IL-6, IL-1 β) compared to air-exposed controls. In contrast, *Ahr* Δ CC mice exposed to EPFRs failed to induce these responses. Histopathological analysis revealed that EPFR exposure induced airspace enlargement, peribronchiolar fibrosis, mucus hyperproduction, and decreased lung function in LM mice, but these pathophysiological changes were absent in *Ahr* Δ CC mice. **Conclusions:** These findings identify club cell-specific AHR signaling as a critical determinant of EPFR-induced epithelial-immune crosstalk, driving neutrophilic inflammation, remodeling, and functional decline in the lung. This work provides mechanistic insight into the pulmonary toxicity of redox-active pollutants and highlights epithelial AHR as a potential therapeutic target in pollutant-induced lung disease.

PS 3484 Exposure to Hazardous Waste Emissions Alters Human Physiology: Findings from a Community-Based Pilot Study

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Background and Purpose: Open burn/open detonation (OB/OD) is used for the disposal of explosive and hazardous wastes, releasing toxic gases and particulate matter into the environment. Colfax, Louisiana, hosts the only commercially operated OB/OD thermal treatment (TT) facility in the United States, raising concerns about its potential impact on surrounding communities. This pilot study aimed to investigate metabolic changes associated with residential proximity to the facility using untargeted urine metabolomics. **Methods:** Urine samples were collected from 51 residents living within a 30-km radius of the facility, with proximity, sex, and race considered as key variables. Samples were analyzed via ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry. Metabolic alterations and potential biomarkers of exposure were identified, followed by pathway enrichment analysis. **Results:** A total of 217 metabolites were identified. Individuals residing closer to the facility exhibited significant metabolic shifts, particularly in pathways linked to energy metabolism, amino acid metabolism, and oxidative stress. Elevated glutathione sulfonamide (GSA) levels and altered glutathione/glutathione disulfide (GSH/GSSG) ratios indicated increased oxidative stress and redox imbalance. Pathway enrichment highlighted disruptions in glycolysis, the citric acid cycle, sulfur metabolism, and nucleotide metabolism, which are essential for cellular energy and DNA repair. Differences in metabolite profiles were also observed across sex and racial groups, emphasizing the combined influence of biological and environmental factors. **Conclusions:** Proximity to the TT facility was associated with measurable metabolic alterations and increased oxidative stress in residents, suggesting potential health risks from chronic exposure. Further research is crucial

to understand the long-term health implications of these metabolic alterations and to develop strategies to mitigate the environmental and health risks associated with the TR of hazardous wastes.

PS 3485 Characterization of Murine Microglial Interactions Induced by Chronic Exposure to Diesel Exhaust

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Background and Purpose: Air pollution is one of the leading causes of mortality, contributing to about 6.7 million premature deaths annually. Diesel exhaust (DE) is a significant contributor to particulate matter (PM) in urban traffic-related air pollution, resulting in adverse respiratory, cardiovascular, and reproductive health effects. Research has suggested that PM can also affect the brain and central nervous system, triggering neurodegeneration. Microglia, the brain's resident immune cells, play a crucial role in regulating PM-induced toxicity. To protect the brain from damage, resting microglia are activated to eliminate foreign substances and dead cells, while initiating an inflammatory response. Though initially beneficial to maintain homeostasis and repair damage, prolonged exposure to PM can cause microglia to promote a pro-inflammatory environment due to oxidative stress and cell death, leading to disease escalation. While there is increasing research to uncover how PM impacts neurodegeneration, the specific molecular mechanisms are not well understood. The purpose of this project was to conduct a morphological assessment of the hippocampal region of the brain to distinguish possible microglial change using immunohistochemistry (IHC) and to use proteomics to characterize any possible changes in underlying mechanisms due to exposure in the hippocampus and olfactory bulb. **Methods:** This study examined mechanisms of PM-induced neuroinflammation and cognitive dysfunction, focusing on proteomics and microglia. Six-week-old low-density lipoprotein receptor knockout (Ldlr KO) mice were exposed to freshly-generated DE (~250 μ g/m³) or filtered air (FA) over 18 weeks. Mice (n=12 per sex and exposure) were fed a high-fat (HFD) or chow diet and exposed for 6 h/day, 5 days/week. At the end of 18 weeks, mice were sacrificed and perfused with PBS. The left brain hemispheres were fixed in paraformaldehyde, embedded in optimal cutting temperature compound, cryosectioned at 40 μ m, and used in immunohistochemistry (IHC). Hippocampus and olfactory bulb were isolated from right brain hemispheres, flash frozen in liquid nitrogen, and then processed for proteomics and analyzed via data-independent acquisition liquid chromatography with tandem mass spectrometry (DIA LC-MS/MS). **Results:** Using IHC, microglia, cells expressing TMEM119 and IBA1, and infiltrating macrophages, cells with IBA1 expression without colocalization of TMEM119, were distinguished. Differences in the soma of microglia were identified between the male FA and male DE HFD groups, suggesting possible microgliosis due to DE exposure. Comparing the male chow FA and DE groups showed the least differences in microglial size. Protein analysis revealed significant changes (FDR<0.05) in protein abundance based on exposure (FA or DE), sex, and/or diet (HFD or chow). An initial pathway analysis of the hippocampus identified a dysregulated actin cytoskeleton and Fc gamma R-mediated phagocytosis in HFD DE males, both associated with cognitive decline. In the olfactory bulb, the pathway analysis also revealed impacts on the actin cytoskeleton and calcium signaling in HFD DE females, which are associated with neurodegeneration. **Conclusions:** Overall, this project demonstrates that diet and DE exposure affect microglia and related proteins, exacerbating neuroinflammation that may lead to neurodegeneration. Additional research is underway to understand microglial states and related sub-populations to clarify their role in neurodegenerative diseases.

PS 3486 Co-Exposure of 4th Generation E-Cigarette and Environmentally Persistent Free Radicals Impairs Lung Responses in Male Mice

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Background and Purpose: Fourth-generation electronic cigarette (EC) devices that use the novel formulation of nicotine, i.e., synthetic nicotine salt, may present distinct respiratory hazards due to its formulation. These novel e-liquid formulations raise concerns, particularly given the increasing popularity of such devices among young adults. While EC represents potential risks for pulmonary diseases, environmental sources of airborne toxicants such as particulate matter also contribute to inhalation exposures. Of interest is particulate matter containing environmentally persistent free radicals (EPFR(PM)) formed during the thermal remediation of hazardous waste at Superfund sites. EPFR(PM) has been shown to generate reactive oxygen species (ROS) that drive oxidative stress, which promote pulmonary dysfunction. Given the rising prevalence of EC and the omnipresence of air pollution, including PM, this study investigates the pulmonary consequences of co-exposure to EC aerosol and EPFR(PM). We hypothesized that co-exposure to EC aerosol and EPFR(PM) will synergistically exacerbate lung inflammation and oxidative stress. **Methods:** Male C57BL/6J mice (N=8 per group) were exposed to one of the following conditions: air; blue razz ice-flavored EC aerosol for 1.5 hours/day, 7 days/week for 7 weeks, at

a concentration of 0.32 mg/puff $\pm$ 0.1; EPFR(PM) aerosol, 4 hours/day for 5 days at a concentration of 0.34 mg/m³ $\pm$ 0.1; or a combination of EC aerosol (for 7 weeks) plus EPFR(PM) (during the last 5 days of the 7-week exposure). Exposure concentrations were determined gravimetrically, and real-time exposure concentrations were monitored using a DustTrak II. EC aerosol particle size distribution was measured using a scanning mobility particle sizer (SMPS). At the end of the study, lung function was assessed using whole-body plethysmography and the flexiVent system. Bronchoalveolar lavage fluid (BALF) was collected for cytology analysis. Lung tissue samples were collected for histopathological assessment and for gene expression analysis via PCR, for genes associated with biotransformation, inflammation, oxidative stress, airway remodeling, barrier integrity, and lipid metabolism. **Results:** SMPS data revealed that blue razz ice-flavored EC aerosol had a geometric mean of 143 nm for its particle size distribution. Exposure to this EC aerosol temporarily altered lung function at two weeks, with increased minute volume compared to the air group (83.1 $\pm$ 12.8 vs 66.8 $\pm$ 11.1 mL/min, respectively) followed by recovery, and at 7 weeks, an upward shift in the pressure-volume loop (p <0.05 vs. air). These changes suggest an early transient effect on minute volume and an obstructive pulmonary disease phenotype at 7 weeks. While no BALF inflammation was noted, transcriptomic analysis revealed upregulation of 11 genes following EC exposure: *Mmp9* (4.8-fold), *Mmp12* (2.1-fold), *Col4a1* (2.0-fold), *Ahr* (1.6-fold), *Cyp1a1* (2.9-fold), *Cyp26a1* (1.8-fold), *Hmox1* (1.5-fold), *Il-1 β* (4.3-fold), *Ifn- γ* (1.5-fold), *Tnf* (1.6-fold), and *Cxcl5* (11.9-fold). These dysregulated genes are supportive of the EC aerosol exposure impairing signaling pathways associated with airway remodeling, biotransformation, oxidative stress, and inflammatory responses. In contrast, at the molecular level, EPFR(PM) up-regulated 7 genes: *Mmp9* (6.2-fold), *Cyp1a1* (23-fold), *Cyp1b1* (10-fold), *Nqo1* (2.49-fold), *Il-1 β* (2.7-fold), *Occludin* (1.5-fold), *Sp-d* (1.6-fold). These results are consistent with the known pro-oxidant properties of EPFRs. Regarding the co-exposure group, EC+EPFR(PM) upregulated 5 genes: *Cy1a1* (58.9-fold), *Cyp1b1* (8.6-fold), *Aldh3a1* (1.7-fold), *Nqo1* (1.9-fold), and *Cxcl5* (2.2-fold). Notably, *Cy1a1* showed a synergistic induction, suggesting enhanced activity of lung detoxification pathways following concomitant exposures to both inhaled toxicants. **Conclusions:** These findings reveal distinct lung molecular profiles and underscore the importance of assessing co-exposures in inhalation toxicology. Also, our study highlights the need for studies that account for further investigation into the long-term health effects of emerging nicotine formulations.

PS 3487 Neurotoxic consequences of chronic exposure to concentrated ambient fine and ultrafine matter in a novel mouse model of late-onset Alzheimer's disease

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Background and Purpose: Chronic exposure to ambient particulate matter (PM) is increasingly recognized as a modifiable environmental risk factor for Alzheimer's disease (AD). Epidemiological studies link long-term exposure to fine and ultrafine PM (PM_{2.5} and UFP) with cognitive decline and amyloid pathology, but mechanistic evidence remains limited by the use of overexpression transgenic models that do not recapitulate late-onset Alzheimer's Disease (LOAD). This study addresses that gap by evaluating the neurotoxic consequences of prolonged exposure to environmentally relevant levels of concentrated ambient PM in the humanized amyloid- β knock-in (hA β -KI) mouse, a model that exhibits gradual accumulation of soluble A β , synaptic dysfunction, and age-dependent cognitive impairment. We hypothesize that chronic PM exposure accelerates early synaptic and glial alterations that precede overt plaque deposition in LOAD. **Methods:** Male and female hA β -KI and wild-type littermates were exposed to filtered air or concentrated ambient fine and ultrafine PM for 7 or 13 months using the Versatile Aerosol Concentration Enrichment System (VACES) to simulate long-term exposure. Following exposure, cognition was assessed via object recognition and location memory tasks. Hippocampal long-term potentiation (LTP) was evaluated electrophysiologically to assess synaptic plasticity. Post-mortem analyses included immunohistochemistry such as homer-1 and bassoon staining to quantify post- and presynaptic integrity. Glial activation was characterized using Iba1, GFAP, Clec7a, and C3 immunolabeling. High-resolution confocal microscopy and hyperspectral imaging (CytoViva Imaging Core) were used to assess refractive particle deposition in multiple brain regions. Data were analyzed using two-way ANOVA to evaluate genotype-by-exposure interactions. **Results:** Chronic PM exposure produced age- and genotype-dependent synaptic impairment in hA β -KI mice. After 7 months, LTP amplitude was modestly reduced in hA β -KI mice compared with air-exposed controls, while wild-type animals remained unaffected. After 13 months, PM-exposed hA β -KI mice exhibited a significant 25-30% reduction in LTP magnitude relative to air controls (p < 0.001), indicating cumulative synaptic dysfunction. Behavioral testing revealed corresponding trends toward impaired recognition memory. Immunohistochemical analyses showed increased Iba1-positive microglial density and morphological activation in the hippocampal CA1 and dentate gyrus of PM-exposed hA β -KI mice, along with elevated Clec7a and C3 expression, consistent with a shift toward a neurotoxic glial phenotype. Preliminary Homer-1 and Bassoon staining demonstrated reduced synaptic puncta density, while hyperspectral imaging revealed elevated particle deposition in PM-exposed olfactory bulbs of the hA β -KI mouse cohort. **Conclusions:** Chronic exposure to fine and ultrafine particulate matter accelerates synaptic dysfunction and microglial activation in a physiologically relevant late-onset AD model. These findings support the hypothesis

that air pollution promotes early neurodegenerative processes through maladaptive glial responses and oxidative stress preceding plaque pathology. The integration of synaptic marker analysis and hyperspectral imaging provides mechanistic insight into PM-induced neurotoxicity and positions the hA β -KI mouse as a translational platform for assessing environmental contributions to Alzheimer's disease.

PS 3488 Vascular endothelial dysfunction induced after exposure to environmentally persistent free radicals is mediated by the release of miRNA from the air-blood interface

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Background and Purpose: Particulate matter containing environmentally persistent free radicals (EPFRs) is produced when Superfund wastes are thermally remediated. EPFRs form when organic pollutants are incompletely combusted and adsorb onto the surface of particles containing redox-active metals. Previous studies in mice showed that EPFRs reduce vascular function downstream of the lung. However, this effect was abolished in mice lacking the aryl hydrocarbon receptor (AhR) in alveolar type II (AT-II) cells, which form the air-blood interface in the lung and promote the systemic release of miRNAs. We hypothesize that at the air-blood interface, EPFRs alter the miRNA profile released into the plasma, which, in turn, alters the expression of genes essential for controlling function in peripheral lung vessels. **Methods:** To test our hypothesis, we isolated and cultured mouse AT-II cells at the air-liquid interface (ALI) and exposed them to aerosolized EPFRs (~30 μ g/cm²) using a Vitrocell exposure system. At 24 h after ALI exposure, the basal medium was collected, filtered, and then exposed to commercially sourced murine lung microvascular endothelial cells (mLMVEC). The expression and activation of vascular eNOS, as well as NO production, were then measured. **Results:** Compared to vehicle, activation of the AhR was observed in AT-II cells exposed to EPFRs, evidenced by increased *Ahr*, *Cyp1a1*, and *CYP1b1* mRNA levels. Activation of AT-II-AhR resulted in a decreased release of miR-1906 and miR-542-3p into the basal medium. In mLMVEC exposed to conditioned medium, we observed reduced nitric oxide levels, decreased *Nos3* and *Pik3r2* mRNA expression, and increased *Edn1*. **Conclusions:** Our findings suggest that miRNAs released into basal medium after EPFRs exposure of AT-II cells grown at the ALI regulate endothelial cell gene expression required for maintaining vascular homeostasis, including genes that regulate eNOS expression/function. These findings aid in our evaluation of human health risks of EPFR exposure and could offer an intervention to help communities address the health impacts of EPFRs.

PS 3489 Sex differences in the innate immune response to woodsmoke protect the lung function of female mice

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Background and Purpose: With the increased prevalence of wildfires, understanding long-term health effects of exposure to the smoke is of obvious importance. In both a community exposure study and murine model, delayed decreases in lung function following woodsmoke (WS) exposure were observed, with a greater lung function decline observed in males. Sex differences were likewise observed in alveolar macrophage (AM) functions and phenotype that suggest a tissue remodeling phenotype in males. **Methods:** Mice or murine cells were exposed to WS in our facility. Additionally, female mice were treated with fulvestrant, an estrogen receptor antagonist, to elucidate the observed sex bias. Lung function was measured and *ex vivo* lung tissues samples were assessed for oxidative stress, inflammatory response, and markers of epithelial mesenchymal transition (EMT). **Results:** While fulvestrant treatment did not result in WS-induced changes to lung function, *ex vivo* AM studies showed increased TNF- α levels for male and fulvestrant-treated female mice in response to WS. qPCR analysis of AMs revealed a higher basal NRF2 activation level in females and early markers of EMT in the lung tissue of males. *In vitro* studies showed sex-specific effects on macrophage inflammatory responses including key cytokines IL-33, IL-6, TNF- α and IL-1 β as well as heightened NRF2 activation in response to WS. **Conclusions:** These studies contribute to defining the mechanism of sex as a biological variable on WS-induced adverse health effects. The results suggest that a higher basal level of redox scavengers in the female lung have a protective effect and EMT is attributable to the decrease in lung function observed in males. While fulvestrant treatment did not impact lung function, it did modulate the inflammatory response to WS. This suggests a more temporal or developmental effect of estrogen in the response to WS between AM and parenchyma.

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Background and Purpose: Indoor air quality is increasingly recognized as a key determinant of respiratory health. Among indoor pollutants, volatile organic compounds (VOCs) are of particular concern because they can induce oxidative stress, modulate immune responses, and disrupt the integrity of the airway epithelial barrier. Our previous studies demonstrated that even 0.1 ppm ozone—commonly regarded as a “safe” indoor level—can exacerbate lung injury and allergic inflammation in mice. VOCs are ubiquitous in indoor environments, yet their effects on vulnerable airways at “permissible” concentrations remain unclear. In this study, we investigated whether VOC removal could mitigate pulmonary disease in murine models of chronic allergic asthma and acute lung injury (ALI). **Methods:** Mice were housed under either normal indoor air or air filtered through activated carbon/HEPA chemical filters that reduced VOCs to near the detection limit. In the ALI model, mice received an intranasal instillation of 25 µg lipopolysaccharide (LPS) dissolved in 25 µL phosphate-buffered saline (PBS) 48 h before sacrifice. The allergic asthma model was sensitized intranasally with 25 µg of *Dermatophagoides farinae* (Der f) extract on days 1, 8, and 15, followed by intranasal challenges of 5 µg/day on days 21–23. Mice were euthanized 24 h after the final Der f administration. At the end of the experiment, the lung-draining lymph nodes, bronchoalveolar lavage fluid (BALF), and skin tissues were collected for immunological and histological analyses. **Results:** In both models, exposure to chemically filtered (CF) group significantly improved arterial oxygen saturation and alleviated histopathological lung injury. No significant immunological effects were observed in the ALI model mice. In the asthma model, the number of eosinophils in BALF was significantly lower in the CF group than in controls, indicating attenuated Th2-type airway inflammation. Both CD4⁺ T cells and IgE⁺ B cells in the pulmonary (hilar) lymph nodes were significantly decreased in CF group, accompanied by reduced levels of Th2-related cytokines and type 2 innate lymphoid cells (ILC2s). Notably, these beneficial effects were evident even when VOC concentrations in the control air were below current guideline limits. In the asthma model, combined exposure to VOC-reduced air and treatment with the histamine H1 receptor antagonist fexofenadine further suppressed inflammatory cell infiltration and histamine-induced neurohypersensitivity, demonstrating a synergistic effect between environmental and pharmacological interventions. **Conclusions:** To assess clinical relevance, total VOC concentrations were measured in five animal hospitals differing in building structures and ventilation conditions. Considerable variation was observed, ranging from below 50 µg/m³ in well-ventilated facilities to over 600 µg/m³ in small clinics. Solvent, detergent, and building material-derived chemicals were identified as the major VOC sources in high-concentration facilities. These levels were comparable to or exceeded those shown to aggravate lung pathology in mice. Collectively, our findings indicate that even “low-level” VOC exposure can amplify airway inflammation and impair therapeutic responsiveness. VOC removal not only attenuates disease severity but also enhances drug efficacy. These results highlight indoor air quality as a modifiable determinant of respiratory health and underscore the importance of active VOC management in indoor environments, including medical facilities.

3491 Characterization of combustion emissions from fossil-based vs bio-based plastics

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Background and Purpose: Waste disposal through open-air combustion (OAC) at military burn pit and under-resourced healthcare systems emit complex aerosols. Inhalation exposure to particles and chemicals in these complex aerosols is linked with acute and chronic health effects. Annually, over 1 trillion single-use medical devices made of fossil-based plastics - polypropylene (PP) and polyvinyl chloride (PVC) are disposed in OAC. Bio-based alternatives like polylactic acid (PLA) have been reported to be safer but a lack of comprehensive studies comparing their combustion emissions has been a critical knowledge gap. To address this gap, this study focused on identifying toxicants and their respective amounts. OAC is a dynamic process that loops between material heating, smoldering, and flaming to emit diverse particles and chemicals. Our objectives were to: 1) evaluate chemicals when heating PVC and PLA at different temperatures, 2) combust PVC and PLA and characterize the emitted particles and chemicals in real-time and off-line, and 3) compare respective emissions. **Methods:** The WVU iTOX has established a novel surrogate combustion generator that produces emissions and mimics real-world inhalation exposures with commercial wood-pellets (CWPs). CWPs were fed at 6 gms/min (range: 1–100) to begin and sustain combustion. Three boluses of 10 gms PVC or PLA were added at 10 mins intervals during three trials. Emissions were sampled from the stove flue placed in line between the generator and the exhaust. Combustion emissions were characterized in real-time and off-line for particles (size distribution, and concentrations) and gases (total and speciation) including head-

space analysis with gas chromatography mass spectrometry (GCMS: µg/m³). The number of chemicals was evaluated qualitatively by heating 1 gm of material at 80 °C and 120 °C in GCMS and compared using the NIST library. Primary combustion gases [carbon mono- (CO) and dioxide (CO₂)], and hydrogen cyanide (HCN) in parts per millions (PPM) were analyzed using Fourier transform infrared spectroscopy (FTIR). Total volatiles (PPM) were measured using a photoionization detector. Total particle mass (mg/m³) and number (#/cc) were determined by gravimetric measurements with 37 mm laminated polytetrafluoroethylene filters with a 0.45 µm pore size and a condensation particle counter (CPC), respectively. Particle size distribution (PSD) was reported using an electric low-pressure impactor by count median diameter (CMD: nm) ± geometric standard deviation (GSD). **Results:** Heating at 80 °C and 120 °C released a greater number of chemicals from PVC (38 chemicals) than PLA (1 chemical: Dioxane). Combustion temperature (°C) for PLA (309.1 ± 16.7) and PVC (307.9 ± 26.8) were similar. PLA emitted ~ 8-fold less total aerosol mass (mg/m³: 11.9 ± 0.9 vs 94.1 ± 0.8) and ~ 2-fold less volatiles (PPM: 5.9 ± 0.5 vs 10.5 ± 1.0) than PVC. However, CPC measurements revealed similar total particles from combustion emissions with PLA vs PVC (#/cc: 1.4E7 ± 1.7E5 vs 1.4E7 ± 4.8E5); ~ 3-fold more deviations with PVC. The emissions from both PVC or PLA with the CWPs resulted in respirable particles (<10 µm) with polydisperse PSD (GSD>1.5). Directly after the bolus of material was fed for combustion (0–2 mins), the PSD emitted from the CWPs + PVC was larger (CMD: 129 nm ± 1.98) than the CWPs + PLA (CMD: 48.5 nm ± 1.80). FTIR (PPM) measured 12,034 ± 5000 CO₂, 292 ± 238 CO, and 3.5 ± 1.5 HCN in PVC compared to PLA: 11,435 ± 4828 CO₂, 186 ± 169 CO, and 0.7 ± 0.6 HCN. GCMS analyzed (µg/m³) higher vinyl chloride (1492 ± 57.2), formaldehyde (219 ± 3.4), acenaphthylene (1501 ± 37.1), benzene (558 ± 29.6), furan (2205 ± 56.9), and phenol (1312 ± 20.6) than in PLA. However, emissions from PLA resulted in higher ethane (1407 ± 71.5), and decane (1456 ± 256.7) than in PVC. Chemicals/derivatives identified by GCMS are human carcinogens/possibly carcinogenic as per the IARC. **Conclusions:** Whether heating or combusting, bio-based PLA emits fewer toxicants (qualitatively + quantitatively) than fossil-based plastic PVC. Future studies will include PLA from different sources and other commonly used plastics PP. These assessments will allow categorization by rank order of the materials based on their toxic potentials and to develop mixtures that scale least-to-worst emissions from OAC. **Funding:** NIH P20-GM103434, U54-GM104942, R21-ES034942 (TRN), T32 ES032920 (TRN, VTN)

3492 Open-air combustion emissions simulation and characterization of various wood species and synthetic materials

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Background and Purpose: Open-air combustion (OAC) is prevalent in many parts of the world that lack adequate waste management infrastructure, and during one-off events, such as wildfires. Unlike modern incineration, OAC typically occurs at lower temperatures where smoldering can occur. The resulting incomplete combustion emissions significantly add to air pollution and promote the formation of toxic chemicals which pose significant risks to human health and the environment. To better understand these emissions and to study their health effects, it is important to recreate the OAC process in a laboratory setting. Because of the dynamic process of combustion and the myriad of items burned in OACs, this can be challenging to adequately replicate and analyze. Extensive chemical and aerosol measurements of the emissions are needed to comprehend the dangers present from the various constituents of an OAC. The objective of this study was to utilize our custom combustion emissions generator to simulate and characterize OACs that occur in various settings such as military burn pits, wildfires, wildland-urban interface fires or during waste disposal of plastic materials, such as those used to fabricate medical supplies. **Methods:** The WVU iTOX surrogate combustion generator was developed to burn custom pellets made from wood sawdust + synthetic materials of interest. Wood types used in the pellets included: 1) red oak, 2) loblolly pine, 3) Douglas fir, and 4) ponderosa pine. Synthetic materials included 1) rubber, 2) acrylic, 3) oriented strand board (OSB), 4) foam board insulation (FB), 5) vinyl flooring (VF), 6) polyvinyl chloride (PVC), and 7) polylactic acid (PLA). Pellets were fed into the combustion generator at 6 g/min and combusted with an airflow rate of 12 LPM. Sampling was conducted from the exhaust flue of the generator and measurements included: 1) total aerosol mass concentration (TAMC), 2) particle size distribution (PSD) reported in terms of count median diameter (CMD), and 3) gas chromatography-mass spectrometry (GC-MS) for speciation and concentration levels of specific chemicals. **Results:** All wood types and synthetics were successfully combusted, and their emissions were analyzed. Wood-species-only pellets produced TAMCs in the range of (17–49 mg/m³) with lower CMDs (37–54 nm). Chemicals released included formaldehyde (318–1229 µg/m³) and acetaldehyde (102–373 µg/m³). Some synthetics produced additional larger particles which increased the CMDs of the PDS and TAMC, respectively. Some notable examples include PVC (129 nm, 94 mg/m³) and VF (69 nm, 119 mg/m³). Additionally, the synthetics added high levels of toxic chemicals to the emissions (presented as: chemical, highest concentration in µg/m³). These included OSB (acrolein, 923), FB (styrene, 241), VF (bromochloro-

methane, 315), rubber+acrylic (methyl-methacrylate, 56), PVC (vinyl chloride, 1492). **Conclusions:** The combustion of wood pellets resulted in fairly similar emissions across the wood species. This included aerosols with smaller (nano-size) PSDs with relatively lower TAMCs. Chemicals released in the emissions included formaldehyde and acetaldehyde. As synthetics were added, larger particles increased the CMDs of the PSDs and, in some cases, increased the TAMC significantly. Additional chemicals and much higher concentrations of potentially harmful toxicants were released in the synthetic emissions. This included chemicals such as styrene, methyl-methacrylate, vinyl chloride, and others which have been classified as human carcinogens/possibly carcinogenic by the International Agency for Research on Cancer. **Support:** NIH P20-GM103434, U54-GM104942, R21-ES034942 (TRN), T32 ES032920 (TRN, VTN), UL Research Institutes

PS 3493 High-throughput toxicity assessment of ambient particulate matter enabled by a microfluidic cellular exposure platform

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Background and Purpose: Particulate matter (PM) exposure is a top environmental risk factor affecting public health and is associated with both acute respiratory and chronic lung disease. PM exposure is linked to oxidative stress, inducing downstream inflammatory response and cell damage. Ambient PM is chemically complex and physically diverse, and the mechanisms of aerosol damage depend on the chemical composition. Thus, there is a need to screen a wide range of aerosol compounds for their toxicity. However, traditional 96-well plate-based assays are low-throughput and hard to implement in large-scale screens due to their time and resource intensiveness. In this study, we utilize microfluidics to develop a high-throughput platform with a 10-fold reduction in sample and reagent requirements. We use this platform to assess the impact of ambient PM samples on murine alveolar macrophage cells, an *in vitro* model of immune response in the lungs upon PM exposure. **Methods:** We designed a microfluidic platform with sixteen individual microfluidic devices for unique dose response experiments. All devices have continuous perfusion, driven simply by gravity, enabling equipment-free cell loading and PM delivery. Each device has an upstream mixing module that allows for the automatic dilution of the PM solution into eight different doses. Downstream of the mixing modules, hundreds of cells are compartmentalized for accurate detection and temporal monitoring. As the precise dilution is device specific, each device is calibrated with a fluorescent dye to estimate the delivered dose. We recorded cellular activity in all devices through automated timelapse imaging on an imaging plate reader. We simultaneously measured three cellular responses, including intracellular reactive oxygen species (ROS), mitochondrial membrane potential (MMP), and cell death. For ROS and MMP, we used fluorescent probes H2-DCFDA and TMRE, respectively. For cell death, we trained a neural network [Shappell bioRxiv 2025] for identifying single cells and classifying them as live or dead in a label-free manner from brightfield images. Ambient filter samples were collected during 2017-2018 at the Jefferson Street site in Atlanta. This sampling site has been extensively used in characterizing urban Atlanta air quality [Xu PNAS 2014]. The water-soluble parts of the PM samples were extracted into cell media for exposure to cells in the microfluidic devices [Gao ACP 2020]. **Results:** Caspase 3/7 apoptosis reporter was used as the ground truth fluorescent label to help train the neural network. The classification model was trained on hundreds of annotations with nearly equal amounts of live and dead (killed by H2O2) cells making up the training set. The model was also tested on hundreds of annotations of similar quality but different images from those used for training. The resulting model performance approached 90% precision and 80% recall. We validated the performance of the model with a control experiment with 200 μ M H2O2 exposure while tracking Caspase response and dead cell count over time. We found that the increase in dead cell count aligned to the increase in dead cells until the entire population of cells was dead. Finally, we demonstrated the operation of the devices with exposure to ambient PM samples. ROS response and cell viability were dose-dependent, following sigmoidal trends. Intracellular ROS production activated 1-2 hours before the onset of cell death. Key metrics reported include area under the dose-response curve and activation threshold for ROS and TMRE as well as LC50 for cell death. **Conclusions:** This microfluidic platform is a rapid and cost-effective alternative to traditional well plate assays. Small assay volumes reduce the use of expensive probes and extensively characterized aerosol samples. Single cells monitoring of hundreds of cells exposed to the same experimental condition provides statistical power in detecting differences in cellular response and forgoes the need for large bulk cell culture. This new technology serves to increase the feasibility of cellular toxicity screening for predicting adverse health effects from PM exposure. Future work includes running a large-scale screen of a comprehensive set of ambient PM samples to study the impact chemical composition on cellular response and associated toxicity.

PS 3494 HEPA Filtration Modifies Pulmonary Responses to Episodic Eucalyptus Smoke Exposure in Male Long-Evans Rats

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Background and Purpose: As wildland fires increase in frequency and severity, their threat to both human and ecosystem health grows. Epidemiological studies have shown associations between wildland fire smoke inhalation and increased hospitalizations for cardiorespiratory-related health effects. Smoke from wildland fires is a complex mixture made up of two distinct chemical phases, gas and particulate. The gas phase is primarily comprised of carbon monoxide, carbon dioxide, nitrogen oxides, and a variety of other volatile organic compounds. The particle phase is made up of suspended carbonaceous particulates, with the primary concern for health effects being fine particulate matter (PM_{2.5}). Understanding the contribution of each phase to pulmonary toxicity provides critical information that can be used to inform effective solutions to better protect human health from wildland fire smoke exposure. As such, the purpose of the current study was to characterize the toxicity of HEPA filtered smoke, in comparison to whole smoke in a rodent model. **Methods:** 12-week-old male Long-Evans rats were exposed to filtered air, whole eucalyptus smoke (3.52 mg/m³ PM), or HEPA filtered smoke (0.2 mg/m³ PM) for 1 hour per day for 4 consecutive days, followed by a 3-day break, then 3 more consecutive days (7 days total across a ~2-week period). Ventilatory function was assessed using whole body plethysmography (WBP) on exposure days 1, 4, 8, and 10, both 0- and 2-hours post-exposure. Bronchoalveolar lavage fluid (BALF) was collected for analysis of lung injury, immune cell differentials, and cytokines. RNA from lung tissue was sequenced to evaluate changes in response to smoke exposure. **Results:** Immediately following exposure, WBP revealed an overall decrease in breathing frequency for the whole smoke group ($p < 0.05$). Exposure to filtered smoke had the opposite effect, increasing breathing frequency ($p < 0.05$). Expiratory time was increased overall due to whole smoke exposure ($p < 0.05$), while the filtered group experienced an overall decrease ($p < 0.05$). 2-hours post-exposure, tidal volume was increased due to whole smoke exposure ($p < 0.05$), while exposure to filtered smoke resulted in an overall decrease in tidal volume ($p < 0.05$). A significant reduction in BALF n-acetyl-glycosaminidase, a marker for macrophage activation, was detected following exposure to both whole and filtered smoke ($p < 0.01$). Furthermore, exposure to filtered smoke reduced neutrophils in the BALF ($p < 0.05$) for the filtered group only. This was consistent with reductions in some of the inflammatory cytokines measured, namely IL13 ($p < 0.01$) and KC/GRO ($p = 0.05$). Such effects were not present in BALF from rats exposed to whole smoke. A limited number of differentially expressed genes were detected between the whole smoke- and air-exposed lung tissue ($n = 2$). However, 69 differentially expressed genes were detected in the lungs of rats exposed to filtered smoke, compared to air-exposed controls. These included fatty acid binding protein 1 (FABP1), FXRD domain-containing ion transport regulator 5 (FXRD5), as well as high mobility group box 2 (HMGB2), all of which have been associated with lung disease or injury. **Conclusions:** This data demonstrates exposure to filtered eucalyptus smoke results in worsened pulmonary outcomes when compared to whole smoke from both a molecular and physiological standpoint. However, further study is needed to understand the dynamic relationship between the gaseous and particle phases that drive adverse health effects. (This abstract does not necessarily reflect US EPA policy.)

PS 3495 Predicting Mixture Toxicity of Volatile Organic Compounds in Air-Liquid Interface Cultures Using a Component Based Approach

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Background and Purpose: Volatile organic compounds (VOCs) are a persistent air pollutants in both indoor and outdoor environments. There are limited studies on the respiratory effects of most VOCs, and even less knowledge on how VOC mixtures affect the lung epithelium. **Methods:** Human bronchial epithelial (16HBE) cells cultured at the air liquid interface were exposed to four gases commonly detected following environmental disasters: acrolein, acetone, acetaldehyde, and formic acid. After an acute 2-hour exposure to occupationally-relevant doses of an individual VOC or our designed VOC mixture, each with matched, filtered air control, cells were allowed to recover for 24 hours before being harvested for further analysis. Media was collected for a cytotoxicity assay. Cell lysate was collected to evaluate gene expression using Taqman qPCR, and live cells were used to conduct barrier permeability assays. Additionally, benchmark concentration (BMC) modeling was conducted for all endpoints where a significant effect was observed to compare derived points of departure (PODs) to predicted PODs using a component-based approach. **Results:** Individual VOCs, acrolein, acetone, and formic acid all induced the release of lactate dehydrogenase (LDH), indicative of cytotoxicity, and decreased barrier integrity as shown by increased FITC-Dextran permeability. Acetaldehyde did not appear to affect the viability or barrier function of 16HBE cells. Genes that were consistently upregulated between VOCs were the pro-inflammatory cytokines *IL-6*, *IL-8*, and *TNF α* . However, acetaldehyde was an exception to this trend. *IL-6* and *IL-8* were significantly downregulated, and *TNF α* showed no significant change in expression compared to the filtered air controls. Concentration-response modeling was employed to predict

the effective concentration of the VOC mixture that would induce a 10% change in viability and barrier permeability in exposed lung epithelial cells. This approach led to a prediction that a 10% change in barrier permeability would be observed in cells exposed to 1326 ppb of the VOC mixture, and a 10% change in cytotoxicity would be observed in cells exposed to 1419 ppb of that same mixture. However, following data collection of the actual VOC-mixture exposed cells, the BMC modeling proved to be a conservative prediction. The toxicity of the mixture was slightly over-predicted for each endpoint as visualized with a potency ratio (actual BMC/predicted BMC). The potency ratio for barrier permeability was 1.04, and the potency ratio for cytotoxicity was 1.13. **Conclusions:** While both endpoints resulted in a ratio close to 1, barrier permeability appeared to be the most suitable endpoint for conducting BMC modeling when investigating this set of VOCs. These findings demonstrate that acute exposure to select VOCs can disrupt epithelial integrity and induce cytotoxicity and inflammatory signaling in bronchial epithelial cells, with acrolein, acetone, and formic acid exerting the greatest effects. The component-based approach provided a conservative yet accurate prediction of mixture toxicity, supporting its utility for estimating mixture effects in the absence of direct experimental data.

PS 3496 Effects of exposure to Canadian wildfire particulate matter on airway reactivity, mucus production, and inflammation in advanced *in vitro* and *ex vivo* models.

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Background and Purpose: Wildfires have been rapidly increasing in both frequency and intensity over the last four decades due to climate change, and are expected to continue to escalate. In the US each year, over 63,000 wildfires burn an average of 7 million acres of land. Exposures to wildfire smoke has adverse impacts on respiratory, cardiovascular, and other diseases, but their specific mechanistic effects remain unknown. In June 2023, smoke from wildfires in Quebec drifted into the US east coast, resulting in a 3-day event with PM_{2.5} (particulate matter ≤ 2.5 μm) at concentrations > 700 μg/m³, from NYC to central NJ. During this event, we deployed instruments to collect real-time and time-integrated data on PM and gaseous co-pollutants, and to collect size fractionated PM for offline physico-chemical characterization and toxicological assessment. In this study we sought to investigate (1) the impact and mechanisms of PM-induced effects on human small airways; (2) the potential activation of the SPDEF promoter (which regulates airway mucus production) by PM exposure; and (3) the effect of PM exposure on the lung inflammatory response to an allergen. **Methods:** The PM_{0.1} (≤ 0.1 μm) fraction of the collected Canadian wildfire PM (WFPM_{0.1}) collected during the June 2024 event were employed in these studies. To assess effects of WFPM_{0.1} exposure on small airways, precision-cut human lung slices (hPCLS) prepared from human lung donors were treated with WFPM_{0.1} for 24 hours, followed by measurement of carbachol-induced bronchoconstriction, isoproterenol-induced bronchodilation, and expression of selected alarmin gene expression levels. Assessment of SPDEF activation by WFPM_{0.1} exposure was performed in HEK293 cells stably transfected with an SPDEF promoter-luciferase construct. The cells were exposed to WFPM_{0.1} for 6, 24, or 48 hours, and luciferase was measured in cell lysate supernatants. To assess the effects of WFPM_{0.1} exposure on lung inflammatory responses, mice were sensitized intranasally with the house dust mite (HDM) allergen, followed by intratracheal instillation with WFPM_{0.1}, and bronchoalveolar lavage (BAL) fluid was collected for phenotyping after 24 hours. **Results:** In the hPCLS model, WFPM_{0.1} increased CCh-induced bronchoconstriction, following 24 h treatment, with little effect on isoproterenol-induced bronchodilation. In addition, WFPM_{0.1} exposure increased the expression of IL-33 and CYP1A1 transcripts in hPCLS following 24 h exposure. In SPDEF promoter-luciferase expressing HEK293 cells, exposure to WFPM_{0.1} for 6 or 24h resulted in a dose-dependent significant increase in SPDEF promoter activity. In mice pre-sensitized with HDM allergen, tracheal instillation of WFPM_{0.1} significantly augmented the inflammatory response in the lung, as indicated by significantly greater numbers of total leukocytes, eosinophils, macrophages, neutrophils, and lymphocytes in BAL fluid. **Conclusions:** Collectively, our findings indicate that (1) Exposure to WFPM_{0.1} increases bronchomotor tone by increasing agonist-induced bronchoconstriction in human small airways, and increases expression of the 'alarmin' cytokine IL-33 (released in response to cell damage), and of CYP1A1, a key xenobiotic metabolism enzyme; (2) Exposure to WFPM_{0.1} increases activation of the SPDEF promoter (which induces differentiation of airway goblet cells and promotes mucus production), suggesting that exposure to WFPM_{0.1} could increase airway mucus production via this mechanism; and (3) *In vivo* exposure to WFPM_{0.1} can greatly exacerbate inflammation in the lung.

PS 3497 Quantitative Assessment of Indoor Asbestos Fiber Resuspension: Comparing Theoretical Predictions to Empirical Data

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Background and Purpose: For over six decades, regulatory agencies have asserted that asbestos is readily resuspended by indoor activities such as walking and sweeping. Numerous aerosol physicists have challenged this claim for the past 60 years, arguing that based on fundamental particle adhesion theory, resuspension is unlikely, particularly for biologically relevant fibers. Yet, the courts continue to be flooded with claims that asbestos fibers can be easily relaunched back into the air and deposition (Guth JH. 2018. Deposition of Joseph H. Guth, Ph.D. - Volume I. Dated August 27, 2018). Some experts have even indicated that a worker performing clean-up activities such as sweeping with a broom or cleaning with an air hose could receive a much higher concentration of fibers than the original worker who performed the hands-on work with a product that released those fibers (Guth JH. 2018. Deposition of Joseph H. Guth, Ph.D. - Volume II. Dated August 28, 2018). **Methods:** For two types of simulations (walking and sweeping), two grams of pure chrysotile asbestos were distributed over a 3 meter by 3 meter (3 m × 3 m) square area. Wipe sampling before each simulation discovered fiber surface loadings of 2.91 × 10¹¹ and 2.56 × 10¹¹ fibers for the walking and sweeping trials, respectively. Airborne fibers were sampled on the lapels of the volunteer (to represent personal samples) and around the grid (to represent area samples). These samples were characterized across a spectrum of widths and lengths, including fibers as short as 0.5 μm and up to 74 μm, with a range of aspect ratios. Particular attention was given to OSHA-classified fibers (≥5 μm in length: ≥3:1 aspect ratio). **Results:** Despite the presence of nearly 300 billion fibers on the floor surface, only about 100,000 were resuspended into the air. Asbestos fibers, especially those in the size range of concern from a health risk perspective (OSHA-classified fibers), were also not easily resuspended. Roughly four OSHA-classified fibers were resuspended per 10,000,000 originally deposited when walking was performed and roughly one OSHA-classified fiber was resuspended per 1,000,000 originally deposited when sweeping was performed. **Conclusions:** Ultimately, fiber resuspension observed in both walking and sweeping scenarios was minimal or de minimis, even under the study's considerable fiber-loaded floor. This is one of the only studies validating theoretical predictions that small structures/fibers cling tenaciously to flat surfaces—a phenomenon also observed during studies of the World Trade Center. The proportion of resuspended structures greater than 5 μm in length (fibers) was significantly less during walking and sweeping trials than initially deposited. This was predicted, as 5 μm fibers have greater surface areas for contact with the adhesive surface compared to smaller non-OSHA fibers (e.g., <5 μm). This supports the theory that the surface area-to-volume (SA-V) ratio dictates adhesion effectiveness. Typical fiber loadings on floors due to the vast majority of historical (pre-1985) industrial environments are expected to be significantly less than what was used in this study. Consequently, the resulting airborne concentrations and corresponding health risks from the historical scenarios seen would ultimately be *de minimis*.

PS 3498 Sex-Dependent Effects of Chronic Traffic-Related Air Pollution Exposure on Pulmonary Macrophage Polarization and Neuronal Atrophy

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Background and Purpose: Air pollution is a known risk factor not only for respiratory diseases but also neurodegenerative diseases, such as Alzheimer's disease (AD). Clinical studies linking respiratory disease and cognitive deficits have prompted animal studies to investigate mechanisms connecting traffic-related air pollution (TRAP) effects on the lung and brain. One prevailing hypothesis is that inflammatory mediators originating in the lung reach the brain via the systemic circulation where they exacerbate neuroinflammation and neurodegeneration. To test this, we assessed pulmonary inflammation, neuroinflammation, and neuronal atrophy, and compared these changes with pulmonary and plasma levels of TGFβ, a key mediator in fibrogenesis and neuroinflammation, in TgF344-AD rats that overexpress human AD risk genes following chronic exposure to TRAP. We further examined the relative contribution of particulate matter (PM) vs. gases to TRAP effects. **Methods:** Leveraging the Caldecott Tunnel Exposure Facility, female and male TgF344-AD rats were exposed to emissions drawn from a highway tunnel and delivered to animals in real-time for 11 months (M) starting at 1M of age. Animals were exposed to TRAP fractionated into gas, PM, or gas+PM from either light- and heavy-duty vehicles (LDV+HDV) or LDV only. Controls housed in the same vivarium were exposed to filtered air (FA). When exposures were completed, animals were transported back to UC Davis to collect brain, lung, and plasma. Quantitative immunohistochemistry (qIHC) was performed on fixed left lung lobes and brain hemispheres. Lung sections were immunostained for macrophages (CD68), profibrotic macrophages (CD206), and pro-inflammatory phenotype (iNOS), while hippocampal sections were stained

for homeostatic microglia (TMEM119), synaptic markers (MAP2, PSD95, synaptophysin), and tyrosine hydroxylase (TH) as a biomarker of adrenergic innervation. Since TGF β is a profibrotic cytokine, we correlated pulmonary TGF β 1,2 levels with counts of CD206 immunopositive cells using Pearson's correlation. Additionally, pulmonary TGF β was correlated with plasma TGF β . **Results:** After 11M of exposure, PM+Gas(LDV+HDV) significantly (p .adj<0.1) shifted alveolar macrophages towards a profibrotic phenotype in male lungs, evidenced as increased CD206⁺ cells. In contrast, PM+Gas(LDV) had the opposite effects, significantly reducing CD206⁺ cells in the male lung. While all TRAP exposures significantly increased TGF β -2 in male lungs, PM+Gas(LDV+HDV) showed the strongest correlation between TGF β level and CD206⁺ cell counts. However, TRAP effects on plasma TGF β showed inconsistent correlations with lung TGF β . TRAP did not significantly change macrophage phenotype or TGF β levels in females. Similarly, male-specific effects of TRAP were observed in the brain. LDV+HDV emissions reduced hippocampal TH innervation and excitatory synapses in the male hippocampus. Specifically, Gas(LDV+HDV) reduced TH area (p .adj=0.15) while PM(LDV+HDV) reduced excitatory synapses (p .adj<0.1). TRAP exposures did not affect homeostatic TMEM119 microglia; however, disease-associated microglia remain to be analyzed. **Conclusions:** These results suggest sex-dependent effects of TRAP on pulmonary profibrotic macrophage polarization, TGF β , and hippocampal synaptic and noradrenergic atrophy. Because homeostatic balance of macrophages is critical for maintaining a healthy microenvironment in lung, divergent polarization of alveolar macrophages suggests distinct profiles of immune dysregulation occur with different vehicular exhausts. While these changes in the lung did not directly correlate or affect plasma TGF β or expression of homeostatic microglia at this timepoint, they were associated with synaptic and TH atrophy in the brain. Supported by the NIH (grant# R01 AG074709, T32 HL007013, T34 GM136469, P30 ES023513).

PS 3499 Limited PAHs exposure to ESCs leads to neurodevelopmental alterations in human brain organoids

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Background and Purpose: Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental contaminants associated with adverse neurodevelopmental outcomes. However, their precise cellular and molecular effects in the human context remain poorly understood. This study aimed to investigate whether early and transient exposure to PAHs during the pluripotent stem cell stage could cause lasting alterations in brain organoid development. **Methods:** Human embryonic stem cells (hESCs) were transiently treated with benzo[a]pyrene (BaP) or phenanthrene (Phe) for one week during the ESC stage and subsequently differentiated into brain organoids. Morphological, immunohistochemical, and transcriptomic analyses were performed to assess structural and molecular changes induced by PAH exposure. **Results:** Limited PAH exposure did not affect hESC viability or proliferation but markedly disrupted later neurogenesis in the resulting brain organoids. Organoids generated from PAH-treated ESCs exhibited lasting morphological abnormalities, including smaller and fewer neural rosettes with ectopic neuronal protrusions. Immunostaining revealed that the early PAH exposure induced premature neurogenesis and depletion of the neuronal progenitor pool. Transcriptomic profiling of PAH-treated hESCs showed no significant global gene expression changes, suggesting that immediate transcriptional changes are unlikely to be the primary cause of the observed neurogenic defects. **Conclusions:** Transient early-life exposure to PAHs can program lasting defects in later brain development, underscoring the vulnerability of early developmental stages to environmental toxicants. Further analyses focusing on transcriptomic and epigenetic changes will be necessary to fully elucidate the underlying molecular mechanism.

PS 3500 Pathway-Based Assessment of PAH Mixture Toxicity Utilizing Benchmark Dose Modeling

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Background and Purpose: Polycyclic aromatic hydrocarbons (PAHs) are a large family of organic molecules composed of two or more benzene rings that are ubiquitous in the environment, associated with petrochemicals and burning of fossil fuels. PAHs identified from ambient air have been linked to adverse health effects, including lung cancer; however, little is known about how PAHs in mixtures contribute to toxicity in the lung. **Methods:** In this study, we broadly assess global genes and pathways by RNAseq from primary human bronchial cells cultured at the air-liquid interface (ALI-HBEC) after exposure to the PAH mixture (11-275 uM), which is derived from air sampling at a creosote site during wildfires, compared to two primary components, retene and benzo[b]fluorene (BBF), at equivalent concentrations. To identify the genes and pathways most sensitive after treatment with a PAH mixture compared to individual components, benchmark dose modeling was performed on transcriptional dose-response data with BMDExpress3 software. **Results:** Overall, a majority of genes differentially expressed (q <0.05) by retene and BBF at each concentration were not reflected by the mixture. Similarly, unique genes and path-

ways were identified at the threshold level between the mixture and components suggesting that the response of cells to PAH mixtures may not be a simple reflection of its components. **Conclusions:** These studies provide a global assessment of the mechanistic processes modified by PAHs in a dose-dependent manner and support our understanding for how PAHs contribute to toxicity of mixtures in lung cells.

PS 3501 Integrated Dosimetry Quantification for Aerosol Delivery at the Air-Liquid Interface in a Lung Cell Co-Culture System

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Background and Purpose: Exposure to aerosolized environmental contaminants may elicit acute or chronic effects depending on the inhaled dose. Researchers frequently test the effects of aerosolized substances with *in vivo* animal models and *in vitro* lung cell models using a known potential dose; however, the applied or internal dose is often not reported. **Methods:** This work expands upon previous studies by developing laboratory experimental methods to measure U.S. EPA-relevant dose types: (1) potential dose, (2) applied dose, (3) internal dose, and (4) biological effect. A multiple-cell co-cultured human lung model using epithelial cells and macrophages was developed for aerosol exposure to occupationally relevant substances, namely, aluminum oxide nanoparticles. To control the initial dose, a dry particle generator was used for aerosol production of the particles. The resultant aerosol was characterized by particle size distribution and was collected on filter cassettes to determine potential dose via inductively coupled plasma-mass spectrometry (ICP-MS). The applied and internal doses were also determined by using ICP-MS to analyze samples of spent cell culture media and cell lysate. The biological effect is measured through orthogonal methods probing for multiple mechanisms of action. **Results:** The potential dose was demonstrated as significantly different from the applied and internal dose, with each being less than 4% of what was aerosolized. Therefore, this study shows that applied and internal doses are important and should be reported in toxicological studies. **Conclusions:** This work demonstrates methods for dosimetry data collection, which can then be included in human health risk assessments of aerosolized toxicants to increase understanding of uptake and subsequent toxicological effects.

PS 3502 Sex- and Fuel Type-Specific Effects of Wood Smoke Condensate Exposure in Primary Human Airway Epithelial Cells

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Background and Purpose: Wildfire-derived particulate matter (PM) varies in toxicity by fuel source, and sex may be an important mediator in health outcomes from exposure. PM produced by wood burning is associated with reduced lung function and increased infection susceptibility which are potential markers for lung injury and has become a global concern due to escalating prevalence and severity of wildfires. Smoke from fuel types across the United States, such as red oak (eastern and central US), peat (peatlands/coastal, midwestern and southeastern US), ponderosa pine (western US), and eucalyptus (chaparral, southern California), have shown differential mutagenic and toxic effects in animal models, but effects by fuel type in human-derived models have not been investigated. Individual characteristics, such as sex, are also known to modulate the effects of biomass smoke exposure, though the mechanisms remain unclear. We aimed to fill these knowledge gaps by addressing the hypothesis that both fuel type and sex play a role in modulating respiratory health effects of smoke exposure using an *in vitro* model. **Methods:** Primary human bronchial epithelial cell (hBEC) cultures (n=4 Male, n=4 Female) were grown at air-liquid interface, and exposed apically to wood smoke condensates (WSCs) generated from red oak, peat, pine, and eucalyptus biomass at 5 μ g/cm² (low dose) and 25 μ g/cm² (high dose) for 4 hours. Apical washes, basolateral supernatants, and cell lysates were collected and analyzed for cytokine production via multiplex ELISA and transcriptional changes via RNA sequencing. **Results:** Across all WSCs, there was a consistent induction of IL-1 β and IL-8, while eucalyptus and peat exposures were uniquely associated with IL-2 induction. Pine and red oak exposures had unique IL-6, IL-12, and IL-13 induction. When analyzed by sex, male hBECs exhibited a consistent IL-1 β and IL-8 response across all WSCs and a unique IL-2 response to eucalyptus and peat WSCs. In contrast, female hBECs showed IL-8 induction specifically in response to eucalyptus and peat exposures. Overall, *in vitro* WSC exposure results in distinct inflammatory cytokine profiles based on fuel type and sex. Our RNA-seq analysis identified 202 differentially expressed genes (DEGs) across fuel types, with 51 DEGs overlapping between fuel types. Sex-specific analysis revealed 351 DEGs in male cultures and 108 DEGs in female cultures. Transcriptional pathway analysis utilizing GSEA and Hallmark MSigDB revealed upregulation of ROS production and fatty acid metabolism across all exposures, indicating cellular stress responses. Eucalyptus exposure downregulated protein secretion, while peat and red oak upregulated unfolded protein response, indicating endoplasmic reticulum stress. Downregulation of Notch signaling in both high dose pine and red oak exposures may reflect reduced cell proliferation and repair. Eucalyptus and pine exposure both led to upregulation of IL6/STAT3 signaling, while peat exposure caused upregulation of xenobiotic metabolism and cell proliferation. When disaggregated by sex, glycol-

ysis was downregulated only in female hBECs for eucalyptus and peat exposure, while G2M checkpoint was downregulated in females for eucalyptus and peat and in males for eucalyptus and pine. Estrogen response was downregulated in females following eucalyptus and peat exposure, while only downregulated in males for pine exposure. Androgen responses were downregulated for multiple exposures across both sexes. Oxidative phosphorylation had opposite directions of induction for pine exposures between male and female hBECs. **Conclusions:** This research has the potential to advance the field of toxicology by revealing new insights into individual susceptibility and potential geographical variations in the respiratory health effects of wildfire smoke exposure. Mechanistic insight into fuel type or sex-based susceptibility can provide a basis for personalized treatments and targets for mitigating the toxic effects of wildfire smoke.

PS 3503 Investigation of Cellular and Molecular Mechanisms of PFAS-Induced Lung Inflammation in a Human Alveolar Epithelial Cell Line

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Background and Purpose: Per- and polyfluorinated alkyl substances (PFAS) form a class of chemicals used in various commercial products, including non-stick cookware, firefighting foam, furniture, and waterproof clothing. These substances demand significant attention due to their environmental persistence and association with adverse health effects such as immune and thyroid problems, as well as liver, kidney, and pancreatic cancers. From a toxicology perspective, the inhalation of PFAS, particularly its impact on lung cells, remains notably understudied. While much focus has been on PFAS exposure through drinking water and food, emerging evidence indicates that inhalation—mainly occurring in workplaces and household dust—also plays a significant role in introducing PFAS into the human body. Despite existing research, a major knowledge gap persists in how PFAS causes lung toxicity at the cellular and molecular levels. This limits the development of effective prevention and treatment options for PFAS-related respiratory conditions. This study examines three key factors associated with cellular stress and inflammation: reactive oxygen species (ROS), hypoxia-inducible factor 1 alpha (HIF-1α), and the NOD-like receptor protein 3 (NLRP3) inflammasome. Human alveolar epithelial cells (A549) were exposed to PFAS compounds—perfluorooctane sulfonic acid (PFOS) and perfluorobutane sulfonate (PFBS), representing long-chain legacy and short-chain emerging PFAS. It is hypothesized that PFAS causes lung toxicity by increasing ROS, activating HIF-1α, and triggering the NLRP3 inflammasome, leading to inflammation and cell death. **Methods:** A549 cells were cultured in DMEM supplemented with 10% (v/v) FBS and 1% antibiotics at 37°C in a humidified incubator with 5% CO₂. PFOS and PFBS stock solutions were prepared in DMSO, maintaining a final DMSO concentration of 0.01% in the dosing solution, which is non-cytotoxic to A549 cells. The cytotoxicity of PFOS and PFBS was evaluated using an MTT assay after 24 hours of acute exposure at concentrations of 10, 25, 50, 100, 150, and 200 µg/mL. Following viability testing, reactive oxygen species generation was assessed with the DCFH-DA assay. HIF-1α and NLRP3 protein levels were measured via Western blotting, with protein bands quantified and analyzed using Image Lab software. To observe the stabilization of NLRP3 and HIF-1α in both the cytoplasm and nucleus at all concentrations, immunocytochemistry was performed. **Results:** The results showed an IC50 value of 179 µM for PFOS, and all tested concentrations of PFBS were non-cytotoxic to A549 cells; therefore, we only evaluated PFOS for subsequent downstream analyses and assays. PFOS caused a dose-dependent rise in ROS levels, shown by increased green fluorescence, indicating reactive oxygen species. Exposure to PFOS even at low, non-toxic concentrations (10, 50, 100, and 150 µM) caused a temporary increase in nuclear HIF-1α levels, which then decreased in a dose-dependent manner. PFOS exposure resulted in a dose-dependent increase in NLRP3 protein levels, as confirmed by Western blot analysis. Levels increased starting at 10 µM, peaked at 100 µM, and then slightly decreased at 150 µM, suggesting threshold-dependent activation. Immunocytochemistry revealed greater NLRP3 presence in the cytoplasm and around the nucleus. **Conclusions:** This study shows PFOS, even at non-cytotoxic levels, causes oxidative stress and inflammation in human alveolar cells. Increased ROS and HIF-1α stabilization suggest early involvement of oxidative and hypoxia pathways, likely triggering NLRP3 inflammasome activation, inflammation, and cell injury. PFBS showed minimal effects, indicating lower toxicity. Overall, the findings highlight the ROS-HIF-1α-NLRP3 signaling pathway as a key mechanism linking PFOS exposure to lung inflammation.

PS 3504 Maresin 1 Selectively Enhances Pro-reparative IL-22 Production in Alveolar Macrophages Following Organic Dust Exposure

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Background and Purpose: Occupational dust exposure is a significant health concern, driving the progression of chronic lung diseases such as asthma and COPD. Agricultural dust contains endotoxins and microbial components that activate innate immune pathways, leading to sustained inflammation. Recent evidence highlights the importance of specialized pro-resolving mediators (SPMs), derived from dietary omega-3 fatty acids, in counteracting dysregulated inflammatory responses. Notably,

Western diets are often characterized by an imbalanced omega-6 to omega-3 ratio, which may impair the biosynthesis and function of SPMs. Our laboratory has identified that alveolar macrophages are a source of the pro-reparative cytokine IL-22, which plays a protective role in response to dust-induced lung injury. The present study investigates the role of a specific class of SPMs, including maresins and their biosynthetic derivatives, in modulating IL-22 signaling in the context of organic dust exposure (ODE) *in vitro*. **Methods:** MH-S cells (immortalized murine alveolar macrophages) were pretreated for 1 hour with 1nM, 10nM or 100nM dose of Maresin 1 (MaR1), Maresin 2 (MaR2), or Maresin Conjugates in Tissue Regeneration (MCTR1, MCTR2, MCTR3) prior to exposure with 1% dust extract. Cytokine concentrations (IL-10, IL-22, TNF-α, and IL-6) were quantified using sandwich ELISA. Data were analyzed using two-way ANOVA with Tukey's post hoc test, with significance set at $p < 0.05$. **Results:** All SPMs tested led to a significant suppression of proinflammatory cytokines TNF-α and IL-6 in a dose dependent manner. However, MaR1 pretreatment uniquely enhanced IL-10 (MaR1 x organic dust effects, $p=0.0429$) and IL-22 (MaR1 x organic dust effects, $p=0.0368$) production, peaking at 10 nM following ODE, whereas MaR2 and MCTRs did not elicit a similar pro-reparative response. Instead, MCTRs induced a dose-dependent suppression of IL-22. These divergent outcomes suggest that while MaR1 and its other biosynthetic derivatives potentially reduce inflammation, MaR1 appears to selectively promotes anti-inflammatory and pro-reparative IL-10 and IL-22 signaling. **Conclusions:** Our findings highlight distinct functional roles among maresins and their conjugates in shaping alveolar macrophage responses to organic dust. The selective ability of MaR1 to augment IL-22 suggests a receptor-mediated mechanism, potentially through LGR6 activation. Given the critical role of IL-22 in protecting lung tissue from dust-induced injury, delineating the molecular crosstalk between SPMs and IL-22 signaling could inform novel therapeutic strategies. Future directions include extending these findings to mouse models and defining receptor-specific pathways linking dietary omega-3 metabolites to macrophage-driven lung repair.

PS 3505 Toxicity of diacetyl or 2,3-butanedione, a flavor enhancer, and its implications on aberrant bronchial epithelial repair

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Background and Purpose: Diacetyl or 2, 3 - butanedione is a widely used volatile chemical that is used to enhance different types of flavors in vape liquids. In the early 2000's, it is reported to cause the disease, obstructive bronchiolitis which is an irreversible fibrotic disease in populations of popcorn factory workers who were exposed to aerosolized diacetyl. However, the mechanisms of diacetyl toxicity remains poorly understood and explored. The purpose of the current study is to elucidate how diacetyl causes epithelial cell injury, and to evaluate the possible outcomes of long-term exposure to diacetyl. The current study hypothesizes that diacetyl exposure causes the stabilization of the alpha sub-unit of HIF-1 transcription factor, which is responsible for oxygen regulation in the cells, where it is hypothesized as an initial inflammatory response. While HIF-1 alpha gets stabilized under hypoxic conditions in the cells, several other factors such as cytokines (TNF-α and IL-1 beta) and reactive oxygen species (ROS) are reported in recent literature to stabilize the sub-unit. Furthermore, TGF-β is a regulator of cell repair in physiological repair after injury. However, in pathologic repair TGF-β could stimulate aberrant repair in cells through pathologic epithelial-to-mesenchymal transition, where epithelial markers get downregulated and mesenchymal markers get upregulated compromising the epithelial barrier. Moreover, due to continuous inflammation the epithelial cell layer would be in a constant state of repair leading to a compromised barrier. The overall objective is to understand how diacetyl alters the airway epithelium. **Methods:** 5 mM, 10 mM, and 15 mM concentrations were used to dose the immortalized bronchial epithelial cells (BEAS-2B) and 16-HBE cells in submerged culture for 2 or 1 hour respectively. DCFH-DA cellular ROS (reactive oxygen species) assay was used to evaluate the presence of general ROS in the cells after exposures. The fluorescence intensity was measured using Bio-Tek Lionheart instrument. HIF-1 alpha expression was evaluated using western blot. Primary antibody against HIF-1 alpha was used at 1:500 dilution and a secondary HRP antibody was used at 1:10,000 dilution. The membrane was visualized using Bio-Rad ChemiDoc MP imager. The images were quantified using Bio-Rad Image Lab software. The 16-HBE cell line was used for barrier integrity studies. The 16-HBE cells were dosed intermittently for one hour on day 1, 3 and 5. TEER (Trans epithelial electrical resistance) was measured using EVOM instrument. To quantify the expression of epithelial markers (ZO-1 and E-cadherin), and mesenchymal markers western blotting was used. Immunofluorescence studies were performed to visualize ZO-1 and E-cadherin localization in the cells. The cells were incubated with primary antibodies against ZO-1 and E-cadherin at 1:200 dilution. The secondary fluorochrome conjugated antibodies were used at 1:700 dilution. The cells were visualized with a confocal laser scanning microscope. The results were analyzed using GraphPad Prism 10.4.2. with ANOVA test together with Tukey HSD post-hoc test to get the group comparisons. The significance was set at $p < 0.05$. **Results:** The results for western blotting indicated that HIF-1 alpha expression increased up until 10 mM dose dependently. 5 mM and 10 mM concentration were statistically significant. However, 15 mM had a lower expression compared to other experimental concentrations but showed more expression compared to the untreated control. ROS were detected in the cells dose dependently. All three concentrations showed statistically significant increase in the generation of ROS. The TEER values for the dosed cells were lower compared to the untreated cell monolayers. The cells exposed to 15 mM concentration did not recover after

the dosing period. The expression of ZO-1 and E-cadherin proteins in the 16-HBE cells decreased dose dependently and were statistically significant compared to the untreated cells. **Conclusions:** HIF-1 alpha was observed to be stabilized under normal oxygen conditions possibly due to the presence of ROS in the cells due to diacetyl exposures. The barrier integrity of the 16-HBE cell monolayer was compromised as monitored through TEER values and ZO-1, E-cadherin and alpha-SMA, vimentin protein expression levels.

PS 3506 Deriving a Mixture of Combustible Materials in American Homes and Fire-prone Biomes to Elucidate the Toxicity of Wildland-Urban Interface Fires

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Background and Purpose: Wildfires in the Wildland-Urban Interface (WUI) are an increasing concern, with approximately 33% of homes in the U.S. now located in the WUI and thus at elevated risk of burning during wildfires. WUI fires are unique as they emit a complex mixture of inorganic and organic compounds derived from the combustion of anthropogenic materials in addition to those produced by biomass-only wildland wildfires. A standardized fuel mixture is vital to characterizing exposure chemistries and toxicological impacts of WUI-relevant smoke exposures. **Methods:** We developed two novel mixtures representing WUI materials and a mixture of biomass common to California's plant biome. To develop the WUI mixture, we first integrated 61 combustible materials typically found in the average American WUI home, a 2,016 sq. ft. single-family home with four bedrooms and three occupants. Materials included structural, plumbing, wiring, paint, appliances, furniture, and furnishing materials. We found that the total combustible mass of an average American family home was around 46,500 kilograms, including 81% wood materials, 10% petroleum-based products (asphalt shingles), 6% plastics, and 2% metals. To develop the California biomass mixture, we identified the vegetation types and their proportions in the chaparral biome, which represents regions in California where some of the most destructive wildfires have occurred. The chaparral mixture consisted of 60% coastal sage, 30% chamise, and 10% eucalyptus. Each mixture was processed, homogenized, then burned in a lab-scale furnace system to generate WUI and chaparral smoke condensates from smoldering and flaming combustion conditions. The smoke condensates were then evaluated for toxicity in primary human airway epithelial cells cultured at air-liquid interface in 12-well plates. In the initial dose response experiment, 250 μ L of smoldering WUI smoke condensate was applied as an aqueous dilution to the apical compartment for four-hours at concentrations of 1, 3, 10, and 30 μ g/cm² for three donors, following which cytotoxicity and changes in cellular mRNA expression were evaluated. **Results:** Initial gene-specific RT-qPCR indicated that IL-6 mRNA release was significantly increased at a dose of 30 μ g/cm² of WUI condensate. Notably, cellular transcriptional responses occurred in the absence of cytotoxicity. Subsequent studies treated 12 donors to 3 and 30 μ g/cm² doses of smoldering WUI or chaparral condensates for four hours, and results suggest that 30 μ g/cm² doses of WUI or chaparral condensates decrease cell barrier integrity across all donors when compared to vehicle and lower doses. Next steps being pursued include analysis of transcriptomic, proteomic, and lipidomic signatures to inform more global disruptions in cellular biology resulting from WUI fires, including interleukin and immune response signaling changes. Chemical and particle characterization of the mixture condensates are also in progress. **Conclusions:** Treatment of airway epithelium cells with WUI condensates induced changes in interleukin release, suggesting potential modulation of immune pathways, which is an effect that will be further evaluated across multiple -omics endpoints. Collectively, the WUI and chaparral biome fuels can serve as standardized fuel mixtures representing WUI fires that can be used to investigate chemical and mechanistic determinants to health effects of exposure to smoke and to inform future risk assessment and regulatory efforts that will protect our communities. (This abstract does not reflect EPA policy.)

PS 3507 A Novel *in vitro* Toxicity Assay for Predicting Inhalation Toxicity in Rats

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Background and Purpose: The development of alternative *in vitro* methods for assessing acute inhalation toxicity is a critical step toward reducing animal testing and aligns with the principles of the 3Rs (replacement, reduction, and refinement). This study aimed to develop and optimize a neutral red uptake (NRU) assay using human lung adenocarcinoma cells (A549) as a predictive model (A549-NRU assay) for evaluating acute inhalation toxicity. **Methods:** The assay was improved by introducing two key modifications: (1) reducing the incubation time to 15 minutes

to enable rapid and high-throughput screening, and (2) using glass 6-well plates for chemicals reactive with polystyrene to prevent chemical-induced degradation and ensure assay consistency. LC₅₀ values were determined for 49 chemicals and compared with reported LC₅₀ values from 4-hour rat inhalation studies. Correlations between A549-NRU LC₅₀ values and physicochemical parameters (molecular weight, octanol-water partition coefficients) were also analyzed. **Results:** A significant positive correlation was observed between A549-NRU-derived LC₅₀ values and *in vivo* LC₅₀ values for water-soluble compounds and chemicals containing aldehyde, ketone, alcohol, ether, and epoxide functional groups, suggesting predictive potential. In contrast, A549-NRU LC₅₀ values showed significant negative correlations with molecular weight and octanol-water partition coefficients, indicating lower cytotoxicity for larger or more lipophilic compounds. The A549-NRU assay demonstrated a stronger correlation with *in vivo* LC₅₀ values than the conventional NRU assay using mouse 3T3 fibroblast cells. **Conclusions:** The optimized A549-NRU assay provides a rapid, reliable, and human cell-based *in vitro* method for assessing acute inhalation toxicity. It can be applied to estimate starting doses for *in vivo* studies and holds promise as an alternative approach to predict acute inhalation toxicity without animal testing.

PS 3508 A Modular Smoke Exposure Platform for *In Vitro* Modeling of Burn Pit Emissions

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Background and Purpose: Inhalation exposure to open-air burn pits presents serious health risks, particularly for military personnel deployed in affected regions. Many veterans exposed to toxic smoke from these pits continue to suffer from persistent and often unexplained health problems. A clearer understanding of the mechanisms linking burn pit exposure to long-term illness is essential for preventing future health impacts and for developing effective treatments for those already affected. In alignment with the FDA Modernization Act 2.0 and the growing emphasis on human-relevant, non-animal models, we developed a real-time smoke exposure system tailored for exposing airway organ tissue equivalents (OTEs) matured at an air-liquid interface. **Methods:** We engineered a modular burn chamber capable of generating controlled combustion profiles that replicate real-world burn pit emissions. The materials used represent those most burned in a burn pit, which include plastic, cardboard, and wood, combusted at both smoldering and flaming phases. Real-time monitoring of carbon monoxide, carbon dioxide, particle size distribution, temperature, and humidity was achieved using integrated sensors. Smoke from each burn material and burn phase was chemically characterized for polycyclic aromatic hydrocarbons (PAHs) and volatile organic compounds (VOCs). Human airway OTEs were exposed at the air-liquid interface, enabling direct interaction with both gas and particle-phase constituents. Post-exposure, tissues were assessed for biochemical and functional toxicity markers. **Results:** The exposure system successfully delivered reproducible smoke profiles with tunable combustion conditions that matched burn conditions from real-world burn pits. Sensor data confirmed dynamic shifts in particulate and gas-phase composition across burn phases. Airway OTEs tolerated the exposure protocol and exhibited measurable changes in barrier integrity and oxidative stress markers. Preliminary data suggest phase-dependent toxicity signatures and material response differences in airway OTEs. **Conclusions:** This novel exposure platform enables physiologically relevant modeling of burn pit smoke inhalation *in vitro*. By integrating real-time combustion analytics with human airway tissue models, we provide a scalable and reproducible system for toxicological assessment. This approach supports the transition toward non-animal testing strategies and enhances our ability to evaluate complex airborne exposures with translational relevance.

PS 3509 Using computational fluid dynamics to estimate the efficiency of dosing by varying the respiration rate and tidal volume of the rat and changing the position of the rat snout using an inhalation directed flow chamber

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Background and Purpose: • Inhalation administration is a common dose route used in nonclinical development for delivering drugs to the lungs. The standard approach is to evaluate drug safety in both rodent and non-rodent species. • With the continued use of both New Chemical Entities (NCEs) and biopharmaceuticals, rats continue to be the rodent species of choice. • The standard method for delivering the aerosol to rats is using a snout-only inhalation chamber called a direct flow chamber. During the exposure period, the rats are restrained in plastic clear restraint tubes. • Consistent dosing is pivotal in supporting effective study conduct and data interpretation to draw accurate conclusions about the safety of the test article, however, it is known that animals, like humans, have varying respiration rates and tidal volumes • In this study, Labcorp wanted to establish whether varying the breathing respiration rates and tidal volumes and changing the position of the rat compared to the end of the aerosol chamber delivery tube contributed towards the greater level of animal-to-animal dose variability that is observed with the inhalation compared to other routes of administration like oral or intravenous. • This poster presents new data and expansion of the next stage of the initial data published at SOT 2025 (Moore S, Using

computational fluid dynamics to estimate the efficiency of delivery by changing the position of the rat snout and airflow received using an inhalation directed flow chamber, Society of Toxicology, 2025). **Methods:** • Labcorp designed a computational fluid dynamics (CFD) model of a rat head including the snout using 3d scanning software. Details of the 3d scanning methodology have been presented previously (Ford T and Moore S, Development of using 3D scanning, 3D printing and computer modelling techniques to improve inhalation systems, Association of Inhalation Toxicologist, 2024). • The dimensions of the inhalation chamber and standard restraint tube were measured and incorporated into the model. • Five different combinations of respiration rate and tidal volume were chosen to encompass the full range of potential options based on internal rat data • Five positions were evaluated - 1mm from the end of the tube, 10mm from the end of the tube and 5mm to the side, 5mm up and 5mm down. • A fixed airflow rate per port of 0.94 L/min was chosen to ensure a direct comparison across conditions. **Results:** • The highest % delivery efficiency for the CFD model across all head tube positions was with a respiration rate of 190 breaths/min and tidal volume of 1.9 mL with values between 31.8% and 35.5%. • The lowest % delivery efficiency for the CFD model across all head tube positions was with a respiration rate of 110 breaths/min and tidal volume of 1.2 mL with values of 12.9% and 14.2%. • The sequence of the delivery efficiency appears to correlate directly with the respiratory tidal volume. • In all 5 cases, the delivery position of 1mm away from the end of the delivery tube, then 10mm away, then 5mm to the side, 5mm when the position was upwards then finally when the snout position was angled 5mm downwards. **Conclusions:** • In conclusion, the CFD data suggests that the larger the respiratory tidal volume of the animal, the rat will receive a higher delivery efficiency and thus dose • This research is important in assessing the impact on study integrity and data interpretation.

PS 3510 Evaluating Chemical and Clinical Spaces: The Bifunctional Reactivity of Respiratory Sensitizers in Comparison to Skin Sensitizers and Non-Sensitizing Irritants

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Background and Purpose: Respiratory sensitization is a complex condition characterized by a spectrum of clinical symptoms, ranging from mild rhinitis to severe, debilitating asthma. In contrast to skin sensitizers, the chemical space of low molecular weight respiratory sensitizers is minimal, largely dominated by diisocyanate and anhydride functional groups. Currently, no validated methods exist that are specific to the detection of respiratory sensitizers. Although the proposed adverse outcome pathway (AOP) for respiratory sensitization shares similarities with that of skin sensitization, it has been hypothesized that the mechanisms of chemical reactivity involved in hapten formation, identified as the first key event, differ between respiratory and skin sensitizers. In this study, eighteen chemicals (including non-fragrance skin sensitizers, fragrance skin sensitizers, respiratory sensitizers, and non-sensitizing irritants) were reviewed for existing clinical case summaries, evaluated for chemical reactivity mechanisms, and analyzed using the TIMES (Tissue Metabolism Simulator) *in silico* model to predict their metabolic transformations. The goal was to identify differences in chemical reactivity mechanisms, compare metabolic processes in the skin and lung environments, assess their impact on sensitization potential, and align these findings with clinical observations to distinguish between skin sensitizers, respiratory sensitizers, and non-sensitizing irritants. **Methods:** Existing reviews summarizing clinical data on selected eighteen chemicals were evaluated to document clinical cases of skin sensitization and respiratory sensitization. These chemicals were also examined for predicting chemical reactivity mechanisms and metabolic transformations using the TIMES software. Two models were applied: 1. *In vivo*, non-kinetic skin sensitization with autooxidation2. *In vitro*, lung metabolism Both models predicted the most probable metabolic pathways and the relative quantity of metabolites. **Results:** A review of clinical case summaries for the eighteen chemicals revealed that patients with skin sensitization symptoms from skin sensitizers did not exhibit concurrent respiratory issues. Similarly, workers occupationally sensitized to respiratory sensitizers did not exhibit simultaneous skin sensitization. No clinical cases for either skin sensitization or respiratory sensitization were reported from exposure to non-sensitizing irritants. These observations do not take into consideration the patients' medical history of skin or respiratory allergies. The TIMES *in silico* evaluation showed that strong skin sensitizers, included in this study, interacted directly with skin proteins. Prohaptens, such as piperazine (a respiratory sensitizer), require metabolic activation to a reactive metabolite, with a higher probability of formation in the lung model than in the skin model. Non-sensitizing irritants (alcohols, amines, and carboxylic acids) showed high affinity for Phase II conjugation and excretion. The common structural feature of respiratory sensitizers, in this training set, was that they were bifunctional electrophiles. Compounds with highly reactive functional groups—such as diisocyanates, anhydrides, and bisaldehydes—are recognized as respiratory sensitizers, likely due in part to their strong protein crosslinking capacity, which leads to neoantigen formation. **Conclusions:** Observations from chemical space evaluations and clinical case reviews highlight the bi-functional specificity within lung tissue and the importance of lung-specific immune cells in characterizing the AOP for respiratory sensitization. The *in silico* simulations had two key limitations: (1) the skin sensitization model

was trained on a substantially larger dataset (1,405 chemicals) compared to the lung model (150 chemicals), and (2) the skin model was developed using historical *in vivo* data, whereas the lung model relied on *in vitro* data. These observations provide a foundation for developing new approach methodologies (NAMs) for respiratory sensitization assays.

PS 3511 Indole-3-Carbinol Reprograms Endothelial and Immune Cell Dynamics to Suppress SEB-Induced Inflammation: A Single-Cell RNA Velocity and Pathway Modeling Study

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Background and Purpose: Indole-3-carbinol (I3C), a naturally occurring phytochemical derived from cruciferous vegetables, has demonstrated anti-inflammatory and immunomodulatory properties. However, its effects on cellular transcriptional dynamics and signaling pathways during toxin-induced lung inflammation remain unclear. Staphylococcal enterotoxin B (SEB), a potent superantigen, induces acute lung injury through hyperactivation of immune and vascular pathways. This study applied single-cell RNA velocity analysis integrated with PROGENy pathway modeling to define the cell-type-specific mechanisms by which I3C mitigates SEB-induced immunopathology. **Methods:** Single-cell transcriptomic data from SEB-challenged mouse lungs were analyzed across 18 major cell clusters representing immune, endothelial, and stromal lineages. RNA velocity metrics (length and confidence) and delta-velocity differentials were computed for SEB+Veh, and SEB+I3C groups. PROGENy-based signaling pathway scoring was used to infer downstream transcriptional activity changes across clusters. **Results:** I3C markedly reprogrammed transcriptional dynamics in Endothelial Cell 2 (EC2) and CD4⁺ T-cell populations. In SEB+Veh, pro-inflammatory and stress-related genes (Stk40, Tmem147, Pde3a, Il7r) were elevated, while I3C treatment upregulated regulatory and structural genes (Cav1, Itga2, Xab2, Tox). Pathway modeling revealed heightened NFκB, TNFα, and JAK-STAT signaling under SEB+Veh, contrasted by increased TGFβ, PI3K, and p53 pathway activity following I3C intervention. **Conclusions:** These findings demonstrate that I3C mitigates SEB-induced inflammation by reprogramming endothelial and immune cell transcriptional trajectories and shifting signaling balance from pro-inflammatory toward regulatory networks. This integrative single-cell systems approach highlights I3C's therapeutic potential as a molecular modulator of immune and vascular toxicities induced by bacterial superantigens. (Supported in part by NIH grants R01ES030144, P30GM154631, P20GM103641 and R01AI160896).

PS 3512 Development and Validation of a Human Pluripotent Stem Cell-derived Multicellular Alveolar Model for Toxicity Assessment and Disease Modeling

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Background and Purpose: The growing demand for reliable *in vitro* models for inhalation toxicity testing and respiratory systems research has driven the development of platforms that can accurately mimic the complex human alveolar microenvironment. This study aimed to develop a alveolar model with multicellular composition and pathological response capabilities. **Methods:** Human induced pluripotent stem cells (hiPSCs) were differentiated into alveolar cultures at the air-liquid interface (ALI) using a multi-stage directed differentiation protocol. The differentiation process and cellular composition were systematically characterized by immunofluorescence staining and RT-PCR. Fibrosis was induced by stimulation with 10 ng/mL TGF-β1. Epithelial barrier function was assessed by measuring transepithelial electrical resistance (TEER), and the expression of pro-fibrotic genes (ACTA2, COL1A1, FN1) was quantified by qPCR. Pharmacological response was evaluated using the anti-fibrotic drug nintedanib. **Results:** The results demonstrate the successful establishment of an alveolar model containing multiple key cell types, including alveolar epithelial cells (pro-SPC⁺, PDPN⁺), mesenchymal fibroblasts (PDGFRα⁺), and immune cells (CD68⁺). TGF-β1 treatment effectively induced epithelial barrier dysfunction and significantly upregulated the expression of pro-fibrotic genes. Furthermore, the model showed the expected pharmacological response to nintedanib, as evidenced by a significant inhibition of fibrotic marker expression. **Conclusions:** These findings highlight the model's potential for application in compound screening, pulmonary toxicity assessment, and research into the mechanisms of pulmonary fibrosis.

PS 3513 Toxicity after exposure to inhaled amorphous Silicon Dioxide Nanoparticles in Golden Syrian Hamsters

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Background and Purpose: Amorphous SiO₂ NPs are abundant in occupational settings, often used in food, cosmetics, and paint as fillers and anti-caking agents. Such particles are not as commonly studied when compared to their crystalline coun-

terpart. Amorphous SiO₂ NPs are pulmonary toxicants; however, the mechanism of toxicity is uncertain. **Methods:** In the current study, toxicity was assessed using an *in vivo* system, where Golden Syrian Hamsters were exposed by inhalation to different concentrations of amorphous SiO₂ NPs nanoparticles in a full body exposure chamber to determine a possible mechanism of cell death. Animals were divided into 4 groups: 1-room air control, 2-vehicle control, exposure to aerosolized water, 3-low concentration (6 mg/m³), and 4- high concentration (12 mg/m³). Concentrations were selected based on NIOSH's Time Weighted Average (TWA). Vehicle control exposed to distilled H₂O and treatment groups exposed to SiO₂ NPs were treated for 4 hours a day for 8 consecutive days. **Results:** Histopathological analysis found a significant increase in air space quantified by Mean Linear Intercept. The Mean Linear Intercept in the low and high concentration treated groups were increased by 1.9-fold and 1.7-fold respectively, when compared to controls. A TUNEL Assay found a significant increase in TUNEL positive cells in the high concentration group by 6.1-fold when compared to controls. SEM and TEM found structural alterations to the lung tissues including increase in the number of holes in alveolar walls, thinning of the alveolar septum and apoptotic bodies within tissue. Apoptosis markers Caspases 3 and 8 were significantly increased in the high concentration groups by 1.4-fold compared to controls. Cellular inflammation markers TNF- α and HSP70 were measured. TNF- α had a 1.5-fold increase in the high concentration treated group compared to controls and HSP70 was significantly increased by 7.5-fold in the high concentration treatment group compared to controls. **Conclusions:** Results of the study indicate exposure to SiO₂ NPs may induce an extrinsic apoptotic pathway, leading to tissue damage and significant airspace enlargement.

PS 3514 Increased living temperature alters the cardiopulmonary response of male and female mice to benzene-toluene-ethylbenzene-xylene (BTEX)

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Background and Purpose: Benzene, toluene, ethylbenzene, and xylene (BTEX) compounds are ubiquitous in the environment, originating from both natural and anthropogenic sources. Despite the broad presence of BTEX in the environment, extensive use of petroleum products and improper maintenance of underground storage tanks (USTs) have increased the risk of exposure to these compounds. Hazardous substances stored in USTs, like BTEX, may leak into groundwater, soil, and buildings and homes over time. Furthermore, higher temperatures exacerbate the release of VOCs. Therefore, the purpose of this study was to characterize the cardiopulmonary effects of inhaled BTEX in female and male C57BL/6J mice and assess the role of living temperature. **Methods:** Eight-week-old mice were housed at normal temperature (NT - 22°C) or high temperature (HT - 32°C) for five weeks. Mice were then exposed to either filtered air (FA) or BTEX (316ppm, nose-only) for four hours per day for two consecutive days. Ventilatory function was assessed via whole-body plethysmography (WBP) a week before and immediately after the first exposure. High-frequency echocardiography (HF-echo) was performed the week prior to exposure and again immediately following the second exposure. WBP and HF-echo measurements were compared from pre- to post-exposure. **Results:** When compared to NT, HT significantly increased tidal volume (TV), peak expiratory flow (PEF) peak inspiratory flow (PIF), and relaxation time (RT), and decreased enhanced pause (Penh) in female and male mice. BTEX increased frequency (f) and decreased expiratory and inspiratory times (Te and Ti, respectively) as well as Penh in female NT mice but had no effect on male NT mice. At HT, BTEX significantly decreased f and Penh and increased TV, Te, Ti, and RT in female mice, and only increased TV, PEF, PIF in males. Thus, the impact of HT and BTEX on female mice appeared to be greater than on males. Female HT-FA mice had a significant decrease in heart rate (HR) compared to NT, while BTEX caused an increase. On the other hand, male FA mice experienced an increase in HR at HT. No significant differences in cardiac output, stroke volume, fractional shortening, ejection fraction, end systolic volume, or end diastolic volume were observed between the groups. **Conclusions:** These results indicate that higher living temperature alters cardiopulmonary function in female and male mice in a differential manner. Although breathing changes seem to be largely driven by temperature, it is worth noting that HT might influence the inhaled BTEX dose, and, potentially, increase risk. Long-term exposure assessments are needed to clarify these effects further. (This abstract does not reflect EPA policy.)

PS 3515 A Low-Cost, Versatile Cigarette-Style Combustion Generator: System Development and Validation in Mice Acutely Exposed to Simulated Military Burn Pit Emissions

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Background and Purpose: Military personnel and civilians face increasing exposure to combustion emissions from military burn pits and wildfires, yet physiological impacts on cardiopulmonary systems remain incompletely characterized. Traditional combustion exposure systems are complex, expensive, lack versatility, and require specialized infrastructure, limiting controlled studies. We developed a novel, low-cost simulation military burn pit (MBP) exposure system utilizing cigarette-style fuel rods as standardized, reproducible combustion sources usable with conventional cigarette smoking machines. This study established and validated the system to perform acute inhalation exposure and measured immediate cardiopulmonary responses to controlled MBP emission exposure in mice. **Methods:** The MBP fuel mixture composition was based on reported U.S. military base solid waste data: plastics (27wt%), cardboard (49wt%), wood (24wt%). Materials underwent cryogenic mechanical grinding to achieve a relatively uniform size 'shredded' material which was blended, weighed and rolled into standard combustion rods using commercial rolling papers. Prior to ignition, a small amount of diesel fuel (DF2; 10wt%) was added. MBP emissions were drawn into a mixing chamber and diluted with purified air before passing through a rodent nose-only exposure/sampling manifold. All real-time measurements and sample collections occurred at the animal breathing zone. Particle size distribution, mass concentration, and CO were measured using a controlled puff duration and frequency. Metal, total organic and inorganic carbon, and select PAH concentrations were also measured in the collected samples. An acute inhalation study was conducted using male C57BL/6 mice (n=4-5 per group) exposed to either purified air or freshly generated MBP emissions (15 mg/m³) using a nose-only exposure system for 2 hours/day over 3 consecutive days. Respiratory parameters and blood pressure were measured post-final exposure using whole-body plethysmography and non-invasive tail cuff systems. **Results:** The system generated consistent, reproducible MBP emissions with controlled particle characteristics. Particle size, mass, and CO concentrations increased with puff frequency/duration. Combustion achieved 53% total organic carbon efficiency; average particle size (Dp) 0.050-0.30 μ m. MBP exposure significantly altered respiratory mechanics and cardiovascular function versus controls. Enhanced pause (Penh) decreased significantly while peak inspiratory/ expiratory flows (PIF/PEF) increased. Expiratory time (Te) reduced in exposed mice, indicating faster breathing cycles. Systolic blood pressure increased significantly; diastolic showed non-significant elevation. These validated the system's capability to generate biologically relevant emissions and provided preliminary evidence that acute high-concentration MBP exposure induces cardiopulmonary aberrations. **Conclusions:** This study established and validated a novel, low-cost cigarette-style combustion generator producing consistent, reproducible emissions for controlled animal inhalation studies. The system generates biologically relevant emissions inducing measurable physiological stress characterized by upper respiratory irritation and cardiovascular activation. Decreased Penh with elevated inspiratory/expiratory flows indicates increased nasal/upper airway resistance requiring greater respiratory effort. Reduced expiratory time reflects compensatory mechanisms maintaining ventilation despite increased airway resistance. Concurrent systolic blood pressure elevation suggests systemic cardiovascular stress likely mediated by inflammatory responses and sympathetic activation. The cigarette-style fuel rod approach adapts to most cigarette-smoking exposure systems, offering significant advantages for standardizing combustion research through improved reproducibility, reduced costs, and adaptability to various fuel compositions, enabling translatable findings across military/civilian scenarios including wildfire smoke studies. Validation through acute physiological responses confirms this as a reliable, versatile platform for investigating health impacts of diverse combustion emissions.

PS 3516 Transcriptomic characterization of metabolic competency in reconstructed human respiratory epithelium (RHRE)

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Background and Purpose: Reconstructed human respiratory epithelium (RHRE) are differentiated *in vitro* cell cultures that replicate the *in situ* characteristics of the respiratory tract epithelium. Characterizing their metabolic competency is key to using these models to generate data for toxicological assessments of inhaled xenobiotics. Despite RHRE having been extensively studied, their metabolic competency has not yet been fully characterized. To address this, we analyzed the expression of genes relevant to xenobiotic metabolism across RHRE from different airway regions and compared them to human reference samples. **Methods:** RHRE of one to five donors for the nose, bronchi, small airways, and alveoli were purchased from Epithelix. Three tissues were used per donor. Tissues were cryopreserved at the provider, shipped and RNA was extracted for 50bp paired-end RNA sequencing (RNAseq). Sequencing data were processed, aligned to the human reference genome (GRCh38), transcripts were quantified, and read counts were normalized for library size. Bulk RHRE RNAseq data were compared with human single-cell RNAseq from the Human Lung Cell

Atlas (HLCA). Tracheobronchial and alveolar HLCA references were generated by using the pseudobulk approach, which involves aggregating raw counts of corresponding respiratory epithelial cells using and then normalizing for library size. Data analysis included Principal Component Analysis (PCA), hierarchical clustering, and linear regression on log₂-transformed data. For comparing relative gene expression, percentile (P) thresholds (25th, 50th, and 75th) were calculated separately for RHRE and HLCA datasets. Genes were classified as "Not detectable", "Very low" (<P25), "Low" (P25-50), "Moderate" (P50-P75), or "High" (>P75). All analyses were conducted in R (version 4.4.1) using the following packages: dplyr (1.1.4), ggplot2 (3.5.1), Matrix (1.7-0), pheatmap (1.0.12), Seurat (5.1.0), and viridis (0.6.5). **Results:** Tissue type explained most of the variance in the full RHRE expression profiles as observed by PCA (Principal Component (PC) 1=18.67%). Nasal, bronchial, and small airway tissues clustered closely, with nasal and bronchial samples having a larger variance than the small airway samples. While nasal, bronchial, and small airway samples showed substantial overlap, alveolar samples were more distinct, a pattern also confirmed by hierarchical clustering. This was also observed when the analysis focused on metabolic genes only, indicating that nasal and bronchial RHRE share a comparable metabolic profile, followed closely by small airway RHRE. Correlation with HLCA pseudobulk data showed a linear correlation trend for nasal, bronchial, and small airway RHRE (R²>0.6), but was lower for alveolar RHRE (R²=0.365). This is likely due to the cell composition of the alveolar RHRE, which includes endothelial cells not considered in the reference sample, and the limited data depth for alveolar epithelial cells in the HLCA dataset. Gene-level analysis showed nasal RHRE expression patterns aligned well with the human expression of a subset of lung-specific, CYP enzymes (e.g., CYP2A6 or CYP2A13). Nasal, bronchial, and small airway RHRE demonstrated alignment with the human reference sample for highly expressed, non-CYP, phase I enzymes (e.g., NQO1, CES, and aldo-keto reductases). **Conclusions:** In general, nasal, bronchial, and small airway RHRE showed similar expression profiles that largely aligned with the HLCA human reference sample. This work can assist in confirming whether a RHRE selected for xenobiotic testing based on the potential deposition site of a xenobiotic, expresses the human-relevant set of metabolism-associated genes for that region. The analysis pipeline presented in this work can also be used to characterize the metabolic gene expression for other RHRE and non-respiratory *in vitro* systems.

PS 3517 Comparative Histopathological Analyses of the Respiratory Tract in Preclinical Species Exposed to Dry Powder Vehicles in Inhalation Toxicology Studies

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Background and Purpose: Dry powder inhalation (DPI) formulations are increasingly used for local and systemic drug delivery due to enhanced stability and ease of use. Excipients such as lactose, leucine, mannitol, and trehalose are commonly employed carriers and are generally considered inert. However, repeated inhalation exposure in preclinical studies may induce local histopathological changes that can confound interpretation of test-item-related effects. To compare the incidence and severity of respiratory tract histopathological findings in control rats, dogs, and cynomolgus monkeys exposed to commonly used dry powder excipients during nonclinical inhalation toxicology studies. **Methods:** Retrospective analyses were conducted using data from studies performed at ITR Laboratories Canada between 2008 and 2024. Histopathological findings were extracted from Provantis® records and reviewed across the main respiratory tract regions (nasal cavity, larynx, trachea, carina, and lungs). Control groups included air and powder vehicle-exposed animals. **Results:** Across species, vehicle-exposed controls had a higher incidence of microscopic findings compared to air controls. In **rats** (n=1962), the overall incidence was 19% in vehicle controls versus 9% in air controls, and consisted mainly of respiratory epithelial atrophy (loss of cilia with flattening of epithelium) at the carina and eosinophilic globules in the olfactory epithelium of the nasal cavity. In **dogs** (n=473), 19% of tissues from vehicle controls versus 15% from air controls had findings consisting mainly of minimal alveolar macrophage increases and inflammatory infiltrates. In **monkeys** (n=74), the overall incidence was 27% in vehicle controls versus 10% in air controls and consisted mainly of minimal inflammatory infiltrates and mucous cell hyperplasia in lung airways. Across all species, most changes were minimal to mild in severity. **Conclusions:** Powder vehicle exposure was associated with a slight increase in minimal grade histopathological findings across species, with the rat demonstrating the greatest sensitivity. These results define species-specific background respiratory changes attributable to commonly used vehicles and provide reference data for interpretation of nonclinical inhalation toxicology studies.

PS 3518 Safe-by-Design Assessment of Aerosolized Hair-Care Products Using an *In Vitro* Inhalation Model

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Background and Purpose: Occupational exposure to aerosols from hair-care products may contribute to respiratory discomfort among hairdressers. To support product development under a Safe-by-Design strategy, we established an *in vitro* inhalation model enabling comparative assessment of biological effects from product-related aerosols under realistic application conditions. **Methods:** Aerosols were generated according by applying four commercial hair-care products (TM1-TM4) to human hair strands inside a custom-built plexiglass chamber, following manufacturer instructions. Aerosol exposure to air-liquid interface (ALI) cultures of A549 lung epithelial cells was performed using the P.R.I.T.® ExpoCube® system. To determine the surface-specific dose delivered to the cell cultures, aerosol characterization was performed, including measurements of particle size distribution and concentration. This enabled the establishment of dose-response relationships and facilitated comparison of biological effects across different products under standardized exposure conditions. Biological endpoints included cell viability (WST-1), mitochondrial membrane potential (JC-1), cell stress (Hoechst), and inflammatory response (IL-8). A composite Potential Effect Score (PES) was calculated from aerosol release (Exposure Factor, EF) and biological response metrics (Biological Activity Factors, BAF1 and BAF2). **Results:** Results showed high PES values for TM1 and TM3 (>30), correlating with reported respiratory symptoms in occupational settings. TM2 and TM4 yielded low PES values (<1), indicating minimal biological impact. Intensified application of TM2 (scenario TM2A) increased PES to 9, yet remained below TM1/TM3 levels, suggesting that product composition rather than aerosol quantity was the primary driver of biological effects. **Conclusions:** This approach demonstrates the utility of integrated aerosol and biological profiling for early-stage safety evaluation. By enabling product comparison under realistic exposure conditions, the method supports formulation decisions aligned with Safe-by-Design principles to minimize respiratory risks in professional use.

PS 3519 Utilizing Simultaneous Ultra-Sensitive Assessment of Diaphragmatic and Skeletal Muscle Force, Peripheral Train-of-Four Stimulation Predicts Diaphragmatic Recovery with Cisatracurium in a Non-Human Primate Model

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Background and Purpose: Diaphragmatic recovery is more resistant to neuromuscular blocking agents than peripheral skeletal muscles. This has been shown by simultaneous measurement of peripheral skeletal and diaphragmatic muscle via non-invasive techniques such as an accelerometer or diaphragm ultrasonography. To determine the relationship between diaphragmatic and peripheral nerve recovery with better precision, highly sensitive strain-gauges were used and connected to the diaphragm and thumb. **Methods:** Added on to a model development study, four anesthetized (isoflurane, ketamine, and fentanyl) and mechanically-ventilated male/female rhesus macaque non-human primates were acutely instrumented to simultaneously assess systemic/left ventricular hemodynamics (via catheterization) as well as skeletal (quadriceps), diaphragmatic, and peripheral limb (train-of-four, TOF) force (via strain gauge). Data were collected both before, during cisatracurium (3 µg/kg/min for 30 min IV for 20 mins), and after the infusion stopped. **Results:** Cisatracurium elicited a precipitous decline in skeletal, diaphragmatic (respiratory), and peripheral limb (TOF) force until complete paralysis was achieved during the 20-minute infusion. Function recovered over the next 40-60 minutes. Diaphragmatic force recovered the fastest, skeletal muscle recovered the slowest, and peripheral limb train-of-four in between. The max height of the first TOF twitch (T1 Amp) and TOF ratio (TOFr) of the 1st and 4th twitch (T4/ T1) tracked very similarly (r²=0.99). A 25% and 40% TOF recovery from full paralysis correlated with a 70% and 90% recovery in diaphragmatic force. From the diaphragm's perspective, it recovered 25% before TOF recovery was present. A 50%, 75%, and 90% diaphragmatic recovery correlated to a 10%, 30%, and 50% recovery of the peripheral TOF. **Conclusions:** Thus, the use of ultra-sensitive strain gauges provided a full profile of diaphragmatic and skeletal muscle force to better understand their relationships with a non-depolarizing neuromuscular blocker. These data support the concept that adequate recovery of peripheral neuromuscular function, commonly defined as a TOFr (T4/T1) ≥ 0.9, indicates a functioning diaphragm and can be considered safe to extubate. Moreover, this study provides supportive evidence that the ratio of T4/T1 sufficiently reflects peripheral muscle contraction and can be used to infer diaphragmatic recovery.

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Background and Purpose: Environmental toxicants, including ionizing radiation, induce persistent DNA damage and cellular stress that contribute to long-term tissue remodeling culminating in chronic lung disease. A key, yet underrecognized, mediator of this response is the cyclic GMP-AMP synthase (cGAS) pathway, a cytosolic DNA sensor that activates STING (Stimulator of Interferon Genes), triggering type I interferon signaling, senescence-associated secretory phenotype (SASP), and downstream lung remodeling. The role of the cGAS-STING axis in linking genotoxic injury to chronic inflammation and lung remodeling remains a critical gap in toxicological research. **Methods:** We investigated how the cytosolic DNA sensor cGAS activates the STING pathway in response to ionizing radiation (IR) using a human 3D airway epithelial-stromal model (MatTek tissues composed of epithelial cells and fibroblasts), with or without pharmacological STING inhibition (H-151). DNA damage was assessed via γ H2AX immunostaining, while activation of the cGAS-STING cascade and downstream interferon-stimulated genes (ISGs) was quantified using NanoString, qPCR, and immunoblotting. Senescence was measured by SA- β -gal staining, and SASP profiling was performed using ELISA and immunofluorescence. To validate *in vivo*, STING knockout (STING^{-/-}) mice were irradiated, and primary lung fibroblasts were isolated to assess senescence markers and mitochondrial respiration (Seahorse assay). **Results:** In 3D airway tissues, IR triggered persistent DNA damage, downregulated TREX1, and robustly activated cGAS-STING signaling, leading to elevated IFN- β , CXCL10, IL-6, CCL2, and senescence markers (GLB1, CDKN1A, CDKN2A, GDF15, SERPINE1). This was accompanied by epithelial barrier disruption, fibroblast-to-myofibroblast transition, and impaired fibrinolysis via PAI-1 upregulation. Additionally, IR induced fibrosis-like changes with increased TGF- β , COL1A1, ACTA2, and NF- κ B-driven SASP amplification. Notably, STING inhibition or knockout suppressed SASP release, restored TREX1 expression, dampened TBK1 and IRF3 activation, and reversed epithelial-stromal remodeling. **Conclusions:** Together, these findings show cGAS-STING as a key sensor of genotoxic stress that drives senescence, inflammation, and fibrotic reprogramming. Targeting this pathway may offer therapeutic opportunities in chronic lung injury, with downstream SASP and fibrinolytic mediators serving as candidate biomarkers of tissue remodeling and fibrosis risk.

PS 3521 Wood Smoke Particulate Attenuates Antiviral Cytokine Production Against Live Attenuated Influenza Virus in Differentiated Human Nasal Epithelial Cells

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Background and Purpose: Wildfires are occurring with increasing frequency, leading to greater human exposure to woodsmoke and exposure-associated adverse health effects. Epidemiological data suggest that wildfire smoke exposure is associated with worse respiratory infections, including influenza. Our human controlled exposure studies have shown that woodsmoke particles (WSP) can alter antiviral host defense responses in human volunteers inoculated with the live-attenuated influenza virus (LAIV) vaccine, which contains three circulating influenza strains and serves as a model for influenza infections. However, the impact of wood smoke on respiratory tract cell types that influence immune mediator profiles after LAIV has not been well-studied. Further, the order in which influenza and wood smoke exposures occur has not been evaluated. As wildfire season increasingly overlaps with influenza season and vaccination campaigns, this research could inform optimal vaccination timing relative to wildfire exposure and elucidate mechanisms by which wildfire smoke compromises the respiratory epithelium, a critical barrier against infection. **Methods:** We established an *in vitro* nasal epithelial cell WSP exposure infection model using the LAIV vaccine, which replicates at the temperature of the nasal passage and was previously used in our human controlled exposure studies. Primary human nasal epithelial cells (hNECs) from 6 adults (3 males, 3 females) were isolated and differentiated at air-liquid interface. Cells were exposed apically to 22 μ g/cm² of red oak WSP for 2 hours, followed by infection with 10⁶ 2024/2025 FluMist (intranasal LAIV) for 24 or 72 hours. In a reverse design, hNECs were first infected with LAIV for 24 hours before WSP exposure for 48 hours. WSP was created by burning red oak at a smoldering temperature. WSP were collected on Teflon filters throughout two-hour burn sessions utilized for our controlled exposures. WSP was extracted from filters via sonication in methanol and then solvent-exchanged into HBSS using a nitrogen dryer. WSP composition was analyzed by gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS). Barrier integrity was assessed by transepithelial electrical resistance (TEER). Viral replication was evaluated through gene expression analysis of H1N1 hemagglutinin (HA). IP-10 and IL-6 concentrations were measured by ELISA, and IFN α gene expression was evaluated by qPCR. **Results:** Chemical analysis confirmed the presence of polycyclic aromatic hydrocarbons, phenols, and other respiratory irritants in WSP. TEER measurements showed no significant differences between control, WSP-exposed, or LAIV-infected cells. Viral replication analysis showed an increase in HA counts after LAIV infection, indicating cellular infection and replication. IP-10 levels increased following LAIV infection, but this response was attenuated when cells were pre-exposed to WSP. IP-10 is a chemokine that plays a key

role in antiviral defense by recruiting activated T cells and natural killer cells to sites of infection and suppression may impair immune cell trafficking and reduce viral clearance. Infection with LAIV followed by WSP exposure did not significantly alter IP-10 compared to controls. IL-6 concentrations remained unchanged across all conditions. Gene expression analysis showed a significant decrease in IFN α expression when WSP exposure preceded LAIV infection. **Conclusions:** Woodsmoke exposure prior to LAIV infection dampens interferon-mediated antiviral responses in the hNECs, suggesting a potential for decreased effectiveness of intranasal vaccines after wood smoke exposure and potential increased infection rates. These findings suggest that wildfire smoke exposure may impair vaccine-induced immune responses and highlight the need to consider factors such as wildfire timing when planning vaccination strategies. These data also suggest that nasal epithelial cells are likely sources of impaired host defense response mechanisms. Ongoing work will expand our protein and gene expression targets to completely characterize the hNEC response to WSP and LAIV. Future studies are needed to elucidate the molecular pathways altered by woodsmoke and to identify chemical constituents mediating impaired antiviral signaling.

PS 3522 Inhalation route of administration imparts greater variability in systemic toxicokinetics dependent upon of molecular class

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Background and Purpose: Achieved doses for inhaled drug products are calculated mostly based on assumptions, and systemic exposure is one metric for converting the human equivalent dose. Similar to nonclinical inhalation (INH) delivery, intravenous (IV) doses can be administered over short (bolus) or long (infusion) durations. In contrast to IV administration where a drug directly enters circulation, inhaled drugs must pass through the respiratory tract barriers to enter the bloodstream and different molecular classes can permeate to different extents. The purpose of this research was to determine the contributions of route of administration and molecular class to relative variability in exposure. **Methods:** Toxicokinetic (TK) data were extracted from the pharmacokinetic parameters (PP) domain of Standard for Exchange of Nonclinical Data (SEND) database within the company. Results were limited to studies conducted from 2020 to 2025 using rat, mouse, dog, and non-human primate test systems. For studies using a serial TK model, data for up to 59 INH studies, 18 IV bolus, and 20 IV infusion studies were available for analysis. For studies using a composite TK model, data for up to 28 INH and 16 IV (bolus) studies were available for analysis; due to limited data, IV infusion data were not evaluated. TK exposure data analyzed included dose normalized AUC and Cmax values (PPTSTCD=AUCINTD and PPTSTCD=CMAXD within the PP domain, respectively). Relative variability was evaluated using coefficient of variance (%CV) for combined sexes following a single administration. For studies using a serial TK model, %CV was calculated for each study using data from all animals and dose levels. For studies using a composite TK model, %CV was calculated for each study using data from separate sexes at all dose levels, due to the possibility of differences in achieved doses. Exposure data for small and large molecule test articles were also compared. **Results:** For all studies using a serial TK model, %CV values for AUC ranged from 6 to 213% for INH, from 14 to 83% for IV bolus, and from 13 to 104% for IV infusion, and %CV values for Cmax ranged from 17 to 391% for INH, from 9 to 118% for IV bolus, and from 12 to 105% for IV infusion. Distribution of %CV values was skewed to higher values for both parameters for all routes. For small molecules, %CV values for AUC ranged from 24 to 115% for INH and from 30 to 33% for IV bolus, and %CV values for Cmax ranged from 17 to 267% for INH and from 24 to 111% for IV bolus; insufficient data were available for IV infusion. For large molecules, %CV values for AUC ranged from 89 to 151% for INH, from 19 to 29% for IV bolus, and from 20 to 104% for IV infusion, and %CV values for Cmax ranged from 19 to 151% for INH, from 18 to 47% for IV bolus, and from 18 to 105% for IV infusion. For all studies using a composite TK model, %CV values for AUC ranged from 10 to 196% for INH and from 7 to 61% for IV bolus, and %CV values for Cmax ranged from 15 to 211% for INH and from 8 to 45% for IV bolus. Distribution of %CV values was skewed slightly to higher values for IV and bi-modal for INH. For small molecules, %CV values for AUC ranged from 32 to 91% for INH and from 7 to 33% for IV bolus, and %CV values for Cmax ranged from 25 to 211% for INH and from 11 to 26% for IV bolus. For large molecules, %CV values for AUC ranged from 25 to 36% for INH and from 15 to 51% for IV bolus, and %CV values for Cmax ranged from 31 to 35% for INH and from 8 to 26% for IV bolus. **Conclusions:** For studies using a serial and composite TK model, relative variability was greater for the INH route of administration compared to either IV route of administration, where relative variability was generally similar for IV bolus and infusion for serial TK profiles. For the INH route, relative variability was lower for small molecules, although this was skewed by higher %CV values for composite TK profiles. Due to SEND scope, non-linear TK could not be considered, which limits the ability to interpret exposure variability across studies. However, these data demonstrate greater variability in systemic exposure of inhaled drugs, particularly for large molecules.

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Background and Purpose: Fetal development is a sensitive window of vulnerability to toxicant exposures, which can lead to lifelong respiratory health consequences. Electronic-cigarette (e-cig) devices are commonly used by young adults in the U.S., including pregnant women, as recent studies report that 2.2-7.0% of individuals use e-cigs during pregnancy. The mechanisms by which maternal vaping increases the risk of lung diseases in offspring are currently poorly understood. Our previous studies showed that mice exposed *in utero* to e-cig aerosols had upregulated expression of interleukin-10 (IL-10), a crucial immunoregulatory cytokine in lung homeostasis. This study aims to investigate both the impact of gestational e-cig exposure and the role of IL-10 on mouse offspring lung function. **Methods:** C57BL/6 wild type (WT) and IL-10 knockout (KO) dams (N=15-16 dams/group) were exposed to either air or honeywood tobacco-flavored e-cig aerosol (free base nicotine: 24 mg/mL). Total particulate matter (TPM) concentration of the e-cig aerosol was measured gravimetrically, and the stability of the aerosol was monitored in real-time using a Microdust Pro. Physical characterization of the e-cig aerosols included size distribution, evaluated using a scanning mobility particle sizer (SMPS), and number concentration measured with a condensation particle counter (CPC). Exposure consisted of 1.5-hour/day for 14 days prior to conception plus 20 days during gestation. Mouse offspring were sacrificed at 3 time points: at birth (N=11-14/group), at 14-16 days, and following phosphate-buffer saline (PBS) or house-dust mite (HDM) treatment to induce asthmatic responses at 7-8 weeks (N=4-6 mice/sex/group). Lung function testing was conducted in offspring utilizing the flexiVent. **Results:** Pregnant dams were exposed to e-cig aerosols at a TPM concentration of 0.2 ± 0.1 mg/puff. SMPS and CPC revealed that the e-cig aerosol particle geometric mean was 93.8 nm with a GSD of 1.7, and an average number concentration of 46,601 particles/cm³. At birth, WT mouse offspring exposed to e-cig aerosol *in utero* had a significantly reduced birth weight compared to air controls (1.28 ± 0.08 g and 1.38 ± 0.16 g, respectively). IL-10 deficiency improved birth weight with no difference observed between air and e-cig groups (1.33 ± 0.07 g and 1.36 ± 0.07 g, respectively). At 14-16 days of age, *in utero* e-cig exposure significantly ($p < 0.05$) affected the lung pressure-volume (PV) loops, measured via the area under the curve, in WT offspring, reflecting a restrictive pulmonary phenotype, primarily driven by the female offspring. IL-10 deficiency protected from this impairment returning PV loop curvature to air levels in male offspring. Also, in WT mice the *in utero* e-cig exposure significantly increased the respiratory system resistance (Rrs) and lung tissue damping compared to the air group. Removal of IL-10 in the e-cig group kept these parameters to the levels of the air-exposed offspring ($p < 0.05$). IL-10 deficiency was significantly beneficial for female offspring exposed to e-cig *in utero* ($p < 0.05$), highlighting sex-specific effects. Lung structure analysis at birth and at 14-16 days were supportive of underdeveloped lungs in both WT and IL-10 KO offspring exposed to e-cig *in utero*, correlating with the decreased lung function observed in these offspring. Exposure to HDM is commonly used to induce eosinophilic asthma. Following PBS and HDM treatment in adult WT offspring, we found that the impaired lung function parameters, including restrictive PV loop phenotypes and increased Rrs, carried over from the 14-16-day-old time point; however, HDM treated groups displayed exacerbations of those affected parameters. These lung function impairments were absent in IL-10 KO females, but not in IL-10 KO males. **Conclusions:** *In utero* e-cig exposure has significant effects on lung function in early-life in WT offspring, with lungs exhibiting reduced ability to stretch and expand, whereas IL-10 deficiency shielded from these impairments. The protective effect of IL-10 deficiency at birth and at 14-16 days of age remained in adulthood primarily with female offspring, whereas IL-10 KO male offspring were affected by the *in utero* e-cig exposure similarly to WT offspring.

PS 3524 Exceptional Emitted-to-Delivered Aerosol Efficiency of theBuxcoPre-Clinical Inhalation System: Bridging Theory and Experiment

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Background and Purpose: One of the primary obstacles in preclinical inhalation toxicology is the substantial quantity of test article required to achieve target lung doses across multiple subjects, driven predominantly by extensive aerosol losses within conventional exposure systems. These losses inflate research costs, complicate delivery of respirable aerosols under physiologically relevant conditions, and frequently deter investigators from selecting the inhalation route despite its scientific superiority for pulmonary therapeutics. The Buxco Inhalation System overcomes this limitation through a novel low-dead-space, precisely regulated whole-body exposure design that achieves >70% aerosol delivery efficiency. To substantiate this claim, we conducted a rigorous, empirically driven validation study. **Methods:** A 4-tier, 24-port Buxco system was configured at a total flow of 8 L/min and -0.5 cm H₂O negative pressure. Real-time aerosol monitoring included temperature/humidity sensors, a digital photometer, gravimetric filters, and cascade impaction for particle-size analysis. Test aerosol was generated using an Aerogen vibrating-mesh nebulizer (0.384 mL/min output) delivering 5 mL of a 9 mg/mL solution. The delivered dose at individual exposure ports was quantified gravimetrically using a high-sensitivity analytical

balance and compared with theoretical predictions. **Results:** Across eight replicate experiments, measured delivery efficiencies ranged from 71% to 76% (mean 72.8%, SD 1.9%), more than doubling the industry-standard 30% typically achieved with conventional systems. These results remained consistent across diverse exposure environments with relative humidity varying from 30% to 80%, induced by differing aerosol outputs and conditioning methods. Of particular relevance to inhalation toxicology, incorporating a diffuser dryer to mitigate high humidity altered the collected delivery dose by no more than 2% from the mean, demonstrating minimal additional material loss alongside substantial improvements in the inhalation environment. **Conclusions:** These findings confirm that the Buxco Inhalation System reduces test-article consumption, lowers study costs, and ensures reproducible, respirable aerosol delivery.

PS 3525 From Targets to Toxicity: Interpretable Machine Learning for Organ System Risk Prediction

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Background and Purpose: Target-related toxicities remain a major cause of late-stage drug attrition and patient risk, yet systematic approaches for assessing these risks are limited. Current evaluations often rely on manual expert review or heterogeneous datasets whose relevance varies across organ systems. Genetic features such as protein interaction networks, loss-of-function tolerance, and genetic constraint (Razuvayevskaya et al., 2024) were found to correlate with clinical trial discontinuations, but represent only a part of the picture. Moreover, the relevance of specific factors is expected to vary across organ systems. Here, a target-centric ML (Machine Learning) framework is developed to predict toxicity risks across multiple organ systems and identify the most informative data features per system. **Methods:** By converting compound-based clinical data into a target-level representation, a training dataset for target toxicity across 12 organ systems was created, based on organ-level adverse events. Each target was labeled with a safety score per organ system based on the frequency and occurrence of adverse events (AEs), adjusted for placebo groups where available, and combined with an internal AE severity score. A series of interpretable ML models were trained individually per organ system, using input features spanning genetic, experimental, animal, and clinical data. **Results:** Some organ system toxicities can be validated externally. For example, DiCTRank (Qu et al, 2023) permitted evaluation of cardiovascular risk with an area under the curve (AUC) of 0.75, where AUC is a standard metric indicating how well a model distinguishes between high- and low-risk targets. SHAP-based feature importance analysis, which quantifies how much each input factor contributes to a model's prediction, revealed the relative influence of genetic, experimental, animal, and clinical data for each organ system. For instance, SHAP highlighted genetic evidence as a key contributor to predicted risks of pulmonary hypertension, consistent with clinical observations of myocardial damage captured by DiCTRank. Further insights indicate which data sources are most informative for evaluating target-specific safety across organ systems. **Conclusions:** This work demonstrates the feasibility of learning organ-specific safety signals from integrated, multimodal data. The resulting dataset and models provide a framework for understanding how diverse biological and clinical data contribute to target toxicity, supporting more systematic and interpretable investigations into the mechanisms underlying target-related safety risk.

PS 3526 Enhancing Drug Safety: Secondary Pharmacology Screening Interpretation through AI-based scientific literature mining

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Background and Purpose: Secondary pharmacology screening data plays a critical role in new drug applications by characterizing the selectivity profiles of clinical candidates and identifying off-target interactions. Interpreting target-related safety liabilities in the context of safety margins is essential for early risk assessment and successful clinical progression. Although public and commercial databases provide valuable information on target-associated safety risks, there remains a need for systems that can comprehensively mine, consolidate, and contextualize this data. Such systems can support safety teams and regulatory stakeholders by enabling more robust interpretation of secondary pharmacology data, ultimately guiding strategic decisions in the drug development process. **Methods:** We developed a dedicated AI system, *In Vitro Safety Insight*, to visualize biochemical assay results and potential safety liabilities associated with targets in secondary pharmacology screening panels. Adverse event data linked to these targets were sourced from both public and proprietary databases, as well as a novel in-house literature mining platform. Our analysis focused on 77 targets recommended by the IQ-DruSafe consortium (Brennan et al., 2024). The mining system retrieves abstracts and available full-text articles by querying multiple target name variants. Using state-of-the-art machine learning models, we segmented texts into sentences and identified mentions of chemicals and adverse events. Sentences containing (1) a drug and a target, (2) a drug and an adverse event, or (3) a target and an adverse event were further evaluated using a large language model (LLM) to confirm associations and classify the nature of the relationships. Following data aggregation,

we generated a curated list of target-adverse event associations, each supported by literature references. Adverse events were mapped to organ systems and integrated with safety screening results into a scoring framework termed the *Organ System Risk Profile* (OSRP). OSRP highlights organ-level risks based on the functional activity and potency of the tested compound at each target, relative to a predefined safety margin. **Results:** We present Organ System Risk Profiles (OSRP) for an antidepressant compound tested at multiple concentrations using the SafetyScreen™77 Functional Panel. We demonstrate how the safety margin influences the final OSRP by determining which targets are considered relevant for organ specific risks. A more nuanced interpretation is presented by resolving modulated targets according to agonist or antagonist compound activity, enabling a deeper understanding of safety liabilities in the context of pharmacological mechanisms. **Conclusions:** We introduce an AI-driven system designed to mine and prioritize potential adverse events associated with targets in secondary pharmacology screening panels. By leveraging the entirety of public literature, this system enhances the interpretability of secondary pharmacology data. Its application holds promise for improving the predictive power of *in vitro* safety assessments and ultimately reducing reliance on animal models in early-stage drug safety evaluations.

PS 3527 Comprehensive Evaluation of Clinically Discontinued Compounds Using an *In Vitro* Secondary Pharmacology Panel to Predict Safety Liabilities

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Background and Purpose: Clinical attrition of drug candidates is frequently driven by unforeseen safety liabilities, which are often associated with off-target pharmacological interactions. Systematic assessment of these interactions using *in vitro* pharmacological profiling in the preclinical stage may improve early detection of potential adverse effects and inform safer compound design. This study investigates the utility of a standardized *in vitro* secondary pharmacology panel to identify off-target interactions associated with clinical safety concerns in compounds that were previously discontinued during drug development. **Methods:** A set of 52 compounds previously terminated due to clinical safety issues was evaluated using the SAFETYscan®47 panel. This panel contains a wide array of molecular targets with established roles in safety pharmacology, including GPCRs, ion channels, nuclear receptors, transporters, and enzymes. Functional assays were performed to determine activity profiles (EC₅₀/IC₅₀), and selected compounds were further assessed in phenotypic follow-up assays to investigate their effects on integrated physiological systems. Off-target liabilities were analyzed by correlating *in vitro* activity with documented clinical safety outcomes. **Results:** The panel enabled the detection of diverse off-target interactions of the tested compounds across multiple receptor, enzyme, and ion channel families, providing a comprehensive pharmacological profile for each compound. The data offered a framework to connect *in vitro* activity with clinical safety outcomes, facilitating interpretation of how specific molecular interactions may contribute to observed adverse effects. Several compounds demonstrated activity at targets mechanistically linked to their documented clinical liabilities, such as hERG channel interactions correlating with cardiac liabilities, supporting the predictive value of the panel for safety assessment. **Conclusions:** Integration of *in vitro* secondary pharmacology profiling can identify off-target interactions predictive of clinical safety risks. Employing such panels in early drug development may enhance the identification of high-risk candidates, thereby reducing clinical attrition rates and informing safer chemical design.

PS 3528 InVEST44™ is a cost-effective *in vitro* safety panel that identifies off-target effects in early-stage drug discovery

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Background and Purpose: Drug development costs across all therapeutic areas exceed \$172m and these costs increase to over half a billion dollars when clinical trial failures are taken into account (Sertkaya et al., 2024, DOI: 10.1001/jamanetworkopen.2024.15445). It can take as long as 20 years to successfully bring a drug to market so it is critical to identify any safety liabilities as early in the drug discovery process as possible to ensure valuable time and money are not invested unnecessarily in time-consuming and expensive preclinical and clinical trials. *In vitro* safety panels, such as InVEST44™, provide an opportunity to identify potential toxicities of lead compounds in a cost-effective manner and at an early stage in drug development. This is particularly relevant as, from 2006 onwards, the proportion of venture capital investments in early stage (including *in vitro* safety panels) has decreased from 50% (2006) to 16% (2023) while increasing for later stage (including clinical trials) from 20 to 54% over the same time (BIO. (2015). The Biopharma Industry Investment Report 2015. Biotechnology Innovation Organization; Larka, M. (2024). Trends in Venture Capital Funding for Biopharma Startups. GlobalData). This indicates an understandable trend towards expensive, high-risk but potentially lucrative,

clinical trials and away from early drug discovery. The goal is to demonstrate that early-stage *in vitro* safety panels are critical steps in the drug development process both fiscally and scientifically as these identify off-target effects of drug candidates before advancing to expensive and time-consuming animal testing and clinical trials. **Methods:** InVEST44™ is an *in vitro* safety panel designed to identify off-target effects of lead compounds in early-stage drug development, ensuring a more efficient path from discovery to clinical trials. It comprises 44 industry standard, high risk targets based on work by Bowes and colleagues (Bowes et al., 2012, DOI: 10.1038/nrd3845). It incorporates several target types, including GPCRs, transporters, ion channels, nuclear receptors and enzymes, which are crucial components in both core systems (central nervous, cardiovascular, respiratory) and secondary organs (gastrointestinal, renal). Various assay types are utilized, including radioligand binding, fluorescence polarization, enzymatic activity, cell reporter, calcium mobilization and electrophysiology assays. **Results:** We identified 20 drug candidates from a selection of failed clinical trials between 2001 and 2025 (clinicaltrials.org) applying the criteria that the trials were terminated because of reported severe adverse effects. These compounds were screened in the InVEST44™ panel. Testing at a concentration of 10 µM, 17 of the 20 drugs elicited >50% effect on at least 1 target in the InVEST44™ panel, including 8 with 4+ target interactions. One drug candidate interacted significantly with 15 safety targets. **Conclusions:** This study demonstrates the importance of *in vitro* safety panels, such as InVEST44™, by identifying off-targets effect of lead compounds before embarking on time-consuming and expensive animal testing and potentially failed clinical trials. In addition, data from such panels are increasingly being provided to enhance IND applications to the US FDA (Scott et al., 2022, DOI: 10.1016/j.vascn.2022.107205). In summary, by providing cost-efficient, early-stage identification of off-target effects by lead compounds, *in vitro* safety panels, such as InVEST44™, have become integral components of drug discovery and warrant increased early-stage consideration and investment in the future.

PS 3529 A Reproducible, Label-Free Platform for Real-Time Cytotoxicity and Hepatotoxicity Screening

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Background and Purpose: Accurate and reproducible assessment of cell toxicity is essential for high-throughput screening in drug discovery and toxicology. The TrayZ system, a real-time, label-free impedance-based assay platform, enables continuous monitoring of cell health and cytotoxicity without the need for endpoint dyes or destructive labeling. This study demonstrates the use of the TrayZ system to measure cytotoxicity across multiple cell types, evaluate assay repeatability, and assess hepatocyte-specific toxicity, a key concern in safety pharmacology. **Methods:** Cancer cell lines (A549 and SKOV3) were seeded into TrayZ 96-well microplates with embedded electrodes for impedance-based detection of cell attachment, proliferation, and cytolysis. Cytotoxic compounds (paclitaxel, doxorubicin) and HER2-specific CAR T cells were used to induce cell death. The kinetics of cytolysis were quantified in real time, and key parameters such as EC₅₀ and KT₅₀ (time to 50% cytolysis) were calculated. Four independent assay plates were run to assess inter-plate reproducibility. To demonstrate hepatotoxicity screening, HepaRG cells were cultured to confluence for 1 week and exposed to known cytotoxic compounds (tamoxifen, nocodazole, FPL 64176) at multiple concentrations for 48 hours. Cytolysis of the HepaRG cells was calculated by comparing treated wells to no treatment control wells. **Results:** The EC50 for Paclitaxel (13.2, 13.9, 14.5, and 13.9 nM) and doxorubicin (1.02, 0.99, 1.01, and 1.09 µM) was consistent across plates with a coefficient of variation of 3.9% and 4.2%, respectively. In a separate assay, SKOV3 cells were treated with HER2-specific CAR T cells across four replicate plates at three effector-to-target cell ratios (1:5, 1:2, 2:1). A two-way ANOVA analysis was performed and found the greatest source of variation to be different treatment groups as opposed to different plates at 96 hours. Of note, the variation between plates was less than the error (or unexplained variation) in the analysis. At the highest dose (E:T = 2:1), the KT50 was 10.4 hours, with a coefficient of variation of 9.8%. HepaRG cells exhibited dose-dependent cytotoxicity in response to all compounds tested. Tamoxifen at 100 µM induced near-complete cytolysis by 48 hours, while nocodazole and FPL 64176 reached ~85% cytolysis at their highest concentrations (50 and 100 µM, respectively). **Conclusions:** The TrayZ system provides a robust, high-throughput, and label-free platform for real-time cytotoxicity assessment. Its reproducibility across multiple plates and compatibility with diverse cell types, including hepatocytes, supports its use for both compound screening and mechanistic toxicology studies. These findings highlight TrayZ as a powerful tool for evaluating hepatocyte toxicity and other cell-based toxicological endpoints in drug development workflows.

PS 3530 Off-Target Platelet Sensitisation by Therapeutic Oligonucleotides: Validation of a High-Throughput Screening Approach

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Background and Purpose: There is evidence indicating that synthetic oligonucleotides (ONs), including CpG-containing immunostimulatory sequences, can exert unintended effects on platelets. Several phosphorothioate-modified ONs have been reported to promote platelet activation, aggregation, or clearance through off-target interactions with platelet receptors, thereby posing a potential thrombotic risk. Inotersen represents a clinically relevant example where platelet liability has been implicated. Similarly, ODN2395 and ISIS120704, CpG-rich immunostimulatory ONs, have been shown to induce platelet activation. We previously demonstrated that the potency of collagen-related peptide (CRP) in washed platelet aggregation assays increases in the presence of ODN2395 and inotersen. However, the effect can become saturated, requiring the use of multiple concentrations to generate reliable potency data, limiting the assay throughput. Our aim was to establish and validate a high-throughput assay to quantify off-target platelet stimulation by ODN2395, ODN2395PO, inotersen, inotersen Neg, and ISIS120704 by using a single subthreshold concentration of CRP with increasing ONs concentrations and employing pooled donor platelets to increase throughput. **Methods:** Washed human platelets were preincubated with test ONs prior to stimulation with CRP. Aggregation was measured in a 96-well plate by light absorbance. Full CRP concentration-response curves were generated to quantify EC₅₀ shifts in the presence of 3 concentrations of ONs (up to 8 compounds can be assessed per donor sample); and compared with single-point assay using subthreshold CRP concentration with a 7-point concentration-response curve of ON (up to 40 compounds per donor sample). ODN2395 (phosphorothioate CpG ODN), ODN2395PO (phosphodiester bonds), inotersen, inotersen Neg (phosphodiester bonds), and ISIS120704 were tested. Responses from individual donors were compared with those from two-donor pooled platelet samples. **Results:** Preincubation with ODN2395 and inotersen produced a consistent leftward shift in the CRP concentration-response curve, indicating platelet stimulation and enhanced sensitivity to CRP. However, the effect observed with ODN2395 reached a saturable plateau, suggesting the need to use lower concentrations. The use of a single subthreshold CRP concentration with increasing ON concentrations, allowed for the quantification of the EC₅₀ for responses to ODN2395, inotersen and ISIS120704. In contrast, ODN2395PO and inotersen Neg had no detectable effect on CRP-induced aggregation. The results were consistent between pooled and unpooled platelet samples. **Conclusions:** Using 96-well plate-based platelet aggregation we demonstrated the off-target prothrombotic potential of ODN2395, inotersen and ISIS120704 as they enhanced platelet responsiveness to CRP, whereas ODN2395PO and inotersen Neg showed no such effect. The agreement between full CRP concentration-response data and simplified single-point assay validates the latter method as a scalable, high-throughput platform for assessing ON-induced platelet aggregation. Utilising this approach in two-donor pooled platelet samples increases the assay throughput from 8 to 80 compounds and enables integration of platelet safety screening within iterative design make test workflows to identify and mitigate thrombotic risks and support safer and more efficient development of therapeutic oligonucleotides.

PS 3531 Development and Validation of High-Throughput *In Vitro* Assays for Thrombolysis and Thrombolysis Modulation Using Human Whole Blood Clots

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Background and Purpose: Reliable *in vitro* models are essential for evaluating thrombolytic efficacy and potential drug interactions in the context of thromboembolic diseases. This study aimed to develop and validate high-throughput thrombolysis and thrombolysis modulation assays using human whole blood clots formed in 96-well plates. **Methods:** Thrombin-induced halo clots were treated with known thrombolytic agents (t-PA, Tenecteplase [TNK], Urokinase, Streptokinase) and inhibitors (Aminocaproic Acid, Tranexamic Acid, Aprotinin). Thrombolysis was quantified spectrophotometrically over 60 minutes. EC₅₀ and IC₅₀ values were calculated using non-linear regression. Assay reproducibility was assessed across multiple donors and time points. **Results:** All thrombolytic agents demonstrated dose-dependent clot lysis, with EC₅₀ values of 1.6 nM (t-PA), 0.55 nM (TNK), 126 U/mL (Urokinase), and 0.88 U/mL (Streptokinase). Inhibitors showed concentration-dependent inhibition of thrombolysis, with IC₅₀ values of 43.7 μM (Aminocaproic Acid), 3.0 μM (Tranexamic Acid), and 1.45 μM (Aprotinin). Flumazenil, used as a negative control, showed no thrombolytic or inhibitory activity. The assays demonstrated low inter-donor and inter-day variability. **Conclusions:** These validated assays provide a robust platform for screening thrombolytic agents and evaluating potential drug interactions. Their reproducibility and sensitivity support their use in preclinical safety and efficacy assessments relevant to toxicology and drug development.

PS 3532 Low-Input RNA Sequencing of Liver Microtissues to Study Adverse Drug Effects Across Species and Structural Analogs

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Background and Purpose: Drug-induced liver injury remains a critical obstacle in drug development, often appearing late in clinical trials. Moreover, hepatotoxic effects detected in nonclinical species frequently fail to predict human outcomes, complicating regulatory evaluation and risk assessment. Recent advances in comprehensive quantitative analytical methods, together with physiologically relevant and scalable *in vitro* models, provide a valuable opportunity to better understand adverse drug effects before first-in-human studies. **Methods:** Low-input bulk RNA sequencing was performed on a single liver microtissue derived from multiple species and exposed to three well-characterized drugs with differing toxicity profiles across species or structural analogues, to evaluate the ability of this approach to identify adverse liver effects. Liver microtissues are miniaturized physiologically relevant spheroid *in vitro* models composed of primary parenchymal and non-parenchymal liver cells. Human, monkey, dog or rat liver microtissues were treated for seven days with troglitazone, rosiglitazone, or fialuridine, and the corresponding effect on gene expression measured by low-input bulk-RNA transcriptomic analysis. **Results:** Troglitazone and rosiglitazone are both hepatotoxic thiazolidinediones used to treat type 2 diabetes, though they exhibit different clinical patterns of liver injury. Troglitazone was withdrawn from the market due to fatal hepatotoxicity, whereas rosiglitazone remains in use, with liver injury typically resolving upon discontinuation of the drug. Both drugs elicited peroxisome proliferator-activated receptor gamma-related transcriptional responses consistent with their pharmacological activity in human liver microtissues, yet only troglitazone triggered additional pathways associated with rug-induced liver injury such as bile acid homeostasis, oxidative stress response, and apoptosis. Fialuridine, a nucleoside analog discontinued in clinical trials due to fatal liver injury, demonstrated significantly higher cytotoxicity in human liver microtissues compared to those from animal species, consistent with *in vivo* findings. Transcriptomic profiling revealed a human-specific gene expression signature characterized by mitochondrial dysfunction, DNA damage response, altered bile acid metabolism, and impaired detoxification pathways, in line with reported pathological observations. **Conclusions:** Overall, these findings suggest that transcriptomic sequencing of liver microtissues can enhance the understanding of adverse liver effects across structural analogues and between different species. In the context of drug development, this approach can support lead compound selection, aid in interpreting discordant animal study results, and help assess whether findings in animal models are likely to translate to humans.

PS 3533 Human Intestinal Organoids and Derived Barrier Models for High-Efficiency Assessment of Drug-Induced Gastrointestinal Toxicity

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Background and Purpose: Drug-induced gastrointestinal (GI) toxicity is one of the most frequent adverse events in Phase I clinical trials, often manifesting as diarrhea, dehydration, and ulceration mainly due to damage to the intestinal epithelium, functional cells (e.g., goblet, enteroendocrine cells), and finally to the barrier integrity. This toxicity frequently leads to dose-limiting complications for chemotherapeutic and targeted agents, compromising clinical efficacy and contributing to clinical trial attrition. There is a critical need for highly physiologically relevant *in vitro* intestinal models to improve preclinical safety assessment. Compared to traditional 2D cell lines, human intestinal organoids (HIOs), which recapitulate the *in vivo* intestinal architecture, self-renewal capability, and cellular function, represent a promising model. This study demonstrates the unique advantages of HIOs and HIO-derived barrier models in predictive drug safety evaluation. **Methods:** HIOs were established from patient-derived small intestinal crypts embedded in Matrigel. An HIO-derived intestinal barrier model was further developed. Model quality was validated via immunofluorescence (IF) and single-cell RNA sequencing (scRNAseq). The predictive sensitivity of HIO and HIO-derived barrier models was systematically compared to traditional Caco-2 models using clinical reported GI-toxic drugs (e.g., 5-FU, Trametinib). We developed a comprehensive evaluation system assessing: 1) 3D cell viability (CellTiter-Glo® 3D); 2) histopathology (H&E), key cellular components (PAS/MUC2 IHC for goblet cells), and tight junctions (ZO-1 IHC); and 3) barrier integrity detection in both 24/96-Transwell systems (Trans epithelial electric resistance (TEER) reading and permeability coefficient determination). **Results:** IF/IHC staining and scRNA-seq confirmed that HIOs and derived barrier models recapitulated the crypt-villus architecture and contained all major intestinal epithelial cell lineages (stem cells, enterocytes, goblet cells, and enteroendocrine cells), whereas Caco-2 models remained monotypic. **3D HIO Toxicity:** HIOs exhibited significantly greater sensitivity to GI-toxic drugs in viability assays versus Caco-2. Pathological staining revealed that 5-FU and Trametinib impaired bud formation, suggesting disruption of stem/progenitor cell renewal. Furthermore, 5-FU depleted goblet cells, while Trametinib induced their aberrant hyperplasia, mirroring clinical GI adverse effects. **HIO-derived Barrier Model Toxicity:** The HIO-derived barrier model was more sensitive to drug-induced barrier dysfunction than the Caco-2 model at clinically relevant concentrations. Drug treatment resulted in a pronounced, dose-dependent barrier disruption, evidenced by significantly decreased TEER values and increased Lucifer-

Yellow permeability. **Conclusions:** We established a highly biomimetic HIO-based platform and multifaceted detection systems, creating a comprehensive *in vitro* framework for GI toxicity assessment. Our results validate that HIO models robustly predict drug-induced GI toxicity and elucidate underlying mechanisms (e.g., impaired stem cell function, goblet cell dysfunction) that are poorly captured by traditional models. Future integration of immune cells and microfluidic systems will further enhance physiological relevance. This platform provides a reliable and predictive tool for de-risking GI toxicity in drug development.

PS 3534 Investigating the Toxicity of Novel Cannabinoids from a Native New Zealand Liverwort

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Background and Purpose: Regulatory, accessibility and safety concerns have limited the uptake of cannabis-derived medicines such as Sativex and Epidiolex in New Zealand as well as globally, despite approval as add-on treatments for multiple indications. Recently, the cannabinoid-like compounds (-)-*cis*-perrottetene (PET) and (+)-*trans*-perrottetene diol (PTD) were discovered in a liverwort native to New Zealand termed Wairuakohu (*Radula Marginata*), prompting research lead by indigenous Māori groups to explore the therapeutic potential and safety of these compounds. This study investigated the hepatotoxicity of PET and PTD and hypothesized that the Wairuakohu-derived cannabinoids would be less cytotoxic to liver cells than those derived from cannabis. **Methods:** Human HepG2 cells (2×10⁴ cells/well) were treated with cannabis-derived (Δ-9-tetrahydrocannabinol (THC), cannabidiol (CBD)) or Wairuakohu-derived (PET, PTD) cannabinoids for 72 hours. Cell viability across a range of concentrations was determined using a Sulforhodamine B protein assay. Non-linear regression was used to estimate best-fit values from three independent replicates performed in triplicate. **Results:** At a comparable concentrations*, PET and PTD reduced cell viability by 10.8% and 16.9%, respectively, compared with 45% and 93% by THC and CBD. THC and CBD were cytotoxic with IC₅₀ values of 30.2 μM and 19.7 μM, respectively. *Due to commercial sensitivity, the concentrations of PET and PTD tested cannot be reported. **Conclusions:** These early findings indicate PET and PTD may be less cytotoxic to liver cells than cannabis-derived cannabinoids *in vitro*. Due to the limited solubility of PET and PTD, IC₅₀ values could not be generated. Future experiments will utilize primary hepatocytes and a liver-on-a-chip approach to generate translatable data and inform the discovery process for PET and PTD. Furthermore, a comparison of plant-derived, and synthetic PET and PTD will aid in the understanding of any hepatotoxicity.

PS 3535 Beyond osimertinib: SGR-5573, a next generation brain-penetrant EGFR inhibitor, overcomes resistance with a favorable safety profile

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Background and Purpose: Epidermal growth factor receptor (EGFR) gene mutations are found in ~15-40% of non-small cell lung cancers (NSCLC) and several generations of EGFR inhibitors have been developed. A key to clinical safety is selectivity for mutant EGFR over wild-type (WT), as WT-EGFR inhibition is associated with adverse events including skin rash and gastrointestinal tract toxicity. Osimertinib is a third generation, irreversible tyrosine kinase inhibitor (TKI) used as a first-line treatment of NSCLC with EGFR classical mutations. Osimertinib is selective for EGFR classical mutations, but has an active metabolite with high WT-EGFR potency that may contribute to its safety profile. Post-osimertinib treatment, approximately 8-15% of patients develop EGFR on-target mutation C797S, which blocks binding of osimertinib. **Methods:** Leveraging Schrödinger's computational platform including *in silico* fragment screening, physics-based methods such as Free Energy Perturbation (FEP+) and energy of solvation (E-sol), we identified SGR-5573, a highly potent, selective, and brain-penetrant EGFR inhibitor with nanomolar potency on EGFR Del19 and EGFR L858R, as well as the osimertinib-resistant mutants EGFR Del19/C797S and EGFR L858R/C797S. **Results:** SGR-5573 has WT-EGFR selectivity *in vitro* comparable to osimertinib using various cellular models of WT-EGFR activity. Further, it lacks the potent *in vivo* WT-EGFR activity conveyed by the active metabolite of osimertinib. We conducted non-GLP repeat dose exploratory studies with SGR-5573 and established a favorable safety profile with double digit margins in rats and dogs. Adverse findings present at high efficacious margins were consistent with WT-EGFR inhibition. **Conclusions:** The margins, efficacy, and ADME properties of SGR-5573 support its development as a next-generation EGFR inhibitor for NSCLC.

PS 3536 Safety Profile Assessment for IND-enabling Studies: Bispecific Antibodies and Antibody-Drug Conjugates (ADCs)

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Background and Purpose: The development of antibody-based therapeutics, including monoclonal antibodies, bispecifics, and antibody-drug conjugates (ADCs), requires a delicate balance between potency and safety. Off-target interactions represent a significant risk, potentially leading to adverse clinical events and late-stage attrition. **Methods:** To address this challenge, we present a systematic de-risking roadmap designed to identify and mitigate off-target liabilities early in the development process, prior to IND-enabling studies. This integrated platform combines two complementary approaches: (1) Retrogenix® Cell Microarray Technology for comprehensive off-target interaction mapping, and (2) *in vitro* safety profiling using human primary tissue cells to evaluate functional liabilities in physiologically relevant systems. **Results:** The Retrogenix® platform enables high-throughput screening against thousands of human membrane and secreted proteins, providing an unbiased assessment of unintended binding events. This early detection capability reduces development risk by informing candidate selection and guiding engineering strategies to improve specificity. In parallel, *in vitro* safety profiling in healthy human cells offers critical insights into potential cytotoxicity or functional perturbations, ensuring precise target engagement and minimizing safety concerns. Together, these approaches deliver a robust framework for evaluating both on-target and off-target effects. **Conclusions:** The case studies demonstrate the value of this integrated strategy for complex modalities such as bispecific antibodies and ADCs, where structural complexity increases the likelihood of off-target interactions. By combining molecular-level screening with cell-based functional assays, the platform developed by Charles River Laboratories accelerates candidate refinement and enhances confidence in progressing to IND and clinical phases. Ultimately, this systematic de-risking approach supports safer, more effective antibody therapeutics, reducing attrition and improving the probability of clinical success.

PS 3537 Accelerating Immunotherapy Development with Bioinformatics-Driven Target Safety Evaluation

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Background and Purpose: Immunotherapies are emerging as an effective cancer treatment, using the body's immune system to target tumors. Despite the effectiveness of immune checkpoint inhibitors, not all patients experience the same level of benefit, and organ-specific immune-related adverse events (irAEs) may occur that can halt trials early. The aim of this study was to predict potential toxicities of immune checkpoint drug targets before they enter the clinical trials using preexisting data. **Methods:** For this purpose, we have generated a comprehensive database and software, including internally curated and various publicly available data on proteins, biologically-active chemicals, their interactions, pathways and pathologies. We catalogued over 30 million references, supporting each database entry. Molecular interactions are hyperlinked to appropriate PubMed articles as support (over 300 million evidence links), while proteins and chemicals are hyperlinked to EntrezProtein and PubChem, respectively. In order to support data integration and capture different levels of histopathological observations, we developed toxicology ontology with over 9000 diseases and 800 pathology endpoints. We validated the system by exploring PD-1 (PDCD1, programmed cell death 1) a known immune-checkpoint drug candidate for cancer treatment. **Results:** Mapping PD-1 onto Gene Ontology (GO) cluster using Analysis function within the system, identified PD-1 biological function in regulation of tolerance induction, T cell apoptotic process, and T cell mediated immune response to tumor. Mapping PD-1 together with its **very high confidence** protein interaction network onto Diseases module within our system, associated PD-1 with several cancers, including endometrial carcinoma, clear cell renal cell carcinoma, head and neck squamous cell carcinoma, lung squamous cell carcinoma, and mucosal melanoma in which is PD-1 dysregulated, which corresponds to potential indication of PD-1 inhibitors. Indeed literature search confirms PD-1 inhibitors are effective in many of these cancers. The other group of diseases that is prominent are immune diseases including autoimmune myocarditis, autoimmune uveitis, allergic asthma and autoimmune glomerulonephritis, which correspond to potential PD-1 inhibitors' toxicity, i.e. adverse events. This is confirmed by mapping PD-1 with its protein interacting network onto adverse events cluster that reveals association with several pathologies mostly related to immune system (myocarditis, pneumonitis, hypophysitis, and colitis). In addition, it reveals potential PD-1 inhibitors toxicities related to sensory system (sudden hearing loss), urinary system (secondary adrenocortical insufficiency) and muscle (muscular weakness, myasthenia). Mapping PD-1 together with its protein interacting network onto organ and tissue pathologies showed association with T-cell apoptosis and T-cell inflammation as the most relevant, which is expected given that PD-1 is an inhibitory receptor on T cells. Mapping PD-1 with its **very high confidence** chemical interaction partners (potential inhibitors) onto adverse events cluster within our system identified lung inflammation,

colitis, myocarditis, hypothyroidism, sudden hearing loss, muscular weakness and hypercalcemia as potential toxicities following PD-1 inhibitor treatment. The results correspond to literature findings. Namely, treatment with PD-1 monoclonal antibodies nivolumab and pembrolizumab is associated with potential muscle toxicities (myasthenia gravis, myopathy, and myositis). In addition, drug-related adverse events following nivolumab treatment included pneumonitis, vitiligo, colitis, hepatitis, hypopharyngitis and thyroiditis, corresponding to irAEs that can be life-threatening or chronic, requiring immunosuppressants. **Conclusions:** On the example of PD-1 inhibition we showed that our tool can contribute to a better understanding of target role in a disease, therapeutic potential of a target inhibition and quick target toxicological assessment.

PS 3538 *In vitro* assessment of potential hematotoxicity of ADC drugs

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Background and Purpose: Antibody-drug conjugates (ADCs) have emerged as a transformative class of cancer therapeutics, integrating antibody-mediated target specificity with potent cytotoxic payloads. However, their clinical utility is frequently constrained by off-target toxicities, among which hematotoxicity (e.g., neutropenia, thrombocytopenia, anemia) representing a common and dose-limiting adverse effect. This phenomenon arises from unintended targeting of hematopoietic cells, either via cross-reactivity with antigens expressed on immature or mature blood cells or non-specific payload release. The objectives of this study are: 1) To evaluate the hematotoxic potential of a panel of ADCs across human CD34⁺ hematopoietic stem and progenitor cells (HSPCs), erythroid, myeloid, and megakaryocytic lineages, using assessments of cell viability and lineage-specific marker expression. 2) To identify lineage-specific vulnerabilities to ADCs, thereby providing mechanistic insights to inform safer therapeutic design and optimized clinical dosing strategies.

Methods: Primary human CD34⁺ cells were isolated from cord blood using magnetic-activated cell sorting and cultured in StemSpan™ SFEM II medium supplemented with cytokines (SCF, Flt3-L, TPO) to maintain stemness. Erythroid, Myeloid and Megakaryocytic differentiation were achieved with indicated cytokines for desired time. A panel of ADCs was administered to CD34⁺ HSPCs; alternatively, ADCs were supplemented continuously for 72 hours during erythroid, myeloid, or megakaryocytic differentiation. Toxicity assessments included: Cell viability measurement via the CellTiter-Glo® (CTG) luminescent assay, with ATP levels quantified as a surrogate for viable cells. Results are expressed as percentage viability relative to vehicle controls. Lineage-specific marker expression analysis via flow cytometry using the following antibodies: Erythroid (CD71-FITC and CD235a [GPA]-PE), myeloid (CD11b-APC and CD15-PE-Cy7), and megakaryocytic (CD41-PE and CD61-APC). Data are reported as percentage of positive cells and mean fluorescence intensity (MFI) relative to controls. **Results:** Flow cytometric analysis of lineage markers and cell viability revealed that ADCs exerted distinct viability effects across human CD34⁺ HSPCs, erythroid, myeloid, and megakaryocytic lineages, while also impairing differentiation. ADCs exhibited lineage-specific toxicity: erythroid cells were most sensitive to DM1-type ADCs, myeloid cells showed greater susceptibility to MMAE-type ADCs, and megakaryocytes displayed relative tolerance—consistent with clinical reports of mild thrombocytopenia associated with the tested ADCs. **Conclusions:** This study demonstrates that primary human CD34⁺ HSPCs and their differentiated lineages constitute a robust *in vitro* model for assessing ADC-mediated hematotoxicity. This model enables the early identification of lineage-specific risks, providing a foundation for preclinical ADC development by guiding payload selection and target validation to minimize hematotoxicity. Future work will expand the panel of ADCs evaluated and investigate payload release kinetics within lineage-specific niches.

PS 3539 Everolimus Toxicology: Tissue Concentration Effects

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Background and Purpose: Common adverse events to Afinitor (oral Everolimus) include anemia (~60%), lymphopenia (~40%), and thrombocytopenia (~35%), with severe cytopenias ≤10%. Stomatitis is dose-limiting (≤70%), while rash, fatigue, and metabolic effects occur in 15-50%. Non-infectious pneumonitis occurs in ~15%, and infections in ~50%. This study examined the relationship between tissue distribution and toxicity following a single oral everolimus dose in rats. **Methods:** Sprague-Dawley rats (1 male, 1 female/group; n=4) received a single 3 mg/kg oral dose. Whole blood and major organs were collected at 0.083 h, 0.5 h, 8 h, and 24 h. Everolimus concentrations were quantified by LC-MS/MS (LLOQ: 0.30 ng/mL blood; 1.00 ng/mL tissue). Tissue concentrations (ng/g) and tissue-to-blood ratios were calculated, and PK parameters (C_{max}, T_{max}, AUC_{0-∞}) determined by noncompartmental analysis (WinNonlin® 8.5). **Results:** Everolimus was rapidly absorbed and concentrated primarily in gastrointestinal organs, liver, lung, and spleen, with no sex differences. The highest exposures occurred in the stomach, small intestine, and large intestine, detectable within 5 min post-dose. At 0.5 h, C_{max} values were 16,893.64 ng/g (stomach), 4,173.68 ng/g (small intestine), and 1,478.18 ng/g (large intestine), compared with 689.70 ng/g (liver), 266.75 ng/g (lung), and 244.42 ng/g (spleen). Whole-blood C_{max} was 2.22 ng/mL, and brain levels were below LLOQ. Everolimus concentrations declined gradually but remained detectable up to 24 h. The correlation between C_{max} and the organ toxicity reported for patients treated with Afinitor was demon-

strated. Higher C_{max} was found in the GI tract and was associated with the high incidence of stomatitis/oral ulcers (44-70%) and diarrhea (50%). In contrast, lower C_{max} was found in blood and was associated with a low incidence of thromboembolism (3%). **Conclusions:** These findings demonstrate that everolimus toxicology correlates with tissue distribution, paving the way for better therapeutic drug monitoring beyond blood levels alone.

PS 3540 Alterations of Xenobiotic ADME in a Rat Model of Heat Strain

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Background and Purpose: The impact of temperature on chemical action has been well studied for many years. A series of publications in the late 1950s began to investigate this premise *in vivo*: the LD₅₀ of varied chemical compounds were assessed in rats housed at different temperatures—cold, control, and hot—and temperature-dependent changes to toxicity were measured. These were the last published studies to test this phenomenon. While environmental stressors are known to influence xenobiotic metabolism, the impact of thermal stress—particularly exposure to elevated temperatures—on the mechanisms underlying absorption, distribution, metabolism, and elimination (ADME) remains almost entirely unexplored. Of note are two major classes of hepatic enzymes, the members of the Cytochrome P450 superfamily (P450), which account for over 75% of drug metabolism in the human body and have implications in the metabolic pathways of many xenobiotic toxicants, and transporters, which are responsible for the distribution and elimination of many xenobiotics and their metabolites. **Methods:** In this study, we examined the effects of environmentally relevant high-temperature cyclical exposure (34°C, 12 hours/day for 7 days) on the expression and function of hepatic P450s and transporters in male Sprague-Dawley rats. Cytochrome P450 activity was evaluated using LC-MS/MS-based quantification of metabolite formation from isoform-specific probe substrates, e.g. testosterone for Cyp3a4. Protein quantification of hepatic transporters, both uptake and efflux, were assessed via a targeted LC-MS/MS method developed using surrogate peptides. **Results:** Results revealed alterations in the P450 activity of 4 of the 8 most prevalent drug metabolizing P450 isoforms, namely Cyp2b1, 2d1, 2e1, and 3a1. Further, multiple xenobiotic uptake and efflux transporters were demonstrated to change, including a decrease in uptake transporters Ntcp, Oatp2b1, and Oat2, as well as in efflux transporters Bcrp and Bsep, alongside multiple trends in other transporters. **Conclusions:** These changes, accompanied by classical heat strain physiology, including increased body temperature and significant body weight fluctuations, suggests that cyclical thermal stress can induce a transient phenotypic state resulting in altered capacity for xenobiotic disposition and metabolism.

PS 3541 MASH Impairs Renal Anion Transporter Function and Alters Cefazolin Disposition

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Background and Purpose: Metabolic dysfunction-associated steatohepatitis (MASH), a progressive form of liver disease, affects up to 30% of the global population. While hepatic transporter alterations in MASH have been well characterized, far less is known about the effect of MASH on renal transport processes. In particular, the renal handling of organic anions remains poorly understood. Herein, we used cefazolin (CEF), an anionic antibiotic primarily used to prevent surgical site infection, as a model compound to investigate MASH-induced alterations in renal transporter function and their role in pharmacokinetics. **Methods:** MASH was induced in mice using fast food-dieted/thioacetamide (FFDTH) which has been shown to accurately model the transporter changes seen in human MASH. Using LC-MS/MS, transporter expression was evaluated via proteomics, and plasma, urine, and tissue concentrations of CEF were analyzed. **Results:** Expression of Oat3 and Oat5 were significantly reduced (50% and 80%, respectively) in MASH animals compared to controls. In response, plasma concentrations of CEF increased from 83% in MASH compared to controls, while hepatic accumulation rose ~80%. No differences in renal accumulation or excretion of CEF were observed between MASH and healthy mice. **Conclusions:** This data suggests that MASH-induced Oat transporter reduction increases both systematic CEF concentration along with the accumulation in the liver, without differences in renal or urinary excretion. Overall, these findings highlight the risk of increased systemic exposure in MASH patients, specifically those undergoing surgical procedures ultimately suggesting that it would be beneficial to explore dose adjustments in these patients.

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Background and Purpose: With the increasing integration of nanomaterials (NMs) into daily life, their technological advantages have become evident. However, their intricate interactions with biological systems introduce complexities that can lead to unpredictable toxicological outcomes. **Methods:** This study investigated the *in vivo* toxicokinetics and toxicodynamics of single- and multi-component NMs composed of silicon carbide (SiC), titanium dioxide (TiO₂), and a SiC@TiO₂ composite, along with a physical mixture of SiC and TiO₂ in the same ratio as the composite. Rats were exposed to these materials via single intratracheal instillation, and biological responses were assessed over time (1 h to 28 days) to identify the no-observed-adverse-effect level (NOAEL). **Results:** All NMs induced minimal structural alterations in lung tissue and prompted varying degrees of inflammatory cell infiltration. Over time, translocation from the lungs to secondary organs (heart, spleen, liver, kidney) was observed, with distinct distribution patterns between Si- and Ti-containing NMs. Bronchoalveolar lavage fluid analysis revealed a minimal to mild inflammatory response that evolved in a time-dependent manner, even at NOAEL exposure levels, suggesting delayed-onset biological effects. **Conclusions:** SiC@TiO₂ demonstrated a reduced pulmonary toxicological profile relative to its single-component counterparts, likely due to antagonistic effects between its constituents. These findings highlight the need to assess multicomponent nanomaterials as distinct entities and suggest that rational material design may help mitigate adverse biological effects, supporting safer nanotechnology development.

PS 3545 Development of a long-term 3D spheroid mouse hepatocyte culture model for use in drug-induced liver injury (DILI) applications

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Background and Purpose: Rodent models are an important tool for safety and toxicology testing. However, recent FDA guidance indicates a shift from animal models towards cell culture models to minimize the impact to and use of animals in drug safety and efficacy testing. Despite this, rodent systems will continue to play an important role in drug testing for years to come. In order to better align with FDA guidance, advanced rodent cell culture models, especially physiologically-relevant 3D models, can offer better predictivity of *in vivo* results in live animals, ultimately requiring the use of fewer animals throughout the entire testing workflow. Here, we demonstrate a protocol for long-term culturing of two strains of mouse hepatocytes (CD-1 and C57BL/6) in a 3D spheroid format for 21 days in culture. The utility of this model is validated through a DILI assessment of acetaminophen, an exemplar hepatotoxin. **Methods:** Lonza mouse hepatocytes (CD-1 or C57BL/6) were thawed in Lonza Rodent and Monkey Cryopreserved Hepatocyte Thawing Medium. Tubes were centrifuged aspirated of supernatant, and then cells were resuspended in Spheroid Formation Medium (consisting of Hepatocyte Culture Medium (HCM) Medium; Lonza) with 20% FBS and 25 mM HEPES buffer). Mouse hepatocytes were seeded at 1,500 cells / well in an ultra-low attachment 96-well plate and were incubated for 5-7 days. Spheroids formed by day 7, after which spheroids were maintained in serum-free HCM medium for another 14 days in culture (total of 21 days). Spheroid health and functionality was assessed visually using live-cell imaging and by measuring basal and rifampicin-induced (10µM; 72 hour exposure) CYP3A4 enzyme induction using a P450 luminescence assay at time points 3, 7 and 14 days post-spheroid formation (days 10, 14 and 21 in culture). Repeated drug exposure with acetaminophen (a hepatotoxic compound; 0-25 mM) began on day 7 in culture and continued for 14 days (to day 21). Cytotoxicity assessments were made at multiple time points (3, 7 and 14 days after dosing) based on total cellular ATP to generate dose response curves and EC₅₀ values. **Results:** Primary mouse hepatocytes of both strains formed spheroids with well-defined perimeters within 7 days and maintained appropriate morphology for an additional 2 weeks in culture (total of 21 days). CYP3A4 induction by rifampicin was observed across the entire 14-day spheroid culture period by both mouse strains; however, the basal CYP3A4 activity was far higher in strain CD1, resulting in an overall lower induction potential compared to the C57BL/6 strain, but much higher activity in general. Overall, both mouse strains showed robust morphology and functionality across the entire 14-day spheroid culture period - 21 total days in culture. Both mouse strains exhibited the expected dose-response to acetaminophen over time. EC₅₀ values for both mouse strains decreased significantly over time. There were also significant differences between strains. Mouse strain C57BL/6 showed significantly greater toxicity at earlier timepoints when compared with strain CD-1. By day 14 of acetaminophen exposure (21 days total in culture), EC₅₀ values had declined for both strains of mouse nearly to the 10x C_{max} value for acetaminophen in humans, indicating that both mouse strains offer predictive models of DILI toxicity for acetaminophen in humans. **Conclusions:** This study demonstrates a physiologically-relevant and user-friendly 3D spheroid culture model for two strains

of mouse hepatocytes that is validated for use in DILI toxicity testing. In this study, both strains of mouse hepatocytes maintained proper morphology and functionality over the course of a 21-day culture period (14 days of spheroid culture). This long-term culture format allows for the evaluation of acute and chronic DILI toxicity as demonstrated with the exemplar hepatotoxin, acetaminophen. This protocol provides a physiologically relevant initial screening of compounds prior to *in vivo* animal testing, reducing the overall number of live animals needed in the safety testing process.

PS 3546 Enhancing metabolic stability of beagle dog primary hepatocytes via culture optimization

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Background and Purpose: Primary hepatocytes serve as essential *in vitro* models for drug metabolism and toxicology studies. However, during culture—particularly in non-human species such as Beagle dogs and rats—the expression of drug-metabolizing enzymes declines rapidly, resulting in unstable metabolic capacity. Directly applying human-optimized culture methods to other species often yields limited improvement in metabolic activity and causes significant batch-to-batch variability. Our preliminary comparative studies revealed distinct interspecies differences in metabolic performance. Specifically, Beagle dog primary hepatocytes exhibited slower metabolic turnover, reduced CYP enzyme activity, and heightened sensitivity to culture conditions, all of which substantially restrict their utility in *in vitro* research. Therefore, this study aimed to establish a standardized culture system that systematically improves the metabolic stability of primary hepatocytes—particularly Beagle dog hepatocytes—thereby enhancing their *in vitro* metabolic performance and batch consistency, and providing a high-quality cellular model for pharmacokinetic and toxicological evaluation. **Methods:** The culture system for Beagle dog primary hepatocytes was systematically optimized in three aspects: (1) medium composition, (2) temperature regulation, and (3) physical culture conditions. Verapamil, a CYP3A substrate, was used as the metabolic validation compound. Metabolite concentrations and residual parent drug were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS), and metabolic half-life was calculated to evaluate the effects of the optimized system on metabolic capacity. **Results:** Moderate elevation of culture temperature significantly improved the metabolic stability of Beagle dog primary hepatocytes. The addition of sodium thyroxine to the culture medium markedly enhanced CYP3A enzyme activity in Beagle dog hepatocytes, whereas its effect on CD-1 mouse primary hepatocytes was minimal. **Conclusions:** This study established a standardized culture method that systematically enhances the metabolic stability of Beagle dog primary hepatocytes, offering a new technical approach and experimental foundation for developing non-human *in vitro* models for drug metabolism evaluation.

PS 3547 Understanding Mechanisms of PFAS Absorption, Distribution, and Elimination Using a Physiologically Based Toxicokinetic Model

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Background and Purpose: Exposures to some per- and polyfluoroalkyl substances (PFAS), including perfluoroalkyl acids (PFAA), are associated with diverse adverse health effects. Physicochemical properties of PFAA are known to influence their toxicokinetics in mammals, and mechanistic models capable of identifying the key drivers of absorption, distribution, and elimination are limited. Herein, we developed and evaluated a physiologically based toxicokinetic (PBTK) model parameterized to an *in vivo* mouse model using data from *in vitro* studies, addressing the limitations of prior empirical models by incorporating mechanistic data on permeability, protein and lipid binding, and transporter interactions. **Methods:** The PBTK model quantified blood flow, binding to tissue proteins and phospholipids, membrane permeability, hepatic and renal transporters, and fecal and urinary excretion. The model was parameterized using *in vitro* transport data for nine PFAA with fluorinated carbon chain lengths ranging from 4 to 10 (n_{ptc} = 4-10). We simulated PFAA tissue concentrations in wild-type mice, and for perfluorooctanesulfonic acid in knockout mice, following intravenous and oral exposures. Model performance was evaluated by comparing simulated and experimental blood and tissue concentrations. **Results:** The PBTK model reproduced experimental blood and tissue concentrations, with R² values ≥0.67 and relative root mean square errors ≤66% for most PFAA. Model sensitivity analyses showed that permeability and phospholipid binding strongly influenced elimination and distribution of long-chain PFAA (n_{ptc} ≥ 7), while elimination for short-chain PFAA (n_{ptc} ≤ 6) was most sensitive to renal transporters and albumin binding. Blood accumulation was most sensitive to albumin binding, while tissue retention was most positively associated with phospholipid binding. **Conclusions:** This study illustrates that integration of *in vitro*-derived parameters into a PBTK model enables mechanistic evaluation of PFAS toxicokinetics across diverse compounds and physiological conditions.

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Background and Purpose: Integrating toxicological insight into early discovery decisions remains limited despite decades of safety-driven attrition in drug development. Many clinical safety failures arise from mechanisms intrinsic to target biology or compound design—factors that could be identified earlier through systematic analysis of mechanistic data. This gap is evident for emerging modalities such as antibody-drug conjugates (ADCs), where antibody specificity, payload potency, and linker stability jointly determine efficacy and toxicity. ADCs enable targeted delivery of cytotoxic payloads but pose safety challenges. Toxicities may result from off-target binding, premature payload release, or target antigen expression in healthy tissues. These events are difficult to predict using standard models because relevant data—spanning chemical, biological, and clinical domains—are typically siloed. Mechanistic safety science provides a framework for linking molecular events to tissue and organ outcomes, yet its application in discovery has been hindered by data fragmentation and inconsistent contextual representation. To address this gap, Elsevier's Life Science Research Assistant (LSRA) was developed as a multi-agent AI framework that performs cooperative interrogation of diverse scientific information sources. Through coordinated reasoning among specialized agents, LSRA retrieves, evaluates, and synthesizes evidence spanning chemical, biological, pharmacological, and clinical data. Drawing on Elsevier content and third-party databases such as PubMed and ClinicalTrials.gov, LSRA supports hypothesis-driven exploration of mechanistic safety relationships during target identification and lead optimization. **Methods:** LSRA employs autonomous agents specialized in chemistry, biology, toxicology, and clinical science to retrieve and evaluate diverse datasets, iteratively sharing findings through consensus cycles to construct mechanistic hypotheses grounded in experimental and clinical data. LSRA incorporates natural language querying to enable intuitive question formulation and employs computational methods to interpret and connect information across structured and unstructured data sources. A hypothetical ADC program employing tubulin-inhibitor payloads was used to demonstrate the framework's capabilities. Agents examined cytotoxic mechanisms, linker chemistry, and target expression profiles in relation to hepatic and hematologic safety. Computational interrogation included toxicophore mapping and mechanistic substructure inference to identify chemical motifs associated with stress-response pathways. Integrated data encompassed curated toxicology collections, peer-reviewed literature, and clinical trial records. **Results:** The LSRA workflow established mechanistic linkages across preclinical and clinical domains, providing a coherent framework for interpreting biological processes underlying toxicity. Natural language querying enabled discovery of cross-domain relationships that might be overlooked through conventional searches. Findings revealed how linker instability and payload lipophilicity contribute to hepatocyte and bone marrow exposure. Comparison with ClinicalTrials.gov data confirmed concordance between preclinical predictions and clinical adverse event patterns in ADCs with similar design features. Iterative reasoning enhanced the biological plausibility of proposed toxicity mechanisms, refining early safety assessments and informing compound design decisions. **Conclusions:** Integrating mechanistic safety science with molecular, pharmacological, and clinical data enhances early recognition of design factors relevant to safety. The multi-agent approach exemplified by LSRA provides a structured means to integrate mechanistic data across disciplines and evidence sources. Such integration may enhance the interpretability of toxicity mechanisms in complex modalities like ADCs and inform more evidence-based decisions in target selection and lead optimization.

PS 3549 Integrating Physiologically Based Pharmacokinetic (PBPK) Modeling with Intra-Arterial Drug Delivery (I-ADD) for Brain Tumor Therapy

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Background and Purpose: Systemic administration of therapeutics is often hindered by the limited drug distribution in the targeted organ and substantial off-target toxicity. To address this challenge, a bioresorbable polymer-metal intra-arterial drug delivery (I-ADD) device has been developed to enable sustained and localized drug release directly into the cerebral arterial circulation. In this study, we aim to develop a physiologically based pharmacokinetic (PBPK) model to simulate intra-arterial drug delivery (I-ADD) to the brain and to optimize dosing strategies that maximize therapeutic efficacy while minimizing systemic side effects, using dexamethasone (DEX) and cisplatin (CIS) as model drug compounds. **Methods:** A whole-body PBPK model was constructed for multiple species (e.g., rats and mice) comprising blood, liver, kidney, gastrointestinal tract, and a permeability-limited brain submodel consisting of vascular and extravascular (parenchymal) spaces. Model parameters were initialized from the literature and calibrated by published intravenous and oral pharmacokinetic data. To model I-ADD, conventional drug input was replaced with a time-variant intra-arterial release function derived from *in vitro* release experiments. Drug release was described by a Weibull equation and applied as a local input to the carotid artery compartment. This approach enabled simulation of implant-specific

factors (e.g., device composition, drug loading, surface area, degradation rate) on downstream brain and systemic exposure. Sensitivity analyses were performed to identify parameters that most influence brain exposure, and model performance was evaluated against internal pharmacokinetic data. **Results:** The PBPK model successfully reproduced plasma and tissue concentration-time profiles of DEX across collected datasets. Most of the predictive values were within a twofold deviation of experimental observation (>80%), with coefficients of determination (R^2) greater than 0.73. Preliminary simulations indicated that sustained intra-arterial input markedly increased brain exposure while minimizing systemic accumulation compared with intravenous dosing, consistent with expected I-ADD performance. The model will be extended to CIS, and rat physiology is currently in progress to evaluate cross-species and compound-specific applicability. **Conclusions:** This PBPK framework provides a quantitative basis for optimizing I-ADD device design and guiding translational dose selection to maximize brain exposure while minimizing systemic toxicity. By integrating device release kinetics with physiological parameters, the model establishes a mechanistic link between implant design and properties, local hemodynamics, and therapeutic outcomes. The framework will be extended to cisplatin (CIS), rats, and ultimately extrapolated to humans to support rational dose translation and clinical application of I-ADD technologies for brain-targeted therapy.

PS 3550 Transgenerational Implications of Age-Dependent Half Life for Dioxins in Mothers and Breastfed Children

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Background and Purpose: Adult body burdens of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and other dioxin-like dibenzodioxins and dibenzofurans have been declining at an average rate of approximately 4% per year since the early 1980s. This decline correlates with US and international regulatory efforts to reduce emissions due to concerns about potential health risks to children from breastmilk transfer of maternal body burdens. However, pharmacokinetic data analysis since the late 1990s revealed that infants have a far shorter TCDD clearance half-life (e.g., 0.5 y at age 0-1) compared to adults (e.g., 7 y at age 30). Further research showed that this shorter half-life trend applied to all dioxin-like compounds with gradual increasing half-life from infancy through late adolescence, indicating that elevated body burdens acquired *in utero* and from breastfeeding were eliminated within a few years. The current study examines the magnitude of changes in maternal and child body burdens of TCDD toxicity equivalents (TEQ) across three generations of firstborn females born in 1985, 2005, and 2025. **Methods:** A pharmacokinetic model for childhood dioxin body burden was used to integrate concurrent estimates of adult body burden and breastmilk concentrations with age-specific child intake parameters for 12 months of breastfeeding followed by concurrent background dietary intake through age 7 y. All intake and output parameters were calibrated to concurrent TEQ in lipid in maternal and child tissues as well as in breastmilk and other childhood dietary intake. Published data showed an average 4% annual decline in dioxin body burdens on a lipid basis from the 1980s through 2010; this rate of decline was used to estimate child TEQ body burden at birth, maternal breastmilk TEQ, and concurrent childhood dietary TEQ in 1985, 2005, and 2025. The model estimated blood lipid TEQ concentrations monthly for the first 12 months and then annually through age 7. **Results:** The first-generation child (1985) was assumed to ingest breastmilk with 140 pg TEQ/g (lipid basis, first 6 months average) which led to peak blood level of 73 pg TEQ/g lipid at age 6 months that declined to 12 pg TEQ/g lipid at age 7 y. The second-generation child (2005) was assumed to ingest breastmilk with 78 pg TEQ/g which led to a peak blood level of 40 pg TEQ/g at age 6 months that declined to 6.5 pg TEQ/g at age 7 y. The third-generation child (2025) was assumed to ingest breastmilk with 15.5 pg TEQ/g which led to a peak blood level of 8.1 pg TEQ/g at age 6 months that declined to 1.3 pg TEQ/g at age 7 y. **Conclusions:** The childhood dioxin body burden model results illustrate that the shorter age-dependent half-life of dioxins in early childhood rapidly ameliorates the higher dose from breastfeeding and attenuates cumulative intake from background dietary sources through young adulthood. While background dietary dioxins declined in parallel with adult body burdens over time, the shorter age-dependent half-life of dioxins may also contribute to transgenerational body burden declines in reproductive age adults due to more rapid excretion of dietary dioxin intake through early adulthood.

PS 3551 Development of gestational PBK modeling to support dose selection for the developmental neurotoxicity *in vitro* battery testing

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Background and Purpose: Use of the Developmental Neurotoxicity *In Vitro* Battery (DNT IVB) has been endorsed by the OECD as a basis for investigating potential DNT hazard. Appropriate concentration selection is essential, as with any other *in vitro* testing, to ensure that results reflect biologically relevant exposure levels. This enables accurate interpretation of chemical effects and improves the predictive value of the DNT IVB assays at environmentally relevant concentrations. **Methods:** This study addressed challenges in incorporating species-specific and gestational factors by developing rat and human physiologically based kinetic (PBK) models to estimate

fetal exposure during gestation and guide concentration selection. This modeling approach was developed for a specific case study using a pesticide active ingredient. We first developed and evaluated a nonpregnant rat PBK model. Next, a parallel-gram approach was applied to extrapolate rat to human, enabling development of a nonpregnant human PBK model. The nonpregnant rat and human PBK models were used to derive the human equivalent doses (HED) from the doses tested in rat *in vivo* DNT study: 8.2 mg/kg bw/day (low) and 326.2 mg/kg bw/day (high). The nonpregnant human model was then extended to pregnancy by incorporating physiological changes in pregnancy to develop the gestational human PBK model. **Results:** The modelled systemic exposure to parent compound is significantly lower than main metabolite due to its rapid and extensive metabolism. The daily average concentrations of parent compound in blood and brain were predicted to be below 1 µmol/L for all exposure scenarios. For the metabolite, the estimated daily average human fetal blood and brain concentrations were predicted to be < 80 µmol/L at the high HED. In rats, predicted concentration were below 120 µmol/L in blood and under 30 µmol/L in brain at 326.2 mg/kg bw/day. Based on the predictions, a top concentration of 30 µmol/L was chosen for the parent compound in most assays, and the top dose for metabolite was 100 µmol/L (for human cell-based test systems) or 300 µmol/L (for rat-derived assays). **Conclusions:** This study highlights the value of PBK modeling in informing concentration selection for *in vitro* assays across species, particularly when *in vivo* or environmental exposure is known. By predicting fetal brain exposure using gestational human models, we identified biologically relevant concentrations that would ultimately improve the interpretation and human health relevance of DNT IVB results.

PS 3553 Toxicity and biotransformation of trans-1,2-difluoroethene (R-1132-(E))

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Background and Purpose: 1,2-Difluoroethene (R-1132-(E)) is a potential novel refrigerant with favorable environmental properties. Inhalation studies in rats indicate a low toxicity potential, with no observed adverse effect levels (NOAELs) exceeding 15,000 ppm in a 90-day repeat exposure study. In contrast, 28-day whole body exposure of rabbits to 500 ppm resulted in mortality (two of 10 exposed animals), demonstrating pronounced species-differences in R-1132-(E) toxicity. These species-differences may be linked to species-specific biotransformation to toxic metabolites in rabbits. To comparatively study the biotransformation of R-1132-(E) between species, fluorine-containing metabolites were monitored by ¹⁹F-NMR in urine of rats and rabbits following inhalation exposure. **Methods:** Male and female rats (n=5 per sex) were exposed to 0, 2,000, 5,000 and 15,000 ppm of R-1132-(E) for 6 h/day, 5 days/week, for up to 28 days. Male and female rabbits (n=5 per sex) were exposed to 0, 500, 1,000 and 2,000 ppm of R-1132-(E) for the same duration and schedule. Urine of rats was collected in metabolic cages for 16 hours following the 6 h exposure on days 3, 14, 17 and 27. Rabbit urine was obtained by puncture of the bladder 18 hours after the final exposure. **Results:** Besides fluoride, which was also found in urine of untreated rats at comparable levels, ¹⁹F-NMR spectra of urine from rats exposed to 15,000 ppm of R-1132-(E) did not contain any signals indicative of fluorine-containing metabolites. In contrast to rats, trace levels of 2,2-difluoroacetic acid (DFA) were identified in urine of several rabbits exposed to 500, 1,000 and 2,000 ppm of R-1132-(E), with no apparent dose-response. **Conclusions:** The absence of signals of DFA in urine of control rabbits, suggests that DFA may be a potential urinary metabolite of R-1132-(E), even though formation of DFA from R-1132(E) requiring intramolecular 1,2-fluorine migration – similar to the biotransformation of 1,2-dichloroethene – appears unexpected. The at best low levels of biotransformation of R-1132-E is further supported by *in vitro* studies in rat, rabbit and human hepatocytes exposed to 0 or 15,000 ppm of R-1132-(E) for 48 hours, in which no fluorine containing metabolites could be identified by ¹⁹F-NMR. Due to the low levels of DFA detected and the paucity of DFA toxicity data it is currently not possible to draw any conclusions on the role of DFA in R-1132-(E) toxicity in the rabbit. This work was supported by Daikin Industries, LTD.

PS 3554 Bioactivation of alkylfurans to reactive α,β-unsaturated dicarbonyls: Evidence from *in vitro* and *in vivo* studies

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Background and Purpose: Alkyl-substituted furans (alkylfurans) are present in a range of foods and are likely to contribute significantly to overall furan exposure. While furan is a well characterized hepatotoxin and rodent liver carcinogen, there is limited toxicity data on alkylfurans. Based on their structural analogy, there is concern that alkylfurans may produce similar toxicological effects as furan due to CYP450-mediated oxidation of the double bond and subsequent ring opening of the furan moiety to yield reactive dicarbonyls. However, the side-chain may influence the rate of bioactivation, and thus assumption of dose additivity may overestimate health risks. **Methods:** The overall objective of the present work was to inform risk assessment by investigating the biotransformation of selected alkylfurans with different

side-chain length in rat and human microsomes, HepaRG cells and in rats through quantitative analysis of glutathione (GSH)- and N-acetyl-lysine (NAcLys)-adducts of ring-opened metabolites of alkylfurans via stable isotope dilution LC-MS/MS. **Results:** In analogy to furan, GSH- and NAcLys-adducts were detected in incubations of rat and human liver microsomes with 2-methylfuran (2-MF), 2-ethylfuran (2-EF) and 2-pentylfuran (2-PF) in the presence of GSH and NAcLys as trapping agents, confirming CYP450-mediated bioactivation of 2-MF, 2-EF and 2-PF to the reactive α,β-unsaturated dicarbonyls 4-oxo-2-pentenal, 4-oxo-2-hexenal and 4-oxo-2-nonanal, respectively. Quantitative analysis revealed differences in the rate of formation of GSH- and NAcLys-adducts, with overall lower rates with alkylfurans compared to furan (Furan > 2-MF >> 2-EF > 2-PF). Across compounds, higher rates of GSH- and NAcLys-adduct formation were observed in rat vs. human liver microsomes, indicating species-differences. The impact of the side-chain on the extent of bioactivation of furans to reactive dicarbonyls was confirmed in HepaRG cells exposed to furan and alkylfurans (0-10 mM) for 24h and in rats. In male F344 rats administered a single oral dose of 1 mg/kg bw 2-MF, 2-EF or 2-PF, urinary 24h-excretion rates of GSH-adducts of alkylfurans were significantly lower as compared to the excretion rate of the furan derived GSH adduct after furan administration (1 mg/kg bw), with a clear trend with increasing side-chain length (Furan >> 2-MF > 2-EF > 2-PF). Similar to furan, NAcLys-adducts of alkylfurans were also detected in urine of untreated rats, suggesting a yet unknown exogenous or endogenous source. **Conclusions:** Overall, our data support the hypothesis that the rate of bioactivation of alkylfurans to a reactive intermediate is lower as compared to furan, which may suggest that alkylfurans may be of lower toxicological concern. Moreover, the species-differences observed in rat vs. human liver microsomes suggest that humans may be less susceptible to (alkyl)furan toxicity than rodents. This work was supported by Nestlé Research and the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation).

PS 3555 Machine Learning-Enabled Reconstruction of Complete Nanoparticle Biodistribution Profiles in Tumor-Bearing Mice from Incomplete Experimental Data

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Background and Purpose: Most nanomedicine studies in tumor-bearing mice report biodistribution in only 2-3 tissues, limiting cross-study comparability and slowing translation. To address this, we developed a machine-learning (ML) framework to reconstruct full biodistribution profiles across six tissues (tumor, heart, liver, spleen, lung, kidney) from incomplete datasets. Importantly, the model incorporates nanoparticle (NP) dose as a continuous input, enabling dose-aware, cross-study predictions. **Methods:** We curated 1,073 NP biodistribution profiles from tumor-bearing mouse studies, including 505 complete six-tissue datasets. To prevent data leakage, we used profile-level cross-validation and group-based train/test splits, generating 28,280 prediction scenarios with 2-5 tissue inputs. Eight algorithms were benchmarked, including linear regression, tree ensembles, and XGBoost variants. Performance metrics included R², RMSE, MAE, and overfit gap. Model interpretation identified physicochemical and biological drivers of NP fate. **Results:** The tree-based and gradient-boosted models achieved strong performance. For tissues characterized by predominantly passive distribution (tumor, heart, liver, spleen, lung), top test R² values by tissue were tumor 0.695, heart 0.583, liver 0.911, spleen 0.650, lung 0.495 (mean R² = 0.667). In contrast, kidney exhibited lower predictive performance (R² = 0.160), with feature correlations near zero (mean |r| = 0.021 vs. 0.068-0.107 for other organs), indicating renal clearance is driven by unmeasured physiological factors rather than static NP properties. Across all tissues, mean test R² was 0.558, with RMSE 6.32 %ID and MAE 1.63 %ID. Key predictors included NP dose, size, shape, core material, zeta potential, timepoint, and cross-tissue correlations. **Conclusions:** We present the first dose-aware ML framework capable of reconstructing complete 6-tissue NP biodistribution profiles in tumor-bearing mice from partial data. Our findings reveal a fundamental distinction: while static physicochemical properties reliably predict passive organ distribution (R² > 0.49), renal clearance requires additional physiological factors that remain to be determined. This platform enables standardized, cross-study analysis without requiring full experimental reporting, and identifies key physicochemical drivers of NP fate including dose, size, shape, core material, zeta potential, and timepoint. Building on this quantitative foundation, the next step is to integrate these ML predictions with physiologically based pharmacokinetic (PBPK) modeling to create an ML-enhanced PBPK framework. This integrated approach will combine the predictive power of ML with mechanistic pharmacokinetic insights, accelerating rational nanomedicine design and translation.

PS 3556 Major *in vitro* Phase I and Phase II Metabolites of Cannabichromene

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Background and Purpose: Cannabichromene (CBC) is considered one of the most prevalent minor cannabinoids. The U.S. minor cannabinoid market was estimated to be 11.5 billion dollars in 2023 and is expected to triple to nearly 33.3 billion dollars by 2030. The biological effects of CBC are largely uncharacterized. Anecdotal sources targeted to the public claim far-reaching medical benefits including being anti-in-

flammatory, antidepressive, antinociceptive, and having cancer-fighting properties. These broad and unspecific claims coupled with the projected popularity and lack of rigorous scientific research elucidate a critical gap in knowledge. Therefore, characterization of CBC pharmacokinetics, particularly metabolism, will aid in alleviating the current deficiency in research. Previous studies of major cannabinoids (i.e. Δ^9 -tetrahydrocannabinol and cannabidiol) indicate that their respective metabolites maintain biological activity and thus are critical to consider in terms of pharmacokinetic and pharmacodynamic relationships. To date, phase I and phase II metabolic products of CBC have yet to be determined in human *in vitro* or *in vivo* models alike. **Methods:** To study *in vitro* metabolism, CBC was incubated with HepaRG cells and human liver microsomes (HLMs). In HepaRG cells, CBC was incubated for 2 and 24 hours at various concentrations (0.5, 5.0, and 50 μ M). Both cell extract and media were collected from each sample. A semi-quantitative methodology utilizing high-performance liquid chromatography-high resolution mass spectrometry (HPLC-TripleTOF) was used to gauge metabolite generation. To determine HLM enzyme kinetics, incubation time and protein concentration were first optimized for phase I and phase II generation of metabolites. Following optimization, the dose-dependent production of CBC metabolites was calculated wherein K_m and V_{max} values were determined via Michaelis-Menten kinetics. Intrinsic clearance was calculated by the following equation: $CL_{int} = V_{max}/K_m$. **Results:** The metabolic products generated from both HepaRG cells and HLMs were chromatographically separated and analyzed via HPLC-TripleTOF. The major phase I oxidative metabolite was confirmed to be 2'-hydroxycannabicitran, and the major phase II conjugated metabolite was hypothesized to be CBC-glucuronide according to high resolution exact mass. In both HepaRG cells and HLMs, 2'-hydroxycannabicitran was rapidly generated, while CBC-glucuronide exhibited a relatively slower generation. The kinetic patterns of metabolite generation in HepaRG cells were aligned with those of the HLM incubations. In HepaRG cells, these two metabolites constituted the main metabolites detected. There was not any combinatorial oxidation-conjugation type metabolite detected from the given incubations. **Conclusions:** Identification of CBC metabolites is a critical step in elucidating the pharmacokinetics of CBC. Additional experiments characterizing which specific drug metabolizing enzymes namely CYP450s and UGTs are involved in generating 2'-hydroxycannabicitran and CBC-glucuronide, respectively, would be a fundamental step in establishing if there is potential for subsequent drug-drug interactions. Utilizing *in silico* methodologies, further work will probe the potential for biological activity of 2'-hydroxycannabicitran and CBC-glucuronide on various proteins in the endocannabinoidome.

PS 3557 Mechanistic Insights into Liposomal Doxorubicin Distribution in Mice through Physiologically Based Pharmacokinetic Modeling

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Background and Purpose: Cancer remains a major global health challenge, with its incidence and mortality continuing to rise. Nanotechnology offers a promising strategy to improve anticancer drug delivery by enhancing pharmacokinetics (PK) and bioavailability while minimizing systemic toxicity. Liposomal doxorubicin (Doxil®), the first clinically approved nanomedicine for cancer treatment, has been widely studied, yet most preclinical investigations have focused on comparing the PK profiles of native drug versus released drug rather than characterizing the whole-body disposition of intact drug-loaded nanoparticles in tumor-bearing models. Hence, this study aims to develop a joint physiologically based pharmacokinetic (PBPK) model that simultaneously describes the biodistribution of both encapsulated and released doxorubicin in tumor-bearing mice, providing mechanistic insights into nanodrug delivery and disposition processes. **Methods:** The joint PBPK model consists of submodels for encapsulated and released doxorubicin, respectively, sharing an identical physiological structure comprising major organs (lungs, liver, spleen, kidneys, heart, muscle), tumor tissues, blood, and a rest-of-body compartment. Both submodels incorporate plasma albumin binding and excretion via bile, feces, and urine, but differ in kinetic assumptions. The released doxorubicin submodel uses perfusion-limited kinetics for most tissues (permeability-limited for muscle and rest-of-body), includes DNA and cardiolipin binding, and accounts for hepatic metabolism via glycosidase activity. In contrast, the encapsulated submodel applies permeability-limited kinetics to all compartments and includes reticuloendothelial system (RES) subcompartments in the liver, lungs, spleen, and kidneys to describe nanoparticle uptake and clearance. The two submodels are linked by nanodrug uptake and release mechanisms, allowing encapsulated doxorubicin internalized by RES cells or tumor-associated macrophages and cells to be released into the corresponding compartments of the free-drug model. PK profiles for native and released doxorubicin reported from the same studies were used for model calibration, while other available PK data served as evaluation data. Given the numerous parameters in this joint PBPK model, model development proceeded sequentially: first, native drug in healthy and tumor-bearing mouse models to characterize doxorubicin-related parameters; then, liposomal doxorubicin in healthy and tumor-bearing mouse models to characterize parameters related to drug-carrying liposomes. Sensitivity analyses were conducted both before and after parameter fitting to identify essential parameters for optimization and determine which parameters significantly impact organ dosimetry for both released and encapsulated forms. **Results:** Model calibration demonstrated excellent agreement with *in vivo* data over 48 hours, achieving a root mean square error of 13% and an adjusted R^2 of 0.87. Based on preliminary sensitivity analyses and multiple calibration trials, tissue-to-plasma partition coefficients and the Michaelis constant for fecal elimination were identified as requiring

optimization. Compared to default values adopted from previously published PBPK models for doxorubicin, the optimized partition coefficients decreased by an average of two orders of magnitude, while the Michaelis constant for fecal elimination increased 10-fold. Despite these adjustments, the model maintained strong predictive performance, suggesting that the optimized parameters more accurately represent underlying physiological processes captured by the expanded mechanistic framework. **Conclusions:** The model provides a comprehensive framework for understanding liposomal nanotherapeutic pharmacokinetics by capturing critical mechanistic aspects often overlooked in conventional PK models. This approach offers a valuable tool for optimizing liposomal formulations, predicting tissue-specific drug exposure, and enabling rational nanomedicine design. Future applications include extending the model to other nanoparticle-based drug delivery systems and supporting translational efforts to refine clinical dosing strategies.

PS 3558 Safety and Pharmacokinetic Evaluation of a Platelet inspired Hemostatic Nanotechnology in Sprague Dawley Rats and Beagle Dogs

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Background and Purpose: Allogeneic platelet transfusion is a mainstay therapy for bleeding complications in trauma, surgery, hematology/oncology, and congenital disorders, yet its use is limited by short shelf life, contamination risks, and logistical challenges. To address these limitations, we developed SynthoPlate (SP), a lyophilizable, shelf-stable, platelet-inspired nanoparticle that mimics primary hemostatic mechanisms to serve as a potential platelet surrogate for both in-hospital and pre-hospital use. This non-GLP study evaluated SP safety, tolerability, and pharmacokinetics (PK) following intravenous administration in Sprague Dawley rats and Beagle dogs. **Methods:** SP was manufactured using established methods with physicochemical and functional release testing. Non-GLP single ascending-dose studies were performed in healthy Sprague Dawley rats and Beagle dogs, with infusion rate and dose adjusted to assess administration limits. Tolerability was evaluated via clinical observations, hematology, clinical chemistry, cytokine profiling, coagulation panels, gross pathology, and histopathology. Plasma levels of SP were quantified by LC-MS/MS and PK parameters were determined. Lastly, biodistribution in rats was assessed over 72 hours by IVIS imaging. **Results:** As observed with other nanoparticle systems, infusion-related reactions occurred across species and increased in severity with higher doses; however, reducing the infusion rate markedly improved tolerability. In Beagle dogs, complete blood counts showed dose-dependent decrease in white blood cells (neutrophils, lymphocytes, monocytes, eosinophils) and platelets and increase in mean platelet volume (MPV), red blood cells, hemoglobin, hematocrit, and reticulocytes. In rats, a decrease in platelet counts and an increase in MPV was observed with transient dose-dependent recovery. Clinical chemistry in dogs revealed dose-dependent increase in liver enzymes (aspartate aminotransferase [AST], alanine aminotransferase [ALT], alkaline phosphatase [ALP], and gamma glutamyl transferase [GGT]). Furthermore, coagulation analysis in dogs indicated prolonged partial prothrombin time (aPTT) at the highest dose tested without significant effects on prothrombin time (PT) or fibrinogen. Cytokine analysis in dogs showed slight increases in IL-6, IL-10, MCP-1, and TNF- α and decrease in IL-8, consistent with infusion-related immune response. No mechanism-based toxicity was observed; necropsy and histopathology revealed no signs of thrombosis or abnormal clot formation. PK analysis demonstrated biphasic clearance with half-lives of ~3-6 hours in dogs and 2-4 hours in rats. Biodistribution indicated primary accumulation in the liver, moderate uptake in the lungs and spleen, and minimal presence in the brain, heart, and kidneys with near complete clearance from all organs by 72 hours. **Conclusions:** SynthoPlate is a fully synthetic, platelet-mimicking nanotechnology exhibiting dose-dependent, transient, infusion-related inflammatory and hematologic responses consistent with other liposomal platforms. No on-target toxicity was observed at any of the doses tested, which exceeded 250X the effective dose range. The nanoparticles were largely cleared from circulation within 24-48 hours and from major organs by 72 hours. These data establish a favorable tolerability and PK profile and support definitive, IND-enabling GLP toxicology studies in preparation for first-in-human evaluation.

PS 3559 A Regulatory Aligned Dual-Endpoint Human Hepatocyte Platform for Mechanistic and Quantitative CYP450 Induction Assessment and Drug-Drug Interaction Risk Prediction

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Background and Purpose: Cytochrome P450 (CYP) enzyme induction is a key mechanism underlying drug-drug interactions (DDIs) that can affect systemic drug exposure and patient safety. Regulatory agencies, including the FDA and EMA, require robust *in vitro* evaluation of CYP induction potential to support safe clinical progression and regulatory submissions. Despite existing models, variability in hepatocyte responses and reliance on single-endpoint assays can limit mechanistic insight and predictive confidence. To address these gaps, we developed a regulatory aligned, dual-endpoint human hepatocyte platform that quantitatively evaluates both CYP

mRNA expression and enzymatic activity, providing mechanistic insight into induction responses and enabling early, informed decision-making in drug development programs and facilitate compliance with current regulatory guidelines. **Methods:** Cryopreserved primary human hepatocytes are used in two assay formats: a 48-hour induction assay with pooled hepatocytes for screening CYP1A2, CYP2B6, and CYP3A4; and a 72-hour induction assay with hepatocytes from at least three individual donors to assess CYP1A2, CYP2B6, CYP3A4, CYP2C8, CYP2C9, and CYP2C19, capturing donor variability. Media is refreshed every 24 hours during treatment. Following exposure to test compounds, positive controls (omeprazole for CYP1A2; phenobarbital for CYP2B6; rifampicin for CYP2C8, CYP2C9, CYP2C19, and CYP3A4), or the negative control flumazenil, cells are either harvested for gene expression analysis via qRT-PCR or washed and incubated with isoform-specific probe substrates. The substrates used include phenacetin, bupropion, midazolam, amodiaquine, diclofenac, and S-mephenytoin, with their respective metabolites acetaminophen, hydroxybupropion, 1-hydroxymidazolam, N-desethylamodiaquine, 4-hydroxydiclofenac, and 4-hydroxymephenytoin measured. A novel, highly sensitive LC-MS/MS bioanalytical method was developed to simultaneously quantify all six CYP-specific metabolites from a single sample, enabling efficient and accurate assessment of catalytic enzyme activity. Test compound dosing is guided by cytotoxicity assessment (LDH and ATP viability assays). Data are analysed to calculate fold induction relative to vehicle controls, Emax, and EC50 values using four-parameter nonlinear regression. **Results:** Positive control compounds omeprazole, phenobarbital, and rifampicin induced CYP1A2, CYP2B6, CYP3A4, CYP2C8, CYP2C9, and CYP2C19 in a dose-dependent manner at both gene expression and enzyme activity levels, demonstrating assay sensitivity and specificity. Fold inductions relative to vehicle controls ranged from 2- to 15-fold depending on the CYP isoform and inducer. EC50 and Emax values calculated using four-parameter nonlinear regression were consistent with literature and regulatory expectations. A strong positive correlation was observed between mRNA fold changes and corresponding catalytic activity measurements across tested CYPs, validating the complementary dual-endpoint readout. Induction profiles were reproducible across multiple individual donors, supporting assay robustness and the ability to capture donor variability. These results confirm the platform's ability to quantitatively assess CYP450 induction and provide mechanistic insight into potential drug-drug interaction risks, supporting confident decision-making during early and late-stage drug development. **Conclusions:** The developed regulatory-aligned dual-endpoint human hepatocyte platform enables sensitive, quantitative assessment of CYP450 induction at both gene expression and enzymatic activity levels. Leveraging pooled and individual donor hepatocytes alongside a novel multiplexed LC-MS/MS bioanalytical method for simultaneous metabolite quantification, this approach provides comprehensive mechanistic insights into drug metabolism. The platform supports early prediction and de-risking of drug-drug interaction potential, streamlines regulatory submissions aligned with FDA/EMA guidelines, and facilitates informed decision-making across drug development stages. This assay offers a robust, reproducible, and efficient solution for evaluating CYP induction, advancing the safety and efficacy assessment of new chemical entities.

PS 3560 Protecting Predators: Developing a Non-Lethal Framework for Toxicological Impacts of Mercury and PFAS in Sharks

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Background and Purpose: Sharks are apex predators critical for maintaining marine ecosystem balance, yet they face growing pressures from overfishing, habitat loss, and chemical pollution. Mercury (Hg) and per- and polyfluoroalkyl substances (PFAS) are two contaminants that bioaccumulate in marine food webs and have been shown to disrupt neurological, hepatic, and reproductive processes in vertebrates. Sharks' long lifespans, high trophic status, and slow reproduction make them particularly susceptible to such stressors. However, the distribution and physiological processing of these contaminants remain poorly characterized due to ethical and logistical limits on lethal sampling. We **hypothesize** that contaminant concentrations in non-lethal tissues, specifically blood and muscle, can reliably predict burdens in internal organs that are critical for shark health and reproduction (e.g., liver, brain, gonads). By establishing quantitative relationships among these matrices, we can describe contaminant transport and accumulation across tissues using physiologically based toxicokinetic (PBTK) modeling. This project addresses this gap by developing a way to predict tissue-specific contaminant burdens using only blood and muscle samples. Integrating contaminant data, trophic ecology, and shark-specific physiological parameters, this approach establishes a scalable and ethical framework for assessing chemical risk and informing conservation strategies for sharks. **Methods:** Blood and muscle samples were collected from finetooth (*Carcharhinus isodon*) sharks in Bulls Bay and St. Helena Sound, South Carolina, in collaboration with the South Carolina Department of Natural Resources (SCDNR). Organ tissues (liver, brain, gonads, spleen, stomach) from incidental mortalities were collected to aid model calibration. Hg concentrations were quantified by thermal decomposition-amalgamation atomic absorption spectrophotometry (EPA Method 7473), and PFAS congeners are being analyzed via high-performance liquid chromatography coupled with tandem mass spectrometry. Stable isotope analyses ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$) are being used to assess trophic position and diet. Doppler ultrasound assessments will measure total cardiac output and regional blood flow to vital organs at two physiological states (active and stabilized) to parameterize circulatory dynamics. These data,

along with tissue:blood partition coefficients, organ volumes, metabolic rates, and clearance parameters, will inform Bayesian-calibrated PBTK models developed in R and Berkeley Madonna. **Results:** Preliminary Hg data from neonate finetooth sharks show tissue-specific variability, with the highest concentrations observed in muscle, followed by liver, spleen, stomach, brain, and blood. A significant positive correlation between Hg concentrations in blood and muscle ($R^2 = 0.77$) demonstrates consistent partitioning dynamics between these two matrices. This relationship suggests that both tissues reflect integrated exposure and bioaccumulation processes and can be jointly used to estimate internal contaminant distribution. This correlation supports a core assumption of the PBTK framework, that equilibrium partitioning among vascularized tissues can be inferred from accessible compartments. Ongoing analyses are extending these relationships to PFAS congeners and incorporating stable isotope-derived trophic data to refine model parameterization. Upcoming Doppler ultrasound data will provide the first non-lethal estimates of cardiac output and organ-specific perfusion in sharks, improving prediction of contaminant transport and deposition. **Conclusions:** This research represents one of the first integrated efforts to pair empirical contaminant data with physiological modeling to enable non-lethal toxicological risk assessment in sharks. The developing PBTK model will allow prediction of organ-specific Hg and PFAS burdens from easily obtained blood and muscle samples, enhancing conservation and management for species where lethal sampling is not possible. In partnership with SCDNR and NOAA, this work will advance evidence-based risk assessment, support proactive conservation strategies, and strengthen ecosystem-based management of apex predators in coastal environments.

PS 3561 Streamlining tissue toxicokinetic analysis in dose range finding toxicology studies for small molecules

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Background and Purpose: Tissue toxicokinetic (TK) analysis is typically considered at the discovery stage of small molecules, especially for those with an inherent risk to accumulate in tissues. In an effort to systematically replace ad hoc practices with a pragmatic operating model, the purpose of this analysis was to reframe tissue TK assessment from a default requirement to a case-by-case decision. **Methods:** A concise WHEN-HOW-IF framework was applied with a decision tree that triggers analysis when high drug concentrations could affect the tox profile, target tissue exposure, GLP study design, and/or target engagement only after target organ toxicity is confirmed. **Results:** Leveraging historical pilot toxicity data and robust statistical analyses, it was demonstrated that tissue-to-plasma ratios are largely dose-independent in rodents, enabling a "one-dose-only" approach that preserves scientific rigor while minimizing resources. If tissue TK is determined to add value, tissues of concern are to be collected but quantitation can be deferred until warranted. The mid-dose should be prioritized for tolerability and interpretability, and focusing on rodent TK when cross-species data is available would not change decisions. **Conclusions:** This approach accelerates the advancement of safer and more promising molecules by eliminating potentially non-informative tissue TK assessments and focusing analyses where they clarify mechanisms of toxicity, inform study design, or support target engagement. It reduces bioanalytical burden, animal use, turnaround time to obtain results, and cost, while maintaining decision confidence—"one-dose-level" rodent TK and "analyze-when-warranted" workflows streamline dose range finding studies and is an example of how lean practices can improve outcomes without sacrificing rigor, creating a sustainable model that scales across portfolios and de-risks early development.

PS 3562 Route-Specific Variation of Systemic Bioavailability of Chemical Emissions

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Background and Purpose: Chemical systemic bioavailability, as represented by plasma concentration achieved per unit of external dose, can vary significantly depending upon route of absorption. In the extreme, chemicals might be effectively unavailable by one route but readily absorbed by another. The health risk posed by a chemical therefore depends on the plausible routes of exposure. **Methods:** In this study, we sought to predict via high throughput physiologically-based toxicokinetic (PBTK) models how systemic bioavailability of chemicals varied by route of exposure. Four scenarios were considered: 1) inhalation of fumes, 2) oral ingestion, 3) dermal exposure after eight hours of daily exposure to hands (UK Surrey permeability model), and 4) inhalation of an aerosol of particles with mean size 100 nm and standard deviation 40 nm. Chemicals of interest were compiled from public information on semi- and volatile organic compounds (VOCs) including those with known *in vivo* inhalation toxicological points of departure (PODs), Toxic Substances Control Act (TSCA) high priority substances, aldehydes, chemicals identified in emissions from a 3D printer, and a limited number of chemicals screened with air-liquid inter-

face-based new approach methods (NAMs). High throughput toxicokinetic (HTTK) modeling requires chemical-specific values for intrinsic hepatic clearance (Cl_{int}) and fraction unbound in plasma (f_{up}). Quantitative structure-property relationship (QSPR) models were used to make predictions for Cl_{int} and f_{up} . A three-compartment PBTK model was used to predict steady-state plasma concentration (C_{ss}) for oral and fume inhalation exposures. A dermal PBTK model was used to predict C_{ss} for repeated exposure to hands. An aerosol dosimetry model coupled to a gas-inhalation PBTK model was used for C_{ss} from aerosol exposure. Each PBTK model has been individually evaluated using route-specific plasma concentration vs. time data across multiple reference chemicals. Chemicals were ranked using the Toxicological Prioritization Index (ToxPI) to visualize the total and relative contributions of four potential routes of exposure to systemic bioavailability. **Results:** Out of 2776 unique chemicals of interest, *in vitro* measured HTTK model parameter values were available for 136. 2113 had representative structures. Of these, descriptors could be generated for 1892. QSPR predictions were within domain of applicability for 1479 (78%). With QSPRs, HTTK predictions could be made for a total of 1547 chemicals. The chemical that was predicted to be most bioavailable was 2-tert-butylphenol, which is predicted to be readily absorbed from oral, dermal, fume inhalation, and aerosol routes. Among chemicals with PODs, 2-methyl-4,6-dinitrophenol was the most bioavailable; it has an occupational inhalation lowest effect level (LEL) of 4.7 mg/m³ and a clinical oral lowest observed adverse effect level (LOAEL) of 0.35 mg/kg-d. Among seven chemicals that had both oral and inhalation PODs, the bioavailability predictions explained at least 39% of variation in POD by route. For dermal exposure the most bioavailable chemical was 1,2-dimethyl-3-nitrobenzene. Among the chemicals found in emissions from 3D printers, the most bioavailable from fumes was tridecane while 2,2,4-trimethyl-1,3-pentanediol diisobutylate was the most bioavailable as an aerosol. **Conclusions:** The environment is full of potential sources of chemical exposure - the extent to which these exposures result in meaningful tissue concentrations depends on both inherent chemical properties and the route of exposure. Using generic PBTK models, it is now possible to quantitatively predict how the route of exposure may impact human plasma concentrations. These predictions can help identify the most valuable exposure pathway information to measure or curate, as well as the most informative experimental designs for understanding potential toxicity from chemical exposures.

PS 3563 A Quantitative Synthesis of the Drivers and Magnitudes of Maternal-Child PFAS Transfer

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are persistent environmental pollutants that impair vaccine responses and immune function during vulnerable early-life stages. While breast milk is essential for infant nutrition, it also serves as a major PFAS exposure route, yet the toxicokinetics (TK) of transplacental PFAS transfer remain poorly characterized. We developed a maternal-child TK model to better understand the dynamics of maternal-child PFAS transfer and quantify the contribution of breastfeeding to internal exposures across from early life through adulthood (0-30 years). **Methods:** We applied the TK model to perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), and perfluorohexane sulfonic acid (PFHxS) that incorporates continuous pre-pregnancy, gestational with maternal-fetal transfer, and lactational with breastfeeding transfer periods. The model was parameterized with chemical- and life-stage-specific physiological parameters and estimated PFAS exposure through drinking water, food, dust, and inhalation. Model evaluation used paired biomonitoring data from 16 cohorts with maternal, child, and breastmilk PFAS concentrations. We simulated a range of breastfeeding durations to estimate internal PFAS burdens during sensitive developmental windows. **Results:** Model predictions were broadly consistent with biomonitoring data. Predicted PFAS concentrations in breastfed infants after weaning were 2-4 times higher than those prior to breastfeeding. Sensitivity analyses suggest breastfeeding duration and drinking water PFAS concentration as key drivers of early childhood exposures. Breastfeeding was the primary driver of PFAS accumulation in children between ages 1 and 4, with each additional month of breastfeeding associated with a 1.3%-10% increase in serum PFAS concentrations at age 3. The relative contribution of breast milk increased to 34-49% during breastfeeding and declined after weaning, falling below 20% by 2-6 years of age. Compared with drinking water, breastfeeding contributed more to serum PFAS concentrations until approximately 13 years of age, with peak contributions 3.3-6.0 times higher. **Conclusions:** This quantitative synthesis advances our understanding of how maternal-child PFAS transfer shapes childhood PFAS burden. Future maternal cohort studies should integrate detailed exposure, physiological, and breastfeeding data to refine the developed TK model and enhance its applicability across diverse populations.

PS 3564 A rapid dosimetry model to investigate long-term spaceflight effects on drug disposition

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Background and Purpose: Spaceflight causes a wide range of physiological changes which have potential to affect the pharmacokinetics (PK) of drugs. As NASA seeks a more diverse astronaut corps, the combined influence of altered physiology and changing demographics could increase the risk of reduced drug efficacy or adverse effects. Age, sex and racial diversity also introduce a potential concern for genetic polymorphisms that could impact medical outcomes during long-term missions. Thus, a better understanding of how spaceflight physiology and gene polymorphisms impact the PK of drugs is needed. Although studies have tested the PK disposition of a few medications during spaceflight and under simulated microgravity environments, it is impractical to empirically test the impact of microgravity on all drugs that may be provided to astronauts. This study aims to use physiologically-based pharmacokinetic (PBPK) modeling to evaluate how the physiological changes associated with long-duration exposure to microgravity impact drug disposition of medications that may be included in medical kits for lunar and planetary space missions.

Methods: This study used the US EPA's High Throughput Toxicokinetics (httk), a PBPK model that estimates chemical exposure doses in the body for thousands of drugs simultaneously. Physiological parameters were adjusted to reflect current astronaut demographics, and spaceflight modifiers were applied to measured changes during spaceflight, including glomerular filtration rate (GFR), hepatic metabolism, body weight, hematocrit, cardiac output and fraction of drug absorbed in gut. In addition, to explore the role of genetic diversity in drug disposition, metabolic enzyme polymorphisms were introduced using published prevalence data. Model predictions were compared between terrestrial and spaceflight modified conditions for 74 drugs that were grouped into four classes defined by the Biopharmaceutical Drug Disposition Classification System (BDDCS), and the predicted blood concentrations were evaluated. **Results:** Sensitivity analyses showed that GFR, hepatic metabolism and fraction of drug absorbed in gut had the strongest effects on steady state parent-drug blood concentrations. Conversely, the impact of the cephalic shift of fluids and volume loss, which are commonly associated with microgravity, had a negligible effect. Genetic polymorphisms primarily affected Class 1 drugs, with their Oral Equivalent Doses (OEDs) exhibiting fold changes below -0.5 in 8 out of 30 drugs. High metabolism drugs from Class 1 and 2 were predicted to have a greater negative change in spaceflight, with lower OEDs compared to Earth, than Class 3 and 4 drugs. Specifically, two Class 1 drugs, diazepam and fluoxetine, as well as clobidogrel (Class 2), had the lowest fold change, with OED fold changes below -1.5. GFR spaceflight modifiers affected 13 of the 25 drugs from Classes 3 and 4. **Conclusions:** This study uses a high-throughput PBPK model to determine the effects of spaceflight and genetic polymorphisms on drug dosimetry and highlights how predictive approaches can improve personalized medicine for astronauts and the military in physiology-altering environments. The model is flexible and can be updated as impacts of long-term spaceflight on human physiology and mission parameters become clearer. PBPK modeling predictions can be used to guide medical treatment with drugs that exhibit significant PK changes in space, target drug classes for further study, and support drug provisioning for exploratory missions. The httk model enables exploration into the effect of spaceflight on drug disposition and preliminary evaluation of genetic variability in metabolism. Future steps could involve developing bespoke models which can evaluate physiological effects such as fluid shifts associated with microgravity to better characterize spaceflight PK.

PS 3565 Towards a defined approach to assess pesticides for their potential to cause parkinsonian neurodegeneration

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Background and Purpose: The pathophysiology of Parkinson's Disease (PD) implicates the death of dopaminergic neurons projecting from the substantia nigra pars compacta, which contribute to the control of motor function. Epidemiological studies indicate exposure to pesticides represents a risk factor in acquiring PD. Adverse Outcome Pathway 3 (AOP3) outlines the potential of chemical exposure to inhibit complexes of the mitochondrial respiratory chain in dopaminergic neurons, triggering a cycle of cell death and neuroinflammation leading to Parkinsonian motor manifestations. We developed a test battery of New Approach Methodologies (NAMs) utilizing human cell-based and zebrafish embryo assays to potentially link exposure to pesticides used in Europe to mitochondrial complex inhibition, neurodegeneration, and ultimately Parkinsonian motor deficits. **Methods:** 143 pesticides and reference compounds were tested at a single concentration in permeabilized human dopami-

nergic cells (LUHMES) to assess their potential to inhibit individual complexes of the respiratory chain (complexes 1-3). In parallel, potential effects on neurite architecture and cell viability were evaluated in intact LUHMES cells exposed to eight concentrations of the test compounds using high-content imaging. Exploratory characterization of the zebrafish dopaminergic system at 5 days post fertilization (dpf) was carried out through high-content confocal dorsal imaging of the Tg(*slc6a3:EGFP*) line, in which neurons expressing the dopamine transporter (DAT) are labeled with green fluorescent protein (GFP) and behavior was assessed in the Visual and Acoustic Motor Response (VAMR) NAM, capturing 26 behavior-based endpoints. **Results:** NAMs were anchored to AOP3 which describes inhibition of mitochondrial complex 1 (key event; KE1), linked to mitochondrial disruption (KE2), impaired proteostasis (KE3), degeneration of dopaminergic neurons (KE4), and Parkinsonian motor defects (adverse outcome). An initial prediction model identified 24 likely AOP3 positive compounds that inhibited one or more of the mitochondrial complexes (KE1) and reduced neurite mass at lower concentrations than those necessary to provoke cell death (KE4). An additional 14 chemicals were identified to be inactive in all assays. Ten chemicals inhibited a mitochondrial complex and caused neurite loss but not cell death and were classified as potential AOP3 positives. High-content imaging of the dorsal zebrafish brain resulted in quantifiable DAT-expressing neurons, including the main dopaminergic groups within the subpallium and diencephalon. 24 h exposure to known complex I inhibitors MPTP or 6-OHDA induced widespread behavioral hypoactivity across visual motor and acoustic startle endpoints at 5 dpf, suggesting that these compounds can be used to disrupt mature signaling systems to produce bradykinesia-like behaviors rather than impairing the developmental formation of motor circuitry, a key distinction for modeling PD in larval zebrafish. **Conclusions:** This work demonstrates the ability of *in vitro* and *in vivo* NAMs to identify AOP3 positive pesticides. Compounds identified as hits in cell-based NAMs will be tested for mitochondrial respiration inhibition in intact cells. For selected compounds, experiments in zebrafish will assess whether a loss of dopaminergic neurons results in concurrent PD-like behavioral effects. Finally, an AOP-informed Integrated Approach to Testing and Assessment (IATA) will be developed for one chemical as a best practice case study for the use of NAMs to assess effects associated with Parkinsonian-type neurotoxicity.

PS 3566 Prolonged Low-Dose Paraquat Inhalation Model in Mice Reveals Sex Specific Dopaminergic Neurodegeneration and Behavioral Deficits

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Background and Purpose: Paraquat (PQ) is an industrial herbicide used to kill weeds and green plant matter during harvest season. Long-term exposure to PQ has been epidemiologically associated with increased risk of Parkinson's Disease (PD) and neurodegeneration in agricultural workers and rural communities in the United States (US). While PQ is banned in many countries throughout the globe, it remains widely used in the US, partially due to its efficacy and affordability. While inhalation of PQ has been known to induce systemic organ failure at acute concentrations, prolonged low-dose inhalation from agricultural pesticide drift has not been readily evaluated for its potential to contribute to neurodegenerative diseases *in vivo*. Instead, many animal studies have relied on other routes of exposure, including ingestion and intraperitoneal injections, which do not effectively replicate the most common ways that individuals are exposed to PQ in the environment. **Methods:** Whole-body inhalation exposures were conducted in the University of Rochester Inhalation Exposure Facility. Exposures were conducted 5 days a week for 12 weeks. Animals were exposed to either filtered air or PQ aerosols for 4 hours, during which time the mice were singly housed in wire-mesh chambers inside a 30-L steel-reinforced Lexan exposure chamber. PQ aerosols were produced by adding a 0.2 mg/mL solution of PQ in ddH₂O (made fresh every day) into an ultrasonic nebulizer (Ultrasonic2000, Novag Dental and Medical Equipment). Clean, dry air (approximately 500 mL/min) was passed through the nebulizer at a frequency of 2.4 MHz to produce a PQ mist, which then passed through a heated drying tube and a cold trap to remove moisture. The subsequent PQ particles were mixed with diluting air and passed into the exposure chamber at a rate of 25-30 L/min. A condensation particle counter (CPC Model 3022 A, TSI Inc.) was used to capture real-time exposure measurements, allowing for the quantification of continuous particle count concentrations. The particle size distribution of the aerosol was measured once per day using an electrostatic classifier (SMPS Model 3071, TSI Inc.). Three filters (Pallflex Membrane Filters, Pall Life Science) were collected hourly to determine gravimetric exposure concentration by mass. The concentration of PQ on the filters was quantified chemically using a UV-Vis spectrophotometer (HP-Agilent Technologies) at an absorbance wavelength of 259 nm. Analytical standard grade PQ (PQ dichloride hydrate PESTANAL®, ≥98.0% purity) was purchased from Millipore Sigma (CAS No: 75365-73-0). **Results:** Here, we establish and characterize an environmentally relevant mouse model of low-dose PQ inhalation that captures key behavioral deficits and pathophysiological hallmarks of PD. These include mild locomotor dysfunction, anxiety, and depression-like phenotypes, significant reductions in dopamine in the striatum, along with alterations in dopamine synthesis. We validated that PQ can enter the brain, as it was detected in the olfactory bulb, cortex, and the brain stem. The highest concentration was found to be in the olfactory bulb. These findings support the hypothesis that PQ, when inhaled, can bypass the BBB through an alternative route of entry into the CNS. This

likely occurs through sensory neurons in the olfactory epithelium that innervate the olfactory bulb and are partially exposed to the surrounding environment **Conclusions:** This model can be used to more accurately replicate environmental exposure to PQ, leading to a more accurate recapitulation of its toxic effects in humans. Further research on PQ should emphasize low-dose inhalation, given the strength of the epidemiological literature associating prolonged exposure to PD.

PS 3567 Tiny messengers, big impact: EV-miRNAs induce apoptotic signaling pathways in rotenone-induced Parkinson's Disease model

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Background and Purpose: Parkinson's Disease (PD) is a progressive neurodegenerative disorder characterized by the selective loss of dopaminergic neurons, primarily within the nigrostriatal pathway. Rotenone, a naturally occurring insecticide and pesticide, is a potent inhibitor of mitochondrial complex I and is implicated in PD pathogenesis. *In vivo* rotenone models replicate key PD pathogenesis and neuropathology. Emerging evidence indicates that PD pathology may propagate through intercellular communication, particularly via extracellular vesicles (EVs). miRNAs enriched in the EVs, present in biofluids such as serum, have gained attention as potential early biomarkers and mechanistic mediators of PD progression. Preliminary studies demonstrated consistent downregulation of miR-30a-5p and miR-484 in serum-derived exosomes from rotenone-treated young adult and aged rats, accompanied by mitochondrial dysfunction and neuronal cell death. This consistent pattern across ages suggested that these miRNAs may regulate shared pathological mechanisms contributing to PD progression. Bioinformatic analyses predicted that miR-30a-5p and miR-484 target genes associated with mitochondrial functions and apoptotic signaling pathways. The current study aimed to elucidate how downregulation of these miRNAs contributes to neurotoxicity. We aimed to test the hypothesis that reduced expression of miR-30a-5p and miR-484 upregulates pro-apoptotic genes, BNIP3L and CASP2, respectively, leading to mitochondrial dysfunction-mediated neuronal death. **Methods:** Serum-derived EVs were isolated from 15-month old (aged) rotenone-treated rats (a single dose of 3 mg/kg, i.p.). EVs were collected 8 and 24 hours after rotenone treatment. These EVs were incubated with SH-SY5Y neuroblastoma cells. Cell viability was assessed using MTT and calcein-AM assays. BNIP3L and CASP2 were identified and validated as target genes of miR-30a-5p and miR-484, respectively by RT-qPCR. To further characterize the apoptotic response, downstream gene expression of Bax, Bak, and BCL2 was evaluated to assess pro- and anti-apoptotic signaling balance. Gene expression levels were quantified following miRNA modulation and rotenone exposure using RT-qPCR. **Results:** RT-qPCR analysis confirmed that BNIP3L and CASP2 are direct targets of miR-30a-5p and miR-484, respectively. Treatment of SH-SY5Y cells with serum-derived EVs from rotenone-exposed rats resulted in significant upregulation of BNIP3L and CASP2, indicating activation of the BNIP3L-CASP2 apoptotic pathway. Since EVs from young adult rats previously induced mitochondrial dysfunction and cell death, EVs from aged rotenone-treated rats were further evaluated and found to similarly reduce cell viability under rotenone toxicity. Downstream apoptotic profiling revealed that increased BNIP3L expression correlated with Bax and Bak upregulation and BCL2 suppression, confirming activation of the apoptotic cascade. **Conclusions:** The findings indicate that rotenone-induced downregulation of miR-30a-5p and miR-484 enhances the expression of pro-apoptotic genes BNIP3L and CASP2, leading to mitochondrial dysfunction, Bax/Bak activation, BCL2 inhibition, and subsequent neuronal death. These results provide mechanistic insight into miRNA-mediated apoptotic signaling and identify EV-miRNAs as potential therapeutic targets for mitigating neuronal cell death in PD.

PS 3568 Impact of neuromelanin formation on modeling Parkinson's Disease in rats and its implications in Parkinson's disease neurobiology

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Background and Purpose: Parkinson's Disease (PD) is a chronic, neurodegenerative disease characterized by the death of dopaminergic (DA) neurons in the substantia nigra of the midbrain, producing the condition's hallmark motor symptoms. While genetic and environmental risk factors have been identified, there are critical gaps in the understanding of the biochemical mechanisms at play. Heterocyclic aromatic amines (HAAs) formed during the cooking of red meats are known as dietary carcinogens. Prior studies have indicated that HAAs, specifically 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP), may induce mitochondrial dysfunction and neurotoxicity in DA-neurons, which are susceptible to toxicants. Neuromelanin (NM) is a unique dark pigment found in humans, specifically in dopaminergic neurons, and it is believed to play a significant role in protecting against oxidative stress induced by environmental toxicants, such as HAAs. Cellular studies show that HAAs bind to neuromelanin, and neuromelanin formation increases HAA neurotoxicity. Rodents do not express tyrosinase in dopaminergic neurons, which is the enzyme responsible for producing neuromelanin. Expression of human tyrosinase enables neuromelanin

synthesis, which is a more accurate model reflecting human neurobiology. This study aims to validate a transgenic rodent model in which human tyrosinase is expressed in a rat model, producing neuromelanin, thus allowing for an investigation into interactions between NM and HAAs that induce neuronal mitochondrial dysfunction and neurodegeneration. **Methods:** Transgenic rats expressing human tyrosinase were generated using CRISPR-mediated genome editing. Progeny was genotyped using PCR-based assays to distinguish wild-type, heterozygous, and homozygous animals and confirm tyrosinase expression. Neuromelanin formation in dopaminergic neurons was validated through histological analysis, including Fontana Masson staining. **Results:** Genotyping has confirmed successful insertion of the tyrosinase-expressing gene into the genomes of some rats. Neuromelanin production has been validated through histology in tyrosinase-expressing rodents, which show NM granules resembling those found in human substantia nigra neurons. Ongoing analysis will help determine to what degree NM formation amplifies HAA-induced oxidative stress and dopaminergic neurodegeneration. Early *in vitro* work suggests that NM expression increases intracellular PhIP concentration and cell death. **Conclusions:** The validation of the NM-forming, transgenic rat model is a major step forward in overcoming translational limitations in research aimed at neurodegenerative diseases, including Parkinson's Disease and many others. This model provides the unique opportunity to understand how environmental toxicants, like PhIP, contribute to mitochondrial and autophagic dysfunction relevant to PD. Preliminary work confirms NM production, and ongoing work aims to define how NM interacts with HAAs and results in DNA adduct formation, autophagic impairment, and PD pathogenesis. Beyond Parkinson's Disease, this model may provide a foundation for studying broader types of environmental neurotoxicants and advancing the shared understanding of environmentally induced dopaminergic neurodegeneration.

3569 Caffeic Acid Mitigates α -Synuclein Neurotoxicity by Suppressing Fyn Kinase Signaling and α Syn Aggregation

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Background and Purpose: Parkinson's disease (PD) and related synucleinopathies are characterized by the accumulation of misfolded α -synuclein (α Syn) aggregates that exert neurotoxic and pro-inflammatory effects. Studies from our laboratory and others have reported that α Syn pre-formed fibrils (PFFs) act as proteinaceous neurotoxins that seed fibril propagation and activate inflammatory signaling in microglia via Fyn kinase. Caffeic acid (CA), a naturally occurring phenolic compound abundant in coffee and fruits, is well-characterized for its antioxidant, anti-inflammatory, and anti-fibrillogenic properties. Given its ability to modulate both anti-fibrillogenic activity and inflammatory signaling, CA was investigated as a dual-action modulator of α Syn-induced neurotoxicity. Thus, this study examined whether CA can mitigate α Syn-induced neurotoxicity by suppressing proinflammatory Fyn kinase activation and inhibiting α Syn seeding activity. **Methods:** Recombinant human α Syn monomers were purified and incubated under continuous agitation at 37°C for seven days to generate pre-formed fibrils (PFFs) according to the MJFF protocol. C20 human microglial cells were exposed to 1 μ M α Syn PFFs for 1-24 h, with or without co-treatment of 0-100 μ M CA. Cells were harvested at various time points for RT-qPCR analysis of cytokine expression (IL-6, pro-IL-1 β , and TNF- α) and for immunoblotting of total Fyn and phosphorylated Fyn (p-SrcY416). Immunofluorescence staining for IBA1, Fyn, and p-SrcY416 was also performed to validate CA's effect on Fyn activation. α Syn seeding reactions were initiated in different dilutions of PD brain homogenate and contained 10 μ M ThT (Thioflavin). Fluorescence kinetics were monitored for up to 50 h at 37°C with orbital shaking. Concentrations of 300 nM-100 μ M CA were tested to assess concentration-dependent inhibition of α Syn seeding activity. Reaction end-products were spotted onto nitrocellulose membranes and immunoprobed with an α Syn filament-specific antibody to assess fibril formation. **Results:** Exposing human microglial cells to 1 μ M α Syn PFFs elicited a robust proinflammatory response, evidenced by marked upregulation of TNF α , IL-6, and pro-IL-1 β mRNA levels. Co-treatment with CA significantly suppressed α Syn PFF-induced TNF α and IL-6 expression and showed a downward trend in pro-IL-1 β induction, demonstrating the anti-inflammatory properties of CA. To further elucidate the mechanisms underlying this effect, we examined CA's impact on activation of the tyrosine kinase Fyn, a critical upstream regulator of the PKC δ -NF- κ B inflammatory axis. Western blot analyses revealed that α Syn PFF exposure triggered a rapid Fyn activation (evidenced by increased p-SrcY416 phosphorylation) within 1 h that persisted for up to 24 h, whereas co-treatment with CA markedly attenuated Fyn hyperphosphorylation at all time points examined. Immunocytochemical analysis further confirmed CA-mediated inhibition of Fyn activation. Interestingly, RT-QuIC analysis demonstrated that CA addition dose-dependently inhibited α Syn seeding activity derived from human PD brain and skin samples. This reduction in α Syn fibrillization was further validated by dot-blot analysis, confirming that CA exerts both anti-aggregative and anti-inflammatory effects against α Syn pathology. **Conclusions:** Collectively, these findings identify CA as a promising small-molecule modulator that targets both cellular neuroinflammatory responses and molecular mechanisms underlying α Syn misfolding and aggregation, supporting its translational potential in PD and related synucleinopathies. Supported by NIH/NINDS R01 NS121692 and NIH/NIEHS R01 ES036241.

3570 A Trichloroethylene Metabolite Drives Microglial Aging and Inflammation: Implications for Parkinson's Disease

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Background and Purpose: Parkinson's Disease (PD) is a leading motor neurodegenerative disorder marked by the loss of tyrosine hydroxylase (TH)-positive neurons in the midbrain and the formation of α -synuclein-rich aggregates known as Lewy bodies. While genetics explain only about 10-15% of PD cases, environmental exposures are gaining attention as major contributors. Trichloroethylene (TCE), a common industrial solvent, has been implicated in PD and shown to induce α -synuclein aggregation, dopaminergic neuron loss, and motor dysfunction in animal models. However, the role of TCE's metabolite S-(1,2-dichlorovinyl)-cysteine (DCVC) in promoting neuroinflammation remains largely unknown. Since microglia—the brain's immune cells—are central to neuroinflammation and aging in PD, we examined how DCVC affects microglial metabolism, inflammation, and senescence. We also explored whether senolytics quercetin and dasatinib could reverse these effects. **Methods:** We first conducted an MTS assay to measure microglial metabolic activity across DCVC concentrations. A 10 μ M dose significantly reduced viability and was used for subsequent experiments. Mitochondrial function was then assessed with a Seahorse Mito Stress Test, measuring basal respiration, ATP production, and non-mitochondrial respiration. To probe inflammatory signaling, we used immunocytochemistry (ICC) and qRT-PCR to quantify interleukin-6 (IL-6) expression—a marker of the senescence-associated secretory phenotype (SASP). Cellular aging was confirmed with the senescence-associated β -galactosidase (SA- β Gal) assay. Similar experiments were performed in human astrocytes to evaluate cell-type specificity. To test for therapeutic rescue, microglial cultures were co-treated with quercetin and dasatinib, a senolytic combination known to selectively eliminate senescent cells and suppress chronic inflammation. **Results:** DCVC exposure disrupted microglial metabolism, leading to decreased basal respiration, ATP production, and non-mitochondrial respiration, indicating mitochondrial dysfunction. ICC and qRT-PCR revealed increased IL-6 expression, consistent with activation of the SASP, while the SA- β Gal assay confirmed enhanced microglial senescence. In contrast, astrocytes showed no significant changes, suggesting a microglia-specific vulnerability. Remarkably, co-treatment with quercetin and dasatinib restored mitochondrial function, reduced IL-6 levels, and diminished SA- β Gal staining. These senolytics effectively reversed several DCVC-induced phenotypes, demonstrating their potential to counteract environmentally driven microglial aging. **Conclusions:** Our findings reveal that the TCE metabolite DCVC promotes microglial mitochondrial dysfunction, inflammation, and senescence, contributing to cellular conditions associated with PD. The reversal of these effects by quercetin and dasatinib highlights the therapeutic potential of targeting microglial aging in toxin-induced neurodegeneration. Importantly, this work strengthens our understanding of gene-environment interactions in PD: environmental toxicants like TCE may accelerate neurodegenerative processes in individuals with genetic susceptibility by triggering microglial senescence and chronic inflammation. By uncovering this mechanistic link, our study bridges molecular toxicology and neurogenetics, offering a clearer view of how environmental exposures interact with cellular pathways to shape disease risk.

3571 Whole-Brain Mapping Reveals a Novel Neurogenic Role of Prokineticin-2 Signaling in the SVZ-RMS-OB During Parkinsonian Neurotoxic Stress

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Background and Purpose: Chronic exposure to manganese (Mn) from environmental and occupational settings is linked to the development of parkinsonism, including Parkinson's disease (PD). Prior to the onset of motor symptoms, parkinsonism and PD patients frequently display olfactory dysfunction, but the underlying mechanisms remain poorly understood. Emerging evidence also suggests that the subventricular zone (SVZ) neurogenic niche may be affected in PD pathology. Our lab previously discovered that prokineticin-2 (PK2) mediates a novel neuroprotective response during the early stages of parkinsonian neurotoxic stress by inducing mitochondrial biogenesis and activating anti-apoptotic signaling pathways. PK2 is a secreted neuropeptide that initiates its biological functions by activating two cognate GPCRs, PKR1 and PKR2. Intriguingly, PK2 plays a well-established role in olfactory bulb (OB) neurogenesis by serving as a chemoattractant for migratory neuroblasts in the rostral migratory stream (RMS). Building on these observations, we hypothesized that PK2 serves as an early compensatory neuropeptide against Mn-induced neurotoxic stress in the OB-RMS-SVZ neurogenic niche. A comprehensive understanding of PK2 signaling may enable the development of novel drug discovery platforms targeting non-motor dysfunctions associated with neurodegenerative diseases. **Methods:** We utilized the NIH Accelerating Medicines Partnership PD (AMP-PD) clinical and transcriptomics datasets to determine changes in PK2 mRNA expression in the blood of PD patients. For *in vivo* experiments, transgenic PK2-eGFP reporter mice were intranasally exposed to 300 μ g of MnCl₂ for 5, 10, or 30 days. Brain Mn deposition was visualized using preclinical 7T MRI, and behavioral performance was assessed via the open-field, pole, and novel-scent tests. We used iDISCO whole-brain tissue clearing, immunohistochemistry (IHC), and Western blot analyses to map the spatial distribution of PK2 in the brain of Mn-treated mice. PK2-dependent effects in

Mn neurotoxicity were tested by co-treating mice with MnCl₂ (300µg) and the PK2 receptor antagonist PKA7 (5 mg/kg) for 5 days. Additionally, mice were injected with 6-OHDA in the striatum and sacrificed after 3 days for histological analysis. **Results:** We first evaluated whether PK2 serves as a potential biomarker of PD by analyzing the AMP-PD whole-blood transcriptome and clinical datasets. A comparison of the control (n = 1,097) and PD (n = 1,624) cohorts revealed a significant upregulation of PK2 mRNA. Surprisingly, PK2 was further enriched in prodromal patient samples and was positively correlated with motor dysfunction in PD patients. Next, *in vivo*, PK2-eGFP reporter mice intranasally exposed to MnCl₂ showed increased MRI signal intensity in olfactory regions, basal ganglia, and the SVZ, accompanied by olfactory and locomotor deficits following 10 days of Mn exposure. IHC revealed strong PK2 induction in SOX2⁺/GFAP⁺ neural stem cells in the subependymal layer of the OB after a 5-day Mn exposure, coinciding with increased DCX immunostaining, a marker for migrating neuroblasts. These effects were diminished after 10- and 30-day exposure to Mn. Interestingly, we observed PK2⁺ cells in the RMS and nucleus accumbens (NAc) that were contacting neuroblasts in the SVZ and RMS. The iDISCO tissue clearing and light sheet microscopy confirmed PK2⁺ neurons projecting from the NAc to the SVZ and revealed Mn-induced architectural alterations in the SVZ-RMS-OB axis. Notably, PKA7 inhibition of PK2 receptors attenuated the increase in DCX immunostaining in the OB that was observed after 5 days of Mn exposure. Finally, using the 6-OHDA mouse model of PD, we observed a significant increase in PK2⁺/SOX⁺ cells at the lesion site, further suggesting a similar compensatory PK2 response to neurotoxic injury. **Conclusions:** Collectively, PK2 appears to facilitate a novel compensatory neuroprotective signaling response during the early stages of Mn-induced neurotoxicity in the SVZ-RMS-OB axis. These results underscore PK2 as a key mediator linking environmental neurotoxicant exposure to compensatory neurogenic mechanisms and as both a promising therapeutic target and a potential biomarker for addressing environmentally linked neurodegenerative conditions (Supported by NIH/NIEHS R01 ES034196).

PS 3572 Molecular Mechanisms of Nuclear Pore Complex Impairment in Environmental Neurotoxicity Models: Implications for Pathogenesis of Parkinson's Disease

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Background and Purpose: Environmental exposure to neurotoxic chemicals like pesticides, metals, and pollutants has been implicated in the etiology of Parkinson's disease (PD). However, the mechanisms driving neurotoxicant-induced neurodegeneration remain poorly understood. The nuclear pore complex (NPC), which mediates bidirectional nucleocytoplasmic transport of proteins and RNAs, has recently emerged as a critical pathophysiological target of neurodegenerative disorders. Our recent work demonstrated that mitochondria-impairing pesticides activate protein kinase C delta (PKCδ) and induce Lamin B1 hyperphosphorylation, thereby compromising nuclear membrane integrity. Yet, the role of nucleoporins (Nups), functional unit of NPCs, in environmentally linked neurotoxicological processes has not been systematically investigated. **Methods:** Protein levels of Nups were quantified by immunocytochemistry (ICC) and Western blot (WB) in N27 dopaminergic neuronal cells (DAergic cells) exposed to rotenone (1 µM, 3-6 h) or paraquat (400 µM, 12 h) in serum-free RPMI-1640 medium. PKCδ dependence of Nup62 loss was evaluated by WB in CRISPR/Cas9-based PKCδ-knockdown (KD) cells under identical treatment conditions. In addition, nuclear fractions from DAergic cells/DAergic cells were isolated for phosphoproteomic analysis, and Nup153 phosphorylation was validated by proximity ligation assay (PLA). *In vivo*, immunohistochemistry (IHC) for Tyrosine hydroxylase (TH) and Nups was performed in MitoPark mice. Reductions in Nup153 and Nup50 levels were further confirmed by IHC in dementia with Lewy bodies (DLB) patient brain tissues obtained from the UC Davis Health Alzheimer's Disease Research Center. **Results:** Rotenone exposure in N27 DAergic neuronal cells resulted in a time-dependent reduction of the nuclear basket Nup153, the channel Nup62, and the scaffold Nup107 within 3-6 h, without overt cytotoxicity, as confirmed by ICC and WB analyses. Treatment of N27 cells with the redox-cycling pesticide paraquat resulted in a convergent phenotype, characterized by pronounced reductions of Nup62 and 153, increased Lamin B1 phosphorylation, and fragmented perinuclear organization of Nup62 and 153 as revealed by 3D confocal reconstruction, consistent with nuclear envelope destabilization and loss of NPC structural integrity. Further WB analysis identified PKCδ as a key mediator of mitochondrial stress-induced Nup dysregulation, as CRISPR/Cas9-mediated PKCδ-KD significantly rescued Nup62 from rotenone-induced downregulation. Phosphoproteomic profiling of nuclear fractions from rotenone-treated N27 cells identified hyperphosphorylation of Nup153, which was validated by PLA, showing enhanced Nup153-phosphoserine interactions following rotenone treatment. Notably, our transgenic MitoPark mouse model of PD exhibited a drastic decrease in nuclear Nup153, 62, and 107 and accumulation of cytoplasmic Nup62 puncta in intact TH⁺ nigral neurons, accompanied by nuclear membrane invaginations. Human post-mortem brains from DLB patients similarly showed diminished levels of Nup62 and 50, as well as disorganized nuclear morphology, validating clinical relevance. **Conclusions:** Taken together, our findings identify Nup/NPC dysregulation as a major neurotoxicological mechanism mediated by PKCδ activation during pesticide-induced mitochondrial/oxidative stress, provid-

ing a mechanistic link between neurotoxic chemical exposure and selective neuronal loss in environmentally linked PD. Supported by NIH R01 Grants: NS121692 and ES034196.

PS 3573 CeMbio Gut Microbiota Influences the Neuroprotective Effects of Tea Catechins in a Transgenic C. elegans Parkinson's Disease Model

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Background and Purpose: The gut microbiota has a direct influence on the metabolism and neuroprotective potential of dietary polyphenols such as tea catechins (TCs). The study conducted has investigated how defined gut microbial communities (CeMbio consortium) exerts a modulating TC-mediated neuroprotection in the *Caenorhabditis elegans* Parkinson's disease (PD) model UA44, which expresses human α-synuclein in dopaminergic neurons. **Methods:** Twelve bacterial strains from the CeMbio community were tested individually and as a pooled mixture against the standard laboratory diet *E. coli* OP50. L4-stage UA44 worms were exposed to 0-200 µg/mL TCs for seven days at 25 °C. Dopaminergic neuron integrity was assessed by fluorescence microscopy using a neuronal damage scoring scale (0-3), where 0 = all six head neurons intact and 3 = all six neurons degenerated (n = 30 worms × 3 replicates). Dopamine-linked behaviors—area-restricted search (ARS) and basal slowing response (BSR)—were quantified under food and no-food conditions using video-based analysis. **Results:** TC treatment significantly reduced neuronal degeneration in a **dose- and strain-dependent** manner (two-way ANOVA, p < 0.001). In OP50-fed worms, the mean degeneration score decreased from **2.4 ± 0.3 (control)** to **1.6 ± 0.2 at 200 µg/mL TC** (33 % improvement). CeMbio-mix-fed worms showed greater protection, with scores of **1.1 ± 0.2 at 100 µg/mL** and **0.8 ± 0.1 at 200 µg/mL**, corresponding to ~65 % less degeneration than OP50-fed worms (p < 0.001). Among individual strains, *Lelliottia amnigena* (JUB 66) and *Stenotrophomonas maltophilia* (JUB 19) exhibited the lowest damage scores (**0.9 ± 0.2** and **1.0 ± 0.2**, respectively), whereas *Pseudomonas lurida* (MYb11) remained highly susceptible (**2.3 ± 0.3**). Behavioral assays mirrored the neuronal trends: CeMbio-fed worms displayed a **higher basal slowing index (0.68 ± 0.04)** compared with **0.42 ± 0.05 in OP50-fed controls** (p < 0.01) and recovered ARS turning frequency to **14.6 ± 1.2 turns/min** versus **9.5 ± 1.5 turns/min** (p < 0.05). **Conclusions:** CeMbio-associated microbes significantly potentiate the neuroprotective effects of TCs by reducing dopaminergic neuronal degeneration and improving dopamine-regulated behaviors. The protection is strain-specific, with *Lelliottia amnigena* and *Stenotrophomonas maltophilia* providing the most pronounced benefit. These results suggest that microbial enzymatic conversion of catechins into bioactive metabolites (e.g., phenolic acids, valerolactones) underlies the observed reduction in α-synuclein-associated neurotoxicity in the *C. elegans* PD model.

PS 3574 Trichloroethylene metabolites in Parkinson's Disease-related toxicity in C. elegans and mice

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Background and Purpose: Trichloroethylene (TCE) is an industrial solvent associated with Parkinson's Disease (PD) onset in humans. Studies characterizing TCE metabolism in humans indicate that 40-75% of the absorbed dose is metabolized, suggesting that TCE's toxic effects may be attributed to these metabolites. However, there is limited knowledge of TCE metabolites and their neurotoxicity in PD. We performed TCE inhalation studies in mice and then conducted high-resolution mass spectrometry (HRMS) on multiple organs, including the brain, to determine which TCE metabolites are present in the system post-acute exposure to occupationally and environmentally relevant exposures to TCE. We then used *Caenorhabditis elegans* (*C. elegans*) to study how these metabolites disrupt dopamine-dependent behaviors, such as movement and fecundity, and dopamine neuron viability. **Methods:** Mice were exposed to 100 ppm of TCE for 4 hours. Blood, brain, lungs, and liver were collected from each animal at 0, 1, 2, 4, 8, and 24 hours after exposure or 0 hours after no exposure. Using the Thermo Orbitrap IQ-X system, a liquid chromatography-based mass spectrometry system (LC-MS/MS), brain tissue, liver tissue, and plasma from TCE-exposed mice were analyzed for the presence of TCE metabolites. A follow-up untargeted analysis was conducted using brain and liver tissue. In *C. elegans*, wild-type (N2), pdat-1::GFP (BZ555), and pdat-1::α-syn, pdat-1::GFP (UA44) strains were exposed to 5 mM trichloroethanol (TCEOH), 10 mM trichloroacetic acid (TCA), or control doses. Worms were exposed to these compounds at L4 stage and the dose was refreshed every 48 hours until assay completion. Fecundity assay and behavioral assays were conducted using all strains. Area-restricted searching (ARS) assay was conducted, as well as general measures of movement, using the WormLab Behavioral Analysis Software. Using the fluorescent reporter strains, dopaminergic neuron viability was assessed in both the neuron cell bodies and the dendrites. **Results:** HRMS-analysis of TCE-exposed mouse brain, liver, and plasma identified three TCE metabolites, TCA, trichloroethanol glucuronide (TCEOH-gluc), and dichloroacetic acid (DCA). Untargeted analysis of the TCE-exposed mouse brain and liver revealed that the greatest amount of significantly different features occurred at 4

hours post-exposure, when compared to the untreated control. In *C. elegans*, we observed that TCEOH led to the most notable differences across the assays. In particular, the UA44 strain that was treated with 5 mM TCEOH exhibited a significant increase in omega bends and pirouettes, markers of altered dopamine function, as compared to the control-treated worms on day 1, followed by a significant decrease in both by day 5. Additionally, the UA44 strain exhibited more pronounced neurodegeneration as compared to the other doses or strains. **Conclusions:** Together, these findings indicate that TCA, DCA, and TCEOH-gluc are the most prevalent TCE metabolites in the brains of mice exposed to TCE by inhalation. Disruption of metabolic pathways was most dramatic at 4 hours post-exposure. Administration of the metabolites identified in mouse brain to *C. elegans* model indicates that TCEOH can lead to changes in behavioral outcomes, as well as dopamine neuron degeneration. Given that many of the significant results were from a strain that also expresses human α -synuclein, it is possible that TCEOH may exert these significant changes through an interaction with α -synuclein. Additionally, these findings indicate that *C. elegans*-based assays can be used to identify changes in PD-relevant outcomes from TCE metabolite exposure, providing a complement to the mouse studies and enabling the systematic analysis of TCE metabolites.

PS 3575 Inhibition of Mitochondrial Complex III Causes Dopaminergic Neurodegeneration by Redox Stress in *Caenorhabditis elegans*

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Background and Purpose: Environmental factors including chemical exposures are important contributors to Parkinson's disease (PD). Nearly all well-validated chemicals involved in PD also affect mitochondria. However, it is not feasible to test all the chemicals that induce mitochondrial dysfunction. Moreover, most of the chemicals connected to PD inhibit complex I of the mitochondrial electron transport chain, leading to oxidative stress and ATP depletion. We are working to define if other forms of mitochondrial dysfunction result in dopaminergic neurodegeneration, as well as whether oxidative stress and ATP depletion, are required for dopaminergic neurodegeneration. This information should help narrow the focus of efforts to identify chemicals that could contribute to PD. Here, we report results from inhibition of complex III. **Methods:** We are using the model organism *C. elegans* to evaluate the *in vivo* effects of toxic mitochondrial exposures on dopaminergic neurodegeneration (based on morphological changes), ATP levels, and redox state. We exposed *C. elegans* from the embryo stage until the L4 larval stage to complex III inhibitors antimycin A and pyraclostrobin, which are environmentally relevant pesticides. We acquired images of the animals' heads using fluorescence microscopy, capturing the 4 cephalic dopaminergic neurons. Neurodegeneration was quantified by assigning a score of 0 to 6 to each neuron, depending on the type and extent of damage. We used pharmacological (N-acetylcysteine, S3QEL-2, and others) and genetic (superoxide dismutase *sod-2* overexpression, and others) antioxidant interventions to rescue neurodegeneration. We used an optogenetic approach to generate reactive oxygen species (ROS) at complex III to test if this would cause neurodegeneration independent of chemical exposure. **Results:** Complex III inhibitors antimycin A and pyraclostrobin caused dopaminergic neurodegeneration in a dose dependent manner, and significant neurodegeneration was observed for concentrations of 500 nM and 50 μ M respectively. Complex III inhibitors did not induce neurodegeneration in glutamatergic, cholinergic, serotonergic, or GABAergic neurons, nor in neuronal sheath cells. Additionally, antimycin A increased the ratio of oxidized to reduced roGFP, a reporter of redox state in dopaminergic neurons. Rescue experiments with an antioxidant (N-acetylcysteine), and a blocker of ROS specific to Complex III (S3QEL-2) indicated that neurodegeneration linked to complex III inhibitor exposure is caused by an increased production of mitochondrial ROS. Additional rescue experiments with a superoxide dismutase and catalase mimetic (EUK-134) and exposure using a *C. elegans* strain overexpressing the mitochondrial superoxide dismutase gene *sod-2*, strongly confirmed the role of superoxide anion in neurodegeneration driven by complex III inhibition. Finally, artificial production of superoxide anion specifically at the complex III site using an optogenetic approach caused dopaminergic neurodegeneration. This confirmed, in a manner independent of chemical exposure, that ROS generation at the complex III site can cause dopaminergic neurodegeneration. **Conclusions:** PD is the second most common neurodegenerative disease and the most common movement disorder, affecting approximately 10 million people worldwide globally as of 2021. Exposure to environmental pollutants such as pesticides and heavy metals has been linked to the onset of PD, but epidemiological evidence is scarce and causality often unclear. Mitochondrial toxicity is a mode of action for these toxicants, with strong evidence for complex I inhibition as causative of parkinsonism and dopaminergic neurodegeneration. Our work provides robust confirmation that 1) complex III inhibition causes dopaminergic neurodegeneration *in vivo*, and 2) that this is caused by generation of mitochondrial ROS in complex III, providing mechanistic insights on this specific mode of mitochondrial dysfunction. This is important from a regulatory perspective because complex III inhibitors are common in nature, frequently used as pesticides, and increasingly pursued for drug development. From a mechanistic perspective, the knowledge that mitochondrial ROS, in particular superoxide anion, is at the center of dopaminergic dysfunction will be helpful in the development of preventive and therapeutic approaches.

PS 3576 Synaptic and Neurotransmitter Alterations in Canine Cognitive Dysfunction: A Translational Model for Alzheimer's Disease

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Background and Purpose: Alzheimer's disease (AD) is the leading cause of dementia, accounting for 83.6% of cases globally in 2019. Despite its prevalence, effective treatments remain limited underscoring the need for translational models that capture key aspects of human pathology. Canine cognitive dysfunction (CCD) syndrome is a naturally occurring neurodegenerative disorder in aged dogs that mimics AD through shared clinical signs including disorientation, altered social interactions, and neuropathological features including gliosis and protein misfolding. **Methods:** This study examines neurotransmitter and synaptic protein alterations in aged canines with and without presumed CCD to evaluate the presence of synaptic dysfunction and its parallels to AD pathology. Hypothesizing that aged canines with presumed CCD will show similar etiology to Alzheimer's patients, revealing pathological changes at the synaptic and neuronal level. Canines were evaluated using the Canine Dementia Scale (CADES) and divided into CCD- and CCD+ groups, with further subdivision based on amyloid beta pathology. Neurotransmitter concentrations including glutamate, acetylcholine, GABA, dopamine, DOPAC, HVA, serotonin, 5-HIAA, norepinephrine, and epinephrine were quantified across frontal cortex, hippocampus, striatum, and cerebellum using LC-MS/MS. Quantification of pre- and postsynaptic proteins SNAP25, VAMP2, PSD95, and HOMER1, key regulators of neurotransmitter release and postsynaptic signaling, through Western blot analysis. **Results:** Our results show that synaptic proteins are modulated in presumed CCD+ dogs, with region-specific changes across cortical and subcortical areas when compared to aging dogs without CCD. Neurotransmitter analysis revealed slight decreases in glutamate, GABA, and acetylcholine within cortical regions, suggesting early disruption of excitatory and inhibitory balance. **Conclusions:** Together, these findings indicate that CCD involves both biochemical and structural synaptic alterations that parallel AD pathology.

PS 3577 Drug Repurposing of Rapamycin Mitigates Alzheimer's Disease Associated Proteotoxicity in *Caenorhabditis elegans*

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Background and Purpose: Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by the accumulation of misfolded proteins, including amyloid- β ($A\beta_{1-42}$), which disrupt neuronal function and lead to cognitive decline and reduced lifespan. The mechanistic target of rapamycin (mTOR) pathway is a central regulator of aging, proteostasis, and neuronal survival. Its inhibition by rapamycin has demonstrated protective effects against neurodegeneration in several species. Given these findings, rapamycin (RAP), an FDA-approved compound, is being repurposed for Alzheimer's disease therapy due to its ability to attenuate proteotoxicity in multiple model systems. **Methods:** The transgenic *Caenorhabditis elegans* strains expressing human $A\beta_{1-42}$ were used as a model to study Alzheimer's-like proteotoxic stress. Rapamycin was administered across a range of doses to evaluate its neuroprotective potential. Paralysis assays were conducted to measure behavioral outcomes associated with $A\beta$ -induced toxicity, and the timing of paralysis onset was used as the main endpoint for treatment efficacy. **Results:** Rapamycin treatment significantly delayed the onset of paralysis in the transgenic *C. elegans* strains expressing $A\beta_{1-42}$ compared with vehicle-treated controls. This delay indicates a reduction in $A\beta$ -induced proteotoxicity. Dose-response analysis revealed that the neuroprotective effect of rapamycin varied with concentration, providing insight into its optimal therapeutic range. **Conclusions:** These findings demonstrate that rapamycin exerts a neuroprotective effect in a *C. elegans* model of Alzheimer's disease by mitigating $A\beta$ -induced paralysis. The study is among the first to systematically assess rapamycin across multiple doses in this model, offering new insights into its dose-dependent effects on proteostasis and neuroprotection. This work supports further exploration of rapamycin as a candidate for Alzheimer's therapy and highlights the potential for combination treatments and validation in higher model organisms.

PS 3578 Effects of prophylactic tirzepatide treatment in a mouse model of Alzheimer's disease

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Background and Purpose: Glucagon-like peptide-1 receptor (GLP-1R) agonists have been used to treat neurodegenerative diseases such as Alzheimer's disease with overall positive results. This neuroprotection is achieved at least to some extent by GLP-1R's actions on decreasing neuroinflammation, amyloid-beta deposits, and tau hyperphosphorylation, while improving brain energy metabolism and neurogenesis. However, treatment is not often initiated until after diagnosis, when symptoms are already present. Given the neuroprotective value of GLP-1R activity on brain health, we hypothesized that initiating treatment prior to symptom onset may slow disease

progression. **Methods:** Male and female 5XFAD (wild-type and transgenic) mice were administered either saline or tirzepatide (10 nmol/kg) twice daily from postnatal day (P)21-P60. Tirzepatide is a more efficacious formulation, targeting both the GLP-1 and GIP receptors. Mice then underwent a behavioral battery assessing mood-like behaviors, motor coordination, and cognition. **Results:** There were significant differences in anxiety-like behavior (light/dark task, open field test, elevated zero maze) and motor coordination (rotarod) between transgenic and wild-type mice. Prior exposure to tirzepatide did not significantly alter any of these outcomes. These tasks, however, are best suited at detecting gross behavioral deficits and not more subtle changes. To that end, we used a touchscreen-based operant task to assess location discrimination, a hippocampal-based form of episodic memory. Preliminary data suggest that while there were no significant differences in performance between wild-type and transgenic mice, early tirzepatide treatment significantly enhanced performance in the more difficult probe trials. **Conclusions:** Prophylactic treatment with tirzepatide did not significantly alter motor- or mood-related outcomes in 5XFAD mice. However, earlier exposure to the drug enhanced cognitive function in older mice, regardless of disease status. We will next use histology to determine if pretreatment with tirzepatide altered neuroinflammation or beta-amyloid plaques at the neuroanatomical level.

PS 3579 Leveraging Telmisartan to Assess TGFβ's Role in Mediating Traffic Related Air Pollution Effects on Alzheimer's Relevant Neuropathology

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Background and Purpose: Alzheimer's disease (AD) is a complex neurodegenerative disease linked to numerous environmental risk factors. Epidemiological and experimental animal studies have identified traffic related air pollution (TRAP) as an environmental factor that increases AD risk and severity. TRAP is a heterogeneous mixture of particulate matter (PM), volatile organic compounds, and gases from vehicular emissions generated by fuel combustion, brake and tire wear, and resuspension of road dust. Despite TRAP's association with AD, the mechanism(s) by which it promotes AD are unknown. TRAP exposure is associated with fibrosis in the lung, which is promoted by TGFβ. In the brain, TGFβ is known to promote endothelial to mesenchymal transition (EndoMT), a pathological process in which endothelial cells of the blood brain barrier (BBB) dedifferentiate leading to BBB dysfunction. BBB impairment is a key characteristic of AD, which can exacerbate AD-relevant pathology and inflammation. Based on these observations, we hypothesize that pharmacological inhibition of TGFβ signaling will mitigate TRAP-induced AD-relevant pathology. **Methods:** Male and female TgF344-AD rats, which express two human AD risk genes, were exposed to ambient TRAP drawn from a heavily trafficked tunnel and delivered in real-time to exposure chambers adjacent to the tunnel. Controls housed in the same facility were exposed to filtered air (FA). TRAP and FA groups were fed either telmisartan-containing or control chow. Telmisartan, an angiotensin receptor blocker, is a well-established inhibitor of TGFβ signaling. The rats were transported to the tunnel at 1 month of age, telmisartan treatment was initiated at 3 months of age, and animals were euthanized when they were 4, 9, or 15 months old to collect tissues. Neuronal cell loss and amyloid fibrils were assessed using quantitative immunohistochemical (qIHC) analyses of NeuN and OC immunoreactivity, respectively, in AD-impacted brain regions, specifically the hippocampus and entorhinal cortex. Aβ42:40 ratios and phosphorylated tau levels were quantified in the blood and brain via bead-based multiplex immunoassay. **Results:** The Aβ42:40 ratio is used clinically to assess AD progression. Telmisartan decreased Aβ42:40 ratios in both the plasma and cortex from TRAP and FA, with the most robust effects observed at the 15-month timepoint. Phosphorylated tau, another AD biomarker, was increased and decreased by TRAP and telmisartan, respectively. A significant exposure effect was observed in 4-month males, while a significant drug x exposure interaction was found in the 9-month females. IHC indicated that telmisartan decreased neuronal cell count at 4 months in FA and TRAP groups. Neither telmisartan nor TRAP had a significant effect on amyloid deposition in the hippocampus measured by OC immunopositive area. **Conclusions:** These results suggest that telmisartan affects AD pathology with TRAP exposure, and that these effects are age dependent, with ameliorative effects occurring in later stages of the disease. Changes in Aβ42:40 ratios, which we observed precede the accumulation of plaques in the brain measured via IHC, further support a role for TGFβ in promoting the progression of AD phenotypes. Further investigation into the effect of TGFβ inhibition on additional markers of AD and EndoMT will inform the role of TGFβ in mediating TRAP effects on AD-relevant neuropathology.

PS 3580 Neuroinflammation drives CNS metal accumulation: a new perspective on metals, inflammation, and neurodegeneration

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Background and Purpose: Metals play a key role in maintaining homeostasis within the Central Nervous System (CNS), contributing to neurotransmitter synthesis, antioxidant defense, and myelination. Environmental exposure to metal toxicants

is a known risk factor for the development of several neurodegenerative diseases, including Alzheimer's disease (AD). Amyloid-β plaques, tau-neurofibrillary tangles, and reactive glia have increased metal content, contributing to the CNS accumulation of iron (Fe) and manganese (Mn) seen in AD. Previous work has emphasized the role of metal exposure in AD, identifying increased oxidative damage, neurotransmitter disruption, and neuroinflammation as key mechanisms of toxicity. Far fewer studies have directly assessed how AD-related pathology may, in turn, increase susceptibility to adverse effects from environmental metal exposures. This work assesses the effects of neuroinflammation on metal homeostasis in the CNS, and the potential for Mn exposure to exacerbate disease-related changes. **Methods:** Male and female C57BL/6J mice were placed on a control (70 ppm) or high-Mn (2400 ppm) diet from 5- to 11-weeks of age. Following the 6-week exposure, mice received an intraperitoneal (IP) injection of lipopolysaccharide (LPS) (3.5 mg/kg body weight) or saline (0.9% NaCl). Health scores, body temperature, and body weight were recorded over 24 hours. Hippocampal and cortical tissue were collected 24 hours post-injection for biochemical analysis. Bulk RNA was extracted from the hippocampus for use in a Mouse Neuroinflammation Panel on the NanoString nCounter platform. Bulk RNA from both the hippocampus and cortex was converted to cDNA for RT-qPCR experiments. The housekeeping genes for RT-qPCR were 18S and GAPDH. The genes of interest in the RT-qPCR experiments assessed metal import (DMT1, ZIP8, ZIP14, Tfr1), metal storage (H-ferritin, L-ferritin), metal export (FPN1, ZNT10), and cellular stress (NRF2, NQO1, HO-1). Protein homogenate from the hippocampus and cortex were used for Western blot analysis of metal transport and storage proteins and for ICP-MS quantification of metal content. **Results:** LPS treatment resulted in significant changes to sickness behavior, as seen by decreased health scores, body temperature, and body weight. Importantly, the high-Mn diet did not impact the effects of LPS on sickness behavior. There was a robust glial-driven neuroinflammatory response in both the control and high-Mn diets following LPS; however, high-Mn mice exhibited a much more pronounced response. Differential gene expression and pathway analyses revealed greater neuroinflammation in mice on the high-Mn diet, reflected in astrocyte function, inflammatory signaling, and microglial function gene sets. LPS treatment increased expression of metal importer (DMT1, ZIP8, ZIP14, Tfr1) and storage genes (H- and L-ferritin), while decreasing expression of exporters (FPN1, ZNT10). Altered metal transport dynamics following LPS treatment were reflected in Western blot experiments. ICP-MS analysis indicated that Mn and Fe accumulation in the CNS was also increased by LPS treatment. Cellular stress gene expression showed a sex-specific response to LPS; males on the high-Mn diet exhibited the most severe changes compared with control diet males, and females under the same exposure. **Conclusions:** We identified that an inflammatory insult was sufficient to disrupt metal transport in a coordinated manner, with increased metal import and storage, and decreased metal export. These changes reflect an increased capacity for intracellular uptake of metal ions from the systemic circulation and surrounding interstitial fluid. We further confirm that inflammation-induced changes to metal transport do increase the concentration of Mn and Fe in the CNS. We also showed that exposure to Mn exacerbates neuroinflammation and inflammation-induced metal transport changes, ultimately driving an LPS-induced increase in cellular stress. This study highlights an unexplored relationship between inflammation and metal exposure within the CNS, one in which inflammation drives metal accumulation. This relationship has the potential to be further explored in the context of neurodegeneration, to identify how neuroinflammatory changes to metal transport increase susceptibility to environmental exposures throughout the course of disease.

PS 3581 Cholinergic transmission dysregulation and neurodegeneration induced by thyroid signaling disruption following butylparaben single and repeated treatment

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Background and Purpose: Butylparaben (BP), a widely used preservative, was implicated in cognitive impairment, though its neurotoxic mechanisms remain elusive. Basal forebrain cholinergic neurons (BFCN) are selectively lost in dementias, contributing to cognitive decline. **Methods:** To explore different mechanisms related with BFCN loss, we employed BF SN56 cholinergic wildtype or silenced cells for Tau, amyloid-beta precursor protein (βAPP), acetylcholinesterase (AChE), or glycogen synthase kinase-3 beta (GSK3β) genes, exposing them to BP (0.1-80 μM) for 1 or 14 days alongside triiodothyronine (T3; 15 nM), N-acetylcysteine (NAC; 1 mM), or recombinant heat shock protein 70 (rHSP70; 30 μM). **Results:** BP disrupted cholinergic transmission by AChE inhibition, and provoked cell death, through thyroid hormones (THs) pathway disruption, Aβ/p-Tau protein accumulation, AChE-S overexpression and oxidative stress (OS). Aβ/p-Tau accumulation was correlated with HSP70 downregulation, OS exacerbation, and GSK3β hyperactivation (for p-Tau). BP-induced OS was mediated by reactive oxygen species (ROS) overproduction and nuclear factor erythroid 2-related factor 2 (NRF2) pathway disruption. All observed effects were contingent upon TH signaling impairment. **Conclusions:** These findings uncover novel mechanistic links between BP exposure and BFCN neurodegeneration, providing a framework for therapeutic strategies.

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are widely used in daily consumer and industrial products, and human exposure to PFAS has been associated with various neuronal disorders, including Alzheimer's disease (AD), although the underlying mechanisms remain poorly understood. **Methods:** In this study, we investigated the neurotoxic effects of PFOA, a legacy PFAS, and GenX, its proposed "safer" alternative, using human induced pluripotent stem cell (hiPSC)-derived cortical neurons. To assess persistent neurotoxicity, neurons were assessed immediately after exposure and after a 7-day relaxation. Neuronal resilience was evaluated using a secondary challenge assay targeting organelle-specific stressors. **Results:** We identified distinct neurotoxic effects of PFOA and GenX, leading to increased risk of developing AD. PFOA exposure induced transient alteration in neuronal activity, neuron network morphology and synaptic density, but with persistent damage in mitochondria function, further corroborated with increased vulnerability towards mitochondria stress. GenX exposed neurons showed persistent alteration in neuronal network morphology and synaptic density, accompanied by persistent increase in vulnerability towards autolysosomal stress. Both PFOA and GenX triggered transient changes in AT8 and tau expression and conferred persistent vulnerability to tau-preformed fibrils (tau-PFF), implicating impaired proteostasis. Transcriptomic profiling further supported our findings. **Conclusions:** Collectively, our findings reveal divergent neurotoxic mechanisms of PFOA and GenX—mitochondrial damage versus autolysosomal stress—that converge on tau pathology, highlighting increased AD risk induced by PFAS exposure.

PS 3583 Assessing Lead Levels in Human Bone with Portable XRF and Benchtop XRF Technology to Explore Links to Cognitive Performance and Neuropathology

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Background and Purpose: This study investigates the role of cumulative metal exposure—specifically lead (Pb)—in relation to neurodegeneration in an elderly population, focusing on Alzheimer's disease (AD). Lead is a well-established neurotoxin associated with cognitive decline. While animal studies suggest associations between heavy metal exposure and AD-related neuropathology, human studies are limited. This research aims to quantify bone Pb concentrations in elderly donors and assess their relationship with cognitive function, neuropathological markers, and APOE genotype. **Methods:** Skull bone samples from 191 elderly donors, including individuals diagnosed with Alzheimer's disease (AD), were scanned using portable (pXRF, 12 min) and benchtop (7 min) X-ray fluorescence systems. Pb concentrations were processed in MATLAB and analyzed in R using ANOVA, Kruskal-Wallis, Wilcoxon rank-sum, and Spearman correlation tests. Pb levels were compared across sex and APOE genotype, and associations with cognitive diagnoses and neuropathological markers were examined. Longitudinal cognitive data were analyzed using a Linear Mixed Model (SPSS) to evaluate the effect of Pb on cognitive decline over time, adjusting for age and sex. **Results:** Males had significantly higher bone Pb concentrations than females (mean $\pm$ SD: females = 3.37 ± 2.41 μ g/g; males = 5.50 ± 3.26 μ g/g; t-test $p = 0.0016$; Wilcoxon $p = 0.00065$). When stratified by APOE genotype, significant sex differences were observed within APOE3 ($p = 0.0043$) and APOE4 ($p = 0.0007$) groups, but not among APOE2 carriers ($p > 0.05$). APOE genotype was significant in the longitudinal model ($p < 0.05$), indicating an interaction between genetic susceptibility and Pb exposure on cognitive decline. Longitudinal analysis preliminary results revealed a significant $f_{u\_year} \times$ concentration interaction ($F(1, 28.92) = 8.39$, $p = 0.007$), suggesting greater cognitive decline over time among individuals with higher bone Pb levels. **Conclusions:** Preliminary findings suggest potential associations between cumulative bone lead (Pb) exposure, cognitive decline, and modifying effects of sex and APOE genotype. Males showed higher bone Pb concentrations than females, particularly among APOE3 and APOE4 carriers, indicating that biological and genetic factors may influence Pb accumulation and related outcomes. The observed interaction between Pb concentration and follow-up year suggests a possible link between higher Pb levels and greater cognitive decline over time. Further analyses are needed to confirm these trends and clarify the role of genetic and sex-specific factors in Pb-related neurotoxicity.

PS 3584 Investigating the effect of Bisphenol A-induced neurotoxicity in a striatal cell model of Huntington's disease

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Background and Purpose: Exposure to environmental factors such as heavy metals and pesticides can induce neurotoxicity and cell death in Huntington's disease (HD), exacerbating disease severity and age of onset. Bisphenol A (BPA) is a commonly used chemical in the production of numerous widely-used plastics and has been

found at detectable levels in over 90% of the U.S. population's urine. BPA has been shown to cause neurotoxicity and cell death through oxidative stress and mitochondrial disruption in models of Parkinson's disease (PD) and cause a PD-like phenotype in drosophila. Given that similar pathophysiological mechanisms exist between PD and HD, and BPA's demonstrated effect in models of PD, we hypothesize that expression of mutant huntingtin coupled with chronic exposure to BPA will increase neurotoxicity and neurodegeneration through mitochondrial disruption, oxidative stress, and protein aggregation-related pathways. **Methods:** We are using an established immortalized striatal cell model of HD (WT - STHdhQ7/Q7, HD - STHdhQ111/Q111), and exposing cells for 24 and 48 hours, separately. To measure cell viability and death, we use the MTT and SYTOX Green assays, respectively. We measured mitochondrial membrane potential through the use of the TMRE assay. **Results:** We report that 48-hour exposure to BPA resulted in a dose-dependent increase in cell death which trended towards greater susceptibility of the mutant cells in both cell death and mitochondrial capacity assays, with no significant difference at 24 hour exposure. Further investigation into mitochondrial health showed a reduction in mitochondrial membrane potential at both 24 and 48 hour timepoints, with a greater reduction in membrane potential in mutant HD cells. **Conclusions:** Ongoing studies are investigating the impact of BPA exposure on mitochondria biogenesis, oxidative stress signaling pathways, and examining differentially expressed genes via RNA sequencing. Understanding the gene-environment interaction between BPA and HD will elucidate striatal specific effects associated with BPA and help educate at risk-populations.

PS 3585 Investigating cellular mechanisms of prion disease in a primary cervid fibroblast model

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Background and Purpose: The United States is known for a robust cervid population throughout all fifty states. Hunting culture is well established, and the proximity of these wild populations is getting closer to human civilization. Chronic Wasting Disease or CWD was first discovered in captive deer in Colorado State. Since then, it has spread throughout the United States and has traveled to both Europe and Asia via importation of diseased cervid species. CWD is a prion disease characterized by the misfolding of the PrP protein found throughout the central nervous system and surrounding peripheral tissues. This misfolding can happen via contact with an already misfolded PrP protein (PrP^{Sc}) or spontaneously for unknown reasons. Accumulation of PrP^{Sc} in cervids leads to neurological symptoms such as tremors, difficulty walking, drooling and altered behavior like loss of aggressive and fearful tendencies. **Methods:** In this project we use primary fibroblasts isolated from the cheek and ear of White Tail and Mule deer. As this is a primary deer cell line, we needed to optimize the cells maintenance and growth conditions prior to undergoing experimentation. To do this, we tested the growth and death of the cells using various ratios of new to conditioned media. We tested 100:0, 90:10, 75:25, 50:50, 25:75, and 0:100, new to conditioned media for ten days in dishes with normal confluency and low confluency starting with 50,000 and 10,000 cells, respectively. Morphological analysis was performed on day 1, 5, 7 and 10 to determine any changes in the cells with the various ratios of media. On day 11, the cells were trypsinized and percentages of live vs. dead cells we quantified using both trypan blue and flow cytometry to assess quantitative changes in cell viability. **Results:** Based on our morphologic data we found that 100:0 maintained fibroblast morphology however, the 50:50 ratio had the highest percentage of cells that did not undergo cell death and maintained expected morphology in both the low and normal confluency. **Conclusions:** Through these optimization assays we conclude that 50:50 is the best concentration for growth and overall viability in our primary deer fibroblasts. This optimization of cell conditions will allow us to move forward to characterize our primary cell lines and to use them as a tool to understand the cellular mechanisms of prion diseases like chronic wasting disease.

PS 3586 Mechanistic profiling of new potential Parkinsonian toxicants Identified through qHigh-Throughput Screening of MT1G expression in a model of differentiated LUHMES cells

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Background and Purpose: The etiology of Parkinson's disease (PD) remains unresolved; however, cumulative evidence implicates environmental chemical exposure as a major contributor to disease risk. To identify previously unrecognized environmental factors, we developed a quantitative high-throughput screening (qHTS) assay to identify chemicals toxic to dopaminergic neurons. To increase assay sensitivity, a reporter gene was identified, *MT1G*, which was dynamically up-regulated following chemical exposure to seven examples Parkinsonian neurotoxicants. *MT1G* encodes Metallothionein-1, a small protein that sequesters toxic labile metals such as iron and copper, as well as scavenging reactive oxygen species, processes commonly associated with the development of PD. Consequently, LUHMES cells were engineered to express luciferase under the control of endogenous *MT1G* regulatory

elements, enabling a sensitive 3D MT1G-Luciferase reporter assay optimized for qHTS in 1536-well microplates. Screening of the LOPAC library and Tox21 10K library yielded 28 active chemicals, selected for mechanistic characterization of neurotoxicity associated with MT1G induction. **Methods:** To dissect the pathways potentially contributing to neurodegeneration, we employed differentiated LUHMES dopaminergic neurons (dLUHMES) and we plan to evaluate five different mechanistic pathways: (1) Mitochondrial dysfunction, assessed by measuring mitochondrial membrane potential via TMRE fluorescence using flow cytometry at 30 minutes and 3 hours post-exposure, as well as by evaluating cell viability (CellTiter-Glo assay) under glucose- and galactose-based culture conditions at 24 hours; (2) Oxidative stress, determined through quantification of reactive oxygen species and cuproptosis/ferroptosis-associated lipid peroxidation, using viability assays in the presence of pathway inhibitors such as Ferrostatin-1 and metal chelators such as deferoxamine at 24 hours. (3) Dopamine transporter interference, evaluated using functional DAT activity assays; (4) Autophagy and proteasome impairment, assessed by quantification of autophagosomes via flow cytometry and analysis of LC3-II levels by Western blot; (5) Excitotoxicity, examined through intracellular calcium measurements (Fura-2 calcium flux assay), apoptosis analysis using Annexin V assays, and viability assessments in the presence of calcium channel blockers and glutamate transporter antagonists. **Results:** In galactose conditions, dLUHMES cells became more dependent on mitochondrial function. We observed that mitotoxins elicited responses that were approximately three orders of magnitude more sensitive than under glucose conditions, whereas compounds whose primary mechanism of toxicity does not involve mitochondrial dysfunction showed no significant changes in ATP levels. In addition, our preliminary findings after TMRE fluorescence assay indicate that these compounds can be classified into two groups: (i) uncoupling agents that create an alternative pathway for protons and induce mitochondrial membrane depolarization within 30 minutes, and (ii) inhibitors of electron transport chain complexes that lead to mitochondrial depolarization over approximately 3 hours. After standardization of the experimental strategy with the use of known parkinsonian neurotoxins, we observe that some of the compounds do not induce ferroptosis; however, we observed significant differences in cell viability following treatment with iron-chelating agents, indicating that iron availability could modulate the cellular response to these compounds. This finding suggests that iron-dependent processes may still contribute to the observed cytotoxicity. **Conclusions:** Together, these findings underscore the multifactorial nature of environmentally induced dopaminergic neurodegeneration and highlight the value of MT1G-based screening coupled with targeted pathway analyses for uncovering mechanistic liabilities of environmental chemicals relevant to Parkinson's disease risk and even identify potential therapeutic targets.

PS 3587 Evaluation of neurotoxicity of 21 organophosphorus pesticide binary mixtures using rapid behavioral screening in the planarian *Dugesia japonica*

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Background and Purpose: Organophosphorus pesticides (OPs) are a chemically diverse class of compounds that exert their acute toxicity through acetylcholinesterase (AChE) inhibition. However, chronic low-dose exposure to OPs has been shown to cause neurotoxicity and developmental neurotoxicity through other mechanisms besides AChE inhibition. Given the large number of OPs and their frequent use in mixtures, high throughput screening (HTS) methods are needed for comparative OP mixture studies to better understand potential interactions. Because many OPs require bioactivation, HTS-amenable organismal models with human-relevant metabolism are required. Here, we utilized the planarian *Dugesia japonica*, which is well-suited for comparative studies of OP toxicity and amenable to behavioral HTS in developing and adult specimen, to evaluate whether the adverse outcomes of binary mixtures of OPs can be recapitulated by a concentration addition model that assumes a shared mode of action. **Methods:** We screened 21 binary mixtures of 7 OPs (acephate, chlorpyrifos (CPF), diazinon, dichlorvos (DDVP), malathion, parathion, and profenofos) in equimolar ratios across at least 5 concentrations up to 10 μ M in adult and regenerating *D. japonica* planarians using an automated screening platform. We evaluated 22 readouts, quantifying effects on lethality, stickiness, locomotion and responses to light and temperature on days 7 and 12 post-exposure. Using published planarian data on the individual OPs, we determined whether a Loewe concentration addition model describes the experimental OP mixture data. Four OP mixtures were also tested for AChE inhibition, to contextualize the behavioral screening results. **Results:** We found that binary interactions between these OPs can be non-additive and identified both synergistic and antagonistic interactions depending on the OP mixture, readout and developmental stage (adult vs regenerating planarians). For example, in regenerating planarians, except for the binary mixture of DDVP and CPF, all DDVP-containing mixtures showed antagonistic effects on stickiness at day 12. CPF- or profenofos-containing mixtures showed either additive or synergistic effects on stickiness. Regarding AChE inhibition, CPF-malathion and CPF-parathion mixtures showed additive effects, mixtures of diazinon-DDVP showed antagonistic effects, and diazinon-profenofos mixtures showed synergistic effects. These data agree with our previous findings that increased stickiness is correlated with increased cholinergic signaling. In contrast, other readouts did not correlate with AChE inhibition. **Conclusions:** Our results suggest that OP binary mixtures can have both additive and non-additive interactions and that these can vary between the

adult and developing nervous system. Due to the conservation of key enzymes in OP toxicokinetics/toxicodynamics between planarians and humans, the insights gained here are likely to be relevant to human health and can be further verified in targeted mechanistic studies in human cells.

PS 3588 Acute organophosphate intoxication activates the TGF β signaling pathway

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Background and Purpose: Intentional and accidental poisonings with organophosphate (OP) cholinesterase inhibitors can trigger an acute cholinergic toxidrome that includes not only peripheral cholinergic symptoms, but also acute seizures that can rapidly progress to life-threatening status epilepticus (SE). Human and preclinical studies have demonstrated that individuals who survive the acute OP-induced SE have a high risk of developing spontaneous recurrent seizures (SRS) as well as persistent blood-brain barrier (BBB) impairment, neurodegeneration, and neuroinflammation. Other research groups have shown that BBB disruption and the consequential albumin leakage into the brain parenchyma can activate the transforming growth factor β (TGF β) signaling cascade. Data from our lab have shown plasminogen activator inhibitor-1 (PAI-1), a TGF β -responsive gene, is increased following acute OP intoxication and persistently expressed in astrocytes. Phosphorylation of the transcription factors SMAD2/3 (pSMAD2/3) is the signaling molecule that links TGF β receptor activation to upregulation of PAI-1. Our overall goal is to evaluate TGF β signaling as a therapeutic target for mitigating the adverse neurological effects of acute OP intoxication. As a first step towards this goal, we assessed the spatiotemporal expression of PAI-1 and pSMAD2/3 relative to total SMAD2/3 following acute OP intoxication. **Methods:** Adult female Sprague Dawley rats were injected with the OP diisopropylfluorophosphate (DFP, 4 mg/kg, SC; n=36) or PBS (n=22) followed by atropine sulfate (2 mg/kg; IM), 2-pralidoxime (25 mg/kg; IM), and two doses of midazolam (0.65 mg/kg; IM), to improve survival rate. Seizure behavior was scored using a modified Racine Scale (0-5) to monitor the progression and severity of seizures. Animals that had an average behavioral score of 2.5 within the first 40 min (a score that has been shown previously to correspond with electrographic SE) were included in the study. At 3, 6, 12, 24, 72, 168, and 336 h post-exposure, brain tissue was collected for biochemical analysis. The cortex, piriform cortex, and hippocampus were dissected, flash-frozen, and stored at -80°C prior to lysate preparation using a lysis buffer that contained protease and phosphatase inhibitors. PAI-1, pSMAD2/3, and total SMAD2/3 were quantified in 3 technical replicates of each sample using commercially available enzyme-linked immunosorbent assays (ELISAs); values were normalized to total protein concentration. Tests for normality indicated the data were not normally distributed, so Kruskal-Wallis with Bonferroni adjustment was used to assess group differences in target molecule expression. **Results:** Preliminary results indicate that PAI-1 expression was increased in the cortex, hippocampus, and piriform cortex from 12 h through 72 h following DFP intoxication (p<0.05). The ratio of phosphorylated to total SMAD2/3 was increased in all brain regions at 6, 12, and 24 hours post-DFP intoxication (p<0.05) with significant differences persisting at 72 h post-exposure in the piriform cortex and hippocampus (p<0.05). **Conclusions:** Our findings demonstrate that the TGF β signaling pathway is activated following acute DFP intoxication, with the highest activity observed in brain regions previously shown to exhibit severe BBB disruption, neuroinflammation, and neurodegeneration. Converging evidence from multiple studies supports a link between activation of TGF β signaling, BBB disruption, and subsequent epileptiform activity and cognitive deficits. Characterizing the spatiotemporal dynamics of TGF β signaling after acute OP exposure supports further studies evaluating TGF β signaling as a potential therapeutic target for mitigating the adverse neurological sequelae of acute OP intoxication.

PS 3589 Single-nuclei transcriptomics reveal significant glial cell expansion and selective neurotoxicity during the epileptogenic period following organophosphate-induced status epilepticus

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Background and Purpose: Acute intoxication with organophosphate (OP) pesticides or nerve agents inhibits acetylcholinesterase, causing neuronal hyperexcitability that can progress to acute status epilepticus, persistent brain damage, and spontaneous recurrent seizures (SRS). While current standard of care can mitigate acute symptoms, they fail to prevent persistent neurological effects, and 70-80% of intoxicated individuals that experience status epilepticus (SE) develop SRS. Currently, we do not understand the molecular mechanism(s) underlying the development of SRS following acute DFP intoxication, the cell types involved, or why a subset of individuals (20-30%) do not develop SRS despite experiencing acute DFP-induced SE. **Methods:** To address these data gaps, we leveraged a well-characterized rat model of acute intoxication with the OP diisopropylfluorophosphate (DFP). Electrographic profiles predictive of SRS development were used to identify adult male Sprague Dawley rats highly likely to develop SRS (referred to as the SRS group) and which were not (non-SRS group) following DFP-induced SE. snRNA-Seq was performed on nuclei

isolated from the piriform cortex of control animals not intoxicated with DFP (n = 4, 19,165 nuclei), non-SRS DFP animals (n = 4, 29,453 nuclei) and SRS DFP animals (n=4, 25,389 nuclei). Single nuclei analysis showed bidirectional changes in expression of genes regulating neuronal excitability that tracked with seizure outcome. **Results:** Relative to SRS, the glutamatergic neurons of control and non-SRS animals displayed elevated expression of potentially protective genes, *Scn1a* (NAV1.1), *Kcnk2* (TREK-1), *Gabra1/Gabrb2* (GABAA $\alpha 1/\beta 2$ subunits), cell polarity genes *Prickle1/2*, and *Gria3* which encodes an AMPA receptor subunit, which may aid in reducing neuronal excitability. Additionally, we identified increased expansion of glial cell populations in SRS vs. non-SRS DFP rats, including proliferative subpopulations of astrocytes, microglia, and oligodendrocyte precursors cells, with elevated expression of proliferation markers, *Mki67*, *Top2a*, *Cenpe*, *Cenpf*, and *Kif4a*. **Conclusions:** This is the first study to apply single-cell transcriptomic technologies to characterize the impact of acute OP intoxication on individual cell types and subpopulations. The results demonstrate that increased risk of developing SRS was associated with significantly reduced neuronal cell populations and expanded glial cells, including highly proliferative and inflammatory glial subpopulations that were largely absent under homeostatic conditions. Importantly, only glutamatergic neurons showed significant transcriptional shifts between non-SRS and SRS animals. Together, these results suggest that a multipronged treatment strategy that targets both glutamatergic signaling and neuroinflammation during the epileptogenic period is likely necessary to prevent SRS development.

PS 3590 Dose-Dependent Effects of Chlorpyrifos on Embryonic Cortical Neuronal Network

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Background and Purpose: Chlorpyrifos (CPF), a widely used organophosphate pesticide, has been linked to neurotoxic effects in children exposed prenatally, including brain anomalies, behavioral changes, and structural deficits. Previous research has shown disturbance in neuronal networks of postnatal cortical neurons. However, there remains a gap in knowledge regarding the effects of CPF on the embryonic cortical neuronal network. Research has also suggested that embryonic cortical neurons are more vulnerable to CPF exposure than postnatal cortical neurons. Given this previous research, we hypothesize that the embryonic cortical neuronal network is more sensitive to CPF. **Methods:** Primary cortical neurons were prepared from embryonic day 17 (E17) Sprague-Dawley rat embryos. Cells were plated onto multi-well MEA plates, which contain 48 wells per plate, with each well consisting of an array of 16 individual embedded nanotextured microelectrodes. Cells were plated at a density of 120,000 cells/well and cultured in Neurobasal medium. The culture medium was switched to BrainPhys at DIV5, and a full media change was performed at DIV11 to establish baseline activity. At DIV13, a full media change was performed again with increasing concentrations of CPF (0.1 μ M, 1 μ M, 3 μ M, 5 μ M, and 10 μ M). **Results:** We observed a decrease in the number of spikes, number of (network) bursts, (network) burst duration, number of spikes per (network) burst, mean ISI within network bursts and synchronicity in all CPF concentrations in embryonic cortical neurons. **Conclusions:** In contrast to previous studies, which found these effects only occurring at the highest dose in postnatal cortical neurons, our results suggest that embryonic neurons are more vulnerable to the neurotoxic effects of chlorpyrifos. Future work will aim to elucidate the mechanisms underlying CPF-induced neurotoxicity in embryonic cortical neurons.

PS 3591 Substance P Mediates Chlorpyrifos-Induced Airway Hyperreactivity While Neurokinin A and Calcitonin Gene-Related Product Mediate Chlorpyrifos-Induced Lung Tissue Stiffness

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Background and Purpose: Occupational and environmental exposures to organophosphorus pesticides (OP) are associated with increased asthma incidence. The canonical mechanism of OP neurotoxicity is acetylcholinesterase (AChE) inhibition; however, we previously demonstrated that the OP chlorpyrifos (CPF) causes airway hyperreactivity (AHR) in adult rats 24 h and 7 days after exposure at levels that do not significantly inhibit AChE. Neurogenic inflammation has been suggested to play a role in AHR; therefore, we tested the hypothesis that substance P, neurokinin A and calcitonin gene-related peptide (CGRP) contribute to CPF-induced AHR. **Methods:** Eight-week-old male Sprague Dawley rats were administered 30 mg/kg CPF subcutaneously, and airway responsiveness to electrical stimulation of the vagus nerve was assessed at 24 h or 7 d post-exposure using a Flexivent mechanical ventilator. Atropine was administered IP during mechanical ventilation and electrical stimulation to confirm the role of muscarinic receptors in CPF-induced AHR in rats. To test potential contributions of substance P, neurokinin A and CGRP, we determined whether pharmacological antagonism of the receptors activated by these neuropeptides were effective in attenuating CPF-induced AHR. SR140333 (1 mg/kg, IP), which antagonizes the NK1 receptor activated by substance P; olcegepant (1 mg/kg, IP), which blocks the CGRP receptor; and GR159897 (0.2 mg/kg, IP), an NK2 receptor antagonist that blocks signaling by neurokinin A, were injected 1 h before physiological measurements. **Results:** As previously demonstrated, CPF significantly increased

airway resistance and elastance at 24 h and 7 d post-exposure. Atropine, which binds to and inhibits muscarinic receptors, abolished CPF-induced AHR, confirming airway responsiveness was due to activation of the parasympathetic nerves that control contraction of airway smooth muscle. NK1 receptor inhibition significantly decreased vagally-stimulated bronchoconstriction in CPF-exposed animals to levels observed in vehicle controls at 24 h and 7 d post-CPF exposure. Neurokinin A receptor inhibition decreased pressure-volume loops at 24 h and 7 d post-CPF exposure, and CGRP receptor antagonist decreased the pressure-volume loop at 7 d post-CPF exposure, but neither neurokinin A or CGRP receptors antagonists decreased vagally-stimulated bronchoconstriction in CPF-exposed animals. **Conclusions:** These data suggest that substance P signaling contributes to both acute and persistent CPF-induced AHR, which is mediated at the level of the conducting airways, while neurokinin A and CGRP contribute to CPF effects on the elastic properties of the lung tissue, the surface tension of the alveoli, and the compliance of the lung. These findings identify substance P and its receptor as a new therapeutic target for mitigating OP-induced AHR in exposed individuals and further characterize the sites of overall effects of CPF on the mechanics of breathing. This work was supported by the NIH (grant R01 ES017592 and P30 ES023513).

PS 3592 Chlorpyrifos-induced motor deficits are exacerbated in Pon1(-/-) vs wild type.

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Background and Purpose: Chlorpyrifos (CPF) is an organophosphate pesticide widely used in agriculture despite its adverse impacts on human health through acetylcholinesterase inhibition, causing cholinergic effects. Epidemiological and laboratory research has linked environmental exposures, such as CPF, to Parkinson's Disease (PD). However, the impact of genetic variations in PD-related genes, specifically on dopaminergic neurotoxicity, is not well understood. Paraoxonase 1 (Pon1) has a key role in detoxifying neurotoxic CPF Oxon, and genetic polymorphisms in Pon1 may increase susceptibility to PD from CPF exposure. We tested the hypothesis that Pon1 knockout and CPF exposure would represent a PD-relevant gene-environment interaction. **Methods:** We conducted this study on male and female Pon1-knockout (KO) and wild-type Sprague-Dawley rats, aged 3 months. The rats were divided into groups based on their genotype, treatment, and sex, and CPF was administered at a dose of 2.0 mg/kg or a control treatment (Corn oil) for 8 weeks. Behavior was assessed prior to exposures and weekly after exposures began. **Results:** Results from the study revealed that Pon1 KO rats treated with 2 mg/kg CPF demonstrated a decrease in neurobehavioral assessments, including postural instability, vibrissae-elicited forelimb placing, compared to wild-type rats treated with the same CPF dose. These findings suggest that the absence of Pon1 exacerbates the neurotoxic effects of CPF. **Conclusions:** This study highlights the potential for genetic predisposition (Pon1 deficiency) to increase vulnerability to CPF-induced neurobehavioral alterations associated with PD. Future work will involve molecular and neurochemical analyses of isolated brain and liver tissues to elucidate further the mechanisms underlying these effects.

PS 3593 Epigenetic Histone Modifications and Synaptic Plasticity Alterations associated with Chronic Neurobehavioral Deficit Following Repeated Chlorpyrifos Exposure in Adult Rats

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Background and Purpose: Chlorpyrifos (CPF) is one of the most widely used organophosphate (OP) worldwide. Epidemiological studies report long-term depression and cognitive impairments, in OP-exposed individuals. We recently demonstrated that occupational-like CPF exposure in adult rats was associated with significant chronic anxiety and depression. Epigenetic modifications to chromatin are regulatory mechanisms that can adaptively or maladaptively respond to external stimuli. Our previous research identified epigenetic alterations in a rat model of OP DFP-induced depression. In this study we investigated whether occupational-like CPF exposures affect epigenetic pathways that ultimately alter neuronal plasticity mechanisms leading to chronic depression in adult rats. **Methods:** CPF (5 mg/kg, SC, 1x daily for 21-days) was delivered to adult male Sprague-Dawley rats. Following a 12-week washout period, anxiety and depressive outcome were assessed using a battery of rat neurobehavioral assays. Subsequently rats were euthanized and hippocampus was dissected, flash-frozen and used for molecular studies. Another cohort of rats were perfused with paraformaldehyde and brains post-fixed in sucrose for histology. To evaluate CPF-induced changes on histone modifications, antibody-specific western blotting and chromatin immunoprecipitation assays were conducted. Gene expression studies were conducted using RNAseq. Hippocampal spine density was assessed using Dil staining and synapse number was determined by assessing colocalization of PSD95 and synaptophysin using immunohistochemistry. **Results:** CPF exposures in adult rats produced signs of anhedonia, higher anxiety, and increased despair on rodent behavioral tests. These signs were associated significant increase in hippocampal expression of histone deacetylase proteins (HDAC3 and HDAC5) and a decrease in global levels of histone 3 lysine 27 acetylation (H3K27ac). RNA-seq

identified 100 differentially expressed genes (DEGs). Gene Ontology analysis found that these DEGs were enriched in several pathways including regulation of neuronal plasticity. Using Western blotting, we found a significant decrease in BDNF protein expression in the hippocampus of CPF rats and ChIP showed a significant reduction in H3K9ac occupancy at Bdnf promoter IV in CPF rats. Dil staining showed a significant decrease in spine density in CPF-5 rats along with significant reductions in immunohistochemical staining for synaptophysin and PSD-95 in the hippocampal CA1 and dentate gyrus regions **Conclusions:** CPF exposures that mimic human occupational exposures produce chronic depression associated with dysregulations in histone acetylation pathways, a decrease in expression of brain neurotrophic factors, and a reduction in dendritic spine density along with a decrease in synaptic marker proteins indicative of synapse loss. These observations led us to hypothesize that repeated, low-dose OP CPF exposure induces sustained upregulation of HDACs that reduce expression of key genes and proteins involved in the growth and integrity of synapses, manifesting as reduced synaptic plasticity and signs of chronic depression in rats. This study will provide new data on CPF's effects on the brain and deepen our mechanistic understanding of how OPs affect the risk for depression.

PS 3594 Using rodent MRI to assess changes in brain morphology of adult C57BL/6 mice following prenatal chlorpyrifos exposure

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Background and Purpose: Early developmental exposure to the organophosphate pesticide, chlorpyrifos, impairs activity of acetylcholine, disrupts social and motor behavior, and causes heightened oxidative damage in humans and rodent models. Such early life exposure also impacts brain development both in human children and in rodent models. Human children exposed to chlorpyrifos have disrupted white and gray matter density that co-occur with impairment in motor function. In rodent models of exposure, locomotor impairments have been reported, but whether these changes are also met with changes in white and gray matter microstructure has yet to be determined. **Methods:** The current study utilized magnetic resonance histology, i.e. MR microscopy of postmortem specimens to assess changes in brain morphology of C57BL/6J mice exposed prenatally to chlorpyrifos. A total of 42 male and female mice were exposed to 0, 0.5, or 5 mg/kg/day chlorpyrifos from gestational days 6-17. Locomotor behavior was assessed in early adulthood. At 7 months of age, brains were perfused with ProHance and imaged using diffusion tensor images (DTI) acquired on an Agilent 7T scanner. Images were acquired at 35 μ m isotropic resolution (~180,000 times that of clinical DTI). Difference in volume and fractional anisotropy from chlorpyrifos exposure were quantified using multiple ANOVA across control and exposed groups in 231 different regions of interest (ROI). **Results:** Chlorpyrifos exposure resulted in reduced fractional anisotropy in eight ROI, including the sensory regions of the midbrain, regions within the inferior and superior colliculi, the pontine reticular formation, and cochlear nuclei. Volume fraction, i.e. the percentage of volume of a region normalized to total brain volume, increased in 11 ROI including the midbrain reticular formation, subthalamic nucleus, and red nucleus. Exposure also resulted in reduced volume fraction in 8 ROI, including the infralimbic cortex, cerebellum, and orbital cortex. As expected, there were significant differences in fractional anisotropy and volume fraction between males and females, regardless of exposure. **Conclusions:** Alterations seen in this rodent model of prenatal exposure are similar to those reported in humans exposed early in life, showing the potential use of rodent MR microscopy to detect human-relevant changes in neurological endpoints. The observed changes seen with chlorpyrifos exposure are also suggestive of a potential target driving long-term behavioral differences seen in exposed animals. These findings raise potential concern about neurodegeneration occurring due to early life pesticide toxicity that require additional study.

PS 3595 APOE Genotype and Sex Modify the Effects of Organochlorine Pesticide DDT in Humanized APOE Mice

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Background and Purpose: Alzheimer's disease (AD) is the most common neurodegenerative disease in the US with substantial health, social, and economic implications. There is extensive epidemiological data that suggests the environment plays a considerable role in the etiology of the disease. The organochlorine pesticide dichlorodiphenyltrichloroethane (DDT) was used extensively to combat insect-borne diseases in the US until its ban in 1972. Nevertheless, existing dumpsites and DDT's long half-life contribute to persistent trace levels in the environment and food chain. There is limited data on how environmental exposure to DDT is influenced by non-modifiable risk factors. The Apolipoprotein (APOE) ϵ 4 genotype is known to be the strongest genetic risk factor for late-onset AD. Different APOE genotypes have revealed unique effects on lipid metabolism, inflammation, and neuronal function.

Moreover, two-thirds of all AD cases in the US are reported in women, emphasizing the role sex plays as a risk factor. Previous work from our lab suggests that age, sex, and APOE interact to influence basal neuroinflammation and synaptic function. We have also previously demonstrated that DDT-exposed E4 females exhibit the greatest synaptic impairment compared to all groups, as evidenced by immunofluorescence staining and western blot analysis. DDT-exposed E4 females also have the highest levels of serum NFL, a prognostic biomarker of neurodegeneration, compared to all groups. In this study, we sought to investigate the mechanisms behind these findings by investigating how sex and APOE genotype mediate DDT-induced transcriptional and metabolomic changes in humanized targeted replacement (TR) APOE3 (E3) and APOE4 (E4) mice. **Methods:** Male and female, 3-month-old humanized TR mice were exposed to 3 mg/kg of DDT or corn oil every 3 days for 5 months by oral gavage. Brain tissue was dissected and snap frozen following saline perfusion. Hippocampal samples were analyzed using RNA sequencing for transcriptomics and untargeted metabolomics by mass spectrometry. **Results:** Transcriptomics analysis revealed APOE genotype and sex were major drivers of differential gene expression across groups. Interestingly, there was minimal correlation between significantly differentially expressed genes (DEGs) in E4 vs. E3 DDT-exposed males and E4 vs. E3 DDT females, suggesting sex modifies APOE-dependent transcriptional changes under DDT exposure. Functional analysis showed that E4 vs. E3 DDT-exposed males exhibited dysregulation of membrane potential and ion transport, whereas E4 vs. E3 DDT-exposed females displayed alterations in RNA splicing, providing further evidence for a sex-by-genotype interaction in DDT-exposed mice. Furthermore, metabolomics pathway analysis revealed distinct APOE genotype-dependent effects in DDT-exposed mice, where differences in E4 vs. E3 DDT males were primarily associated with inflammatory signaling and energy metabolism, indicating altered mitochondrial function and lipid signaling. In contrast, differentially expressed metabolites in E4 vs. E3 DDT females were enriched for vitamin metabolism and lipid synthesis, reflecting genotype-dependent metabolic differences. **Conclusions:** Overall, these findings suggest that DDT exposure has a multifaceted impact across biological systems, elucidating a complex gene-by-environment interaction modulated by sex. These data identify potential mechanisms by which DDT exposure may interact with the APOE genotype and sex, potentially contributing to Alzheimer's disease risk.

PS 3596 DDT Alters Amyloid β Oligomerization and Synaptic Density in Humanized Amyloid β Mouse Model

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Background and Purpose: Alzheimer's disease (AD) is a neurodegenerative disease characterized by a decline in memory and function. Environmental factors are increasingly recognized as major contributing factors to the onset of Alzheimer's disease and related dementias (ADRD). Dichlorodiphenyltrichloroethane (DDT) is a highly persistent organochlorine pesticide used widely between the 1940s and the 1970s to combat insect-borne diseases. Since the ban in the United States in 1972, DDT levels have been steadily declining, however the persistent metabolite, dichloro-diphenyl-dichloroethylene (DDE) is still detectable. We've previously reported that DDT exacerbates amyloid beta ($A\beta$) plaque pathology and synaptic impairment in the 3xTG-AD mouse model, which overexpresses rare, causal AD genes. Here, we investigated the ability of DDT on $A\beta$ pathology in hA β mice, a humanized $A\beta$ knock-in model (hA β). This model uses a Cre-lox system to humanize the site in mouse amyloid precursor protein to generate human $A\beta$ at physiological levels. This model also produces amyloid burden and related pathologies comparable to those in human late-onset Alzheimer's disease (LOAD) compared to traditional mouse models that overexpress rare, causal genes. **Methods:** Male and female hA β knock-in mice (aged 3 months) were exposed to 3 mg/kg DDT or corn oil (control) by oral gavage every 3 days for 30 days (N=5-7 males and females per treatment). One day after completion of exposure, a Y-maze behavioral test was conducted. The mice were sacrificed 2 days after the final dose. The brains were dissected following saline perfusion. The left hemispheres were fixed in 10% formalin and sectioned at 40 μ m using a microtome. Sections were stained with anti-MOAB (Biosensis, M-1586-100) to visualize oligomeric $A\beta$ and anti-SV2A (Abcam, Ab32942) to assess synaptic loss. Fluorescent images were captured using BZ-X800 and the fluorescence intensity was quantified using BZ-X800 Analyzer. Data were analyzed using a two-way ANOVA followed by Tukey's for multiple comparisons. **Results:** The disruption of working memory was investigated by calculating spontaneous alternations in the Y-maze. Our results revealed that female hA β mice exposed to DDT performed significantly worse compared to control females and fell below chance level (50%) ($p < 0.05$) (Control: 66%; DDT: 51%; $p = 0.026$). Although not statistically significant, DDT decreased percent alternation in males (Control: 62%; DDT: 53%; $p = 0.2398$). The total number of arm entries across groups did not change, suggesting the difference observed in alternations was not due to differences in activity. To assess whether DDT altered synaptic density, immunofluorescence staining was performed for SV2A and the brightness was quantified. DDT exposure decreased synaptic density in the hippocampus (39% in CA3, 27% in CA1, and 38% in the dentate gyrus) and entorhinal cortex (33% decrease) of males compared to the control, indicating reduced connectivity between neurons and thus a potential for cognitive dysfunction. In females, DDT exposure decreased synaptic density in the CA1 (10%) and dentate gyrus (12%), although these differences were not statistically significant. Preliminary

qualitative immunofluorescence images for oligomeric A β suggests that exposure to DDT exacerbates oligomeric A β in hippocampal and cortical regions in male and female hA β mice compared to vehicle-treated mice. However, no plaque pathology was observed. **Conclusions:** These findings demonstrate that DDT exposure induces cognitive deficits and increases oligomeric A β , accompanied by reduced SV2A levels. This evidence highlights the critical role of environmental exposures in altering mechanisms related to AD risk.

PS 3597 Association of Prenatal Pesticide Exposure with Plasma A β -42/40 and Cognitive Function in Midlife is Modified by APOE Genotype: Evidence from the CHDS cohort

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Background and Purpose: Complex gene-environment interactions across the lifespan are increasingly recognized as key contributors to Alzheimer's disease (AD) and related dementias, with studies linking pesticide exposure to a decline in cognitive performance. Dichlorodiphenyltrichloroethane (DDT), an organochlorine pesticide used extensively in the US to combat insect-borne diseases until its ban in 1972, persists in trace amounts due to its long half-life, bioaccumulation, and legacy dumpsites. Although environmental DDT levels have steadily declined, its persistent metabolite, dichlorodiphenyldichloroethylene (DDE), remains ubiquitously detectable and is considered non-neurotoxic. Further, Apolipoprotein ϵ 4 (APOE4) is the strongest genetic risk factor for late-onset AD (LOAD), but it is not entirely predictive. This exemplifies the need to investigate legacy exposures as people highly exposed to DDT between the 1940s and 1970s face a significantly increased risk of developing late-onset AD (≥ 65 years). We have previously reported nearly four times higher serum DDE levels in AD patients compared to healthy individuals, where the APOE genotype modified mini-mental state exam scores and contributed a ~4-fold increase in AD risk. Additionally, we demonstrated that DDE exacerbates amyloid (A β) pathology in cell culture and animal models, and disrupts cognitive function. However, little is known about the effects of DDE exposure on plasma A β -42/40 ratio, a reliable biomarker for prodromal AD and cognitive dysfunction in humans. Here, we sought to assess (i) the effects of human prenatal DDE exposure on cognition and plasma A β -42/40 ratio in mid-life and (ii) the impact of APOE4 genotype on plasma A β -42/40 ratio. **Methods:** To assess the effects of prenatal *p,p'*-DDE exposure, offspring from the Child Health and Development Studies (CHDS) pregnancy cohort (1960-1963) in Oakland, California, were recruited in 2010 for a follow-up study. Participants (mean age: 49.3y) completed cognitive function assessments, including the Wechsler Digit Symbol Substitution Test (DSST), and provided blood samples. A total of 179 individuals were included in this study (56% females and 44% males; 26% black and 74% non-black). Quanterix N3PA kit was used to measure plasma A β -42/40 in mid-life. Genomic DNA was extracted from the buffy coat, and the APOE genotype was determined using PCR-based SNP genotyping. Matched prenatal *p,p'*-DDE, measured in archived maternal pregnancy serum, was available. Associations were estimated in linear, binomial, and multinomial logistic regression models, adjusted for sex, race, and education. **Results:** Higher prenatal DDE levels were associated with lower mid-life DSST scores ($\beta = -0.0219$, 95% CI [-0.0401, -0.0038]; $p = 0.0178$). Associations were robust to multiple alternative modeling approaches. Adjusting for achieved education in mid-life did not alter results. Higher prenatal DDE levels were also associated with lower plasma A β -42/40 ratio in mid-life, where higher prenatal DDE was associated with increased odds of being in the lowest tertile of A β -42/40 ratio (Tertile 3: $\beta = -1.0176$; 95% CI [-2.0212, -0.0140], $p = 0.046$). The test for trend across DDE tertiles was significant ($p = 0.04$). Furthermore, poor cognitive performance in mid-life was associated with lower A β -42/40 ratios ($\beta = 0.0005$; 95% CI: [0.0001, 0.0008]; $p = 0.0069$). Lastly, APOE4 carriers also showed lower A β -42/40 levels ($\beta = -0.62$; 95% CI: [-1.15, -0.08]; $p = 0.02$), with an interaction between DDE and APOE4 ($\beta = 0.01$; $p = 0.04$), suggesting that APOE4 status modifies the relationship between early-life DDE exposure and mid-life amyloid pathology. **Conclusions:** Overall, these data indicate that DDE exposure has a long-lasting neurological impact on amyloid processing and cognitive function. Our translational findings in humans provide evidence that prenatal and early-life environmental exposures increase AD risk, advancing opportunities for early mid-life interventions to prevent disease progression.

PS 3598 Rotenone induced Parkinsonian neurotoxicity in fish alters central catecholamine metabolism in the brain

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Background and Purpose: Rotenone, widely used as an agricultural pesticide and piscicide in aquaculture, is highly toxic to aquatic life and can induce Parkinsonism by causing selective loss of dopaminergic neurons and disrupting catecholamine

metabolism in the brain. This study examines rotenone-induced neurotoxicity and its disruption of central catecholamine metabolism in the fish brain, developing a vertebrate model for Parkinson's-like neurodegeneration. **Methods:** Adult Indian freshwater catfish, (*Mystus cavasius*) were exposed either to a vehicle or water containing mitochondrial complex-1 inhibitor rotenone at 250 μ g/L for three days to induce dopaminergic neurodegeneration. The brain was collected, and catecholamines along with their metabolites were measured using high-performance liquid chromatography (HPLC). **Results:** Neurochemical analysis revealed decreased levels of dopamine (DA), its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), the vesicular storage index (DOPAC/DA), catecholamines (epinephrine and norepinephrine) and aldehyde dehydrogenase (ALDH) activity (DOPAC/DOPAL) with elevated levels of 3,4-dihydroxyphenylacetaldehyde (DOPAL) in the whole brain, indicating disrupted catecholamine metabolism. These alterations were associated with changes in the activities of key enzymes involved in catecholamine synthesis and degradation, including tyrosine hydroxylase (TH) and monoamine oxidase (MAO). Compared to controls, rotenone-treated fish exhibited reduced locomotor activity along with increased anxiety and aggressiveness. Immunohistochemically analysis further confirmed neuronal damages in brain areas controlling movement. **Conclusions:** These findings indicate that rotenone neurotoxicity profoundly disrupts brain catecholamine homeostasis, closely mimicking the neurochemical deficits observed in both mammalian models and human Parkinson's disease. Therefore, the rotenone-treated fish provide a useful model for studying Parkinsonian mechanisms and for screening potential neuroprotective agents targeting catecholaminergic dysfunction.

PS 3599 STING ablation attenuates rotenone-induced astrocytic activation and sex-dependent dopaminergic, motor deficits.

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Background and Purpose: Parkinson's disease (PD) is the fastest growing neurodegenerative disorder. Exposure to environmental toxins has been associated with an increased risk of disease-relevant pathology. A growing body of evidence shows glia are crucial in PD pathogenesis, illustrated by findings that abrogation of glia-mediated inflammation protects against PD-like pathology across various models. In recent years the antiviral cGAS-STING pathway has emerged as a potential target for disease-modifying therapies. A cytosolic dsDNA sensing and IFN-I stimulating system, post-mortem examination of PD patients has associated STING activation with PD pathology. Microglia, considered the primary immune cell of the brain, has been the focus of much of this work. However, astrocytes are crucial partners in responding to neurotropic viral infection and modulating immune responses. This work examines the effects of STING disruption on locomotor functioning utilizing the murine rotenone PD model in addition to interrogating the effect of rotenone exposure on astrocytic cGAS-STING signaling, both utilizing wild-type (WT) and STING-deficient mice. **Methods:** Primary astrocyte-enriched mixed glial cultures were established from neonatal C56BL/6 and golden-ticket STING truncation (Tmem173⁹⁹; GT) strains. We established the IC₅₀[Rotenone] = 78.90nM via dose-response viability assays in WT cultures following 12-hour exposure. Cell survival was again examined at doses at or below the IC₅₀ in GT, WT, and WT cultures co-treated with a STING modulator. Oxidative DNA damage and dsDNA breaks were assessed in DNA isolated from mitochondrial and nuclear fractions using commercially available 8-OHdG and p- γ H2A.X assays, respectively. Proinflammatory signaling was assessed longitudinally using NF κ B-GFP and IFN- β -YFP reporter lines. High-content immunofluorescence microscopy coupled with automated AI-based image analysis was performed against cGAS-STING signaling components. Transcriptomic analysis was performed on RNA extracted from WT and GT cultures following rotenone treatment. To further interrogate STING involvement in glia-mediated neurotoxicity, the viability of primary neuron cultures was determined after exposed to glia-conditioned media (GCM). Locomotor functioning was assessed in male and female WT and GT mice ($n = 5$ /sex/group) administered rotenone (2.5mg/kg/day) or vehicle intraperitoneally for 14 days, followed by a 14 day post-lesioning period. Vehicle-treated mitochondrial polG mutator mice ($n = 4$ /sex/group) served as a positive control. Baseline behavior measures were collected prior to rotenone insult, and every week for 28 days using open-field behavior assessment and automated gait system. Mice were then anaesthetized with isoflurane prior to euthanasia and striatal tissue was isolated for the quantification of catecholamines and monoamines commonly disrupted in PD using HPLC-ECD. **Results:** Rotenone treatment induced significant cytotoxicity in WT astrocytic cultures, which was blunted by both pharmacological and genetic STING disruption, as well robust oxidative damage and double-stranded breaks in both nuclear and mitochondrial DNA. Immunofluorescence microscopy reveals an increase in perinuclear p-STING⁺ puncta in GFAP⁺ and ALDH1L1⁺ astrocytes, and p-IRF3⁺ nuclear area in dual-labeled GFAP⁺/ALDH1L1⁺ astrocytes. Induction of downstream STING signaling partners was attenuated by STING ablation. Notably, co-treatment with the VDAC1 inhibitor DIDS blunted NF κ B-GFP and IFN- β -YFP induction. Further mechanistic interrogation revealed a STING-dependent enrichment of MAPK signaling and ECM remodeling via transcriptomics, as well as the secretion of TNF- α , IFN- β , VEGF, and IL-6 into the supernatant of WT cultures. Locomotor assessment reveal time-, treatment-, and genotype-dependent changes in behavioral parameters in male, but not female, WT mice. Moreover, changes were attenuated in GT mice and neurochemical assessment reveals sex-, treatment-, and genotype-specific alterations in dopamine and dopamine metabolites. **Conclusions:** This data demon-

strates the involvement of cGAS-STING in astrocyte reactivity *in vitro* and protection against locomotor changes *in vivo* following rotenone administration. Future work will assess the contribution of astrocytic cGAS-STING to PD-like pathology *in vivo*.

PS 3600 APOE4 Genotype Modifies Paraquat Effects on α -Synuclein Levels and Ventricular Volume in Aged Humanized Mouse Models

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Background and Purpose: There is increasing concern over the rise of Parkinson's Disease with dementia (PDD) and Dementia with Lewy Bodies (DLB), which collectively form the second most common form of dementia in the United States behind Alzheimer's disease. While age is the most common risk factor for PDD/DLB, presence of the Apolipoprotein E (APOE) 4 allele is one of the most well-established genetic risk factors for PDD/DLB. Studies have shown the APOE4 allele contributes to alterations in synaptic function, lipid and glucose metabolism, mitochondrial dysfunction, and increased neuroinflammation in the brain. Previous studies using transgenic knock-in mouse models containing the humanized targeted replacement APOE3 (TR-APOE3) and APOE4 (TR-APOE4) alleles have shown TR-APOE4 mice have increased PDD/DLB-associated cognitive and motor deficits and α -synuclein aggregation compared to TR-APOE3 mice after AAV-mediated overexpression of α -synuclein or injection of α -synuclein fibrils. While research studying the associations and risks of APOE4 on PDD/DLB pathogenesis has been conducted, there is a gap in the field evaluating the link(s) between APOE4 allele and environmental exposures, such as pesticides. Therefore, this study aims to expand the knowledge in that gap to understand the effects of paraquat (PQ), a widely used herbicide known to cause dopaminergic neuron loss and increase α -synuclein deposition and aggregation, on PDD/DLB-associated pathology and its interaction with APOE genotype. **Methods:** 12- to 14-month-old male TR-APOE3 and TR-APOE4 mice were administered 2 doses, 7 days apart, of either 10 mg/kg PQ or saline (control) via intraperitoneal injections for a total exposure time of 14 days. Following saline perfusion, brains were extracted, divided into hemispheres, and the left hemispheres were coronally sectioned. Fluorescence microscopy was used to assess α -synuclein pathology via immunofluorescence staining in the substantia nigra and striatum using the α -synuclein [MJFR1] antibody and co-localized with dopamine neurons and projections using a tyrosine hydroxylase antibody. Total ventricle volume was calculated by measuring the ventricle area of every 12th nissl-stained section between Bregma +1.44 mm through Bregma -3.84 mm, multiplied by the section thickness and summed. **Results:** In the dopaminergic neurons of the substantia nigra, we observed increased α -synuclein immunofluorescence in saline-administered TR-APOE4 mice compared to TR-APOE3 mice. Furthermore, we observed α -synuclein pathology in dopaminergic neurons of PQ-exposed TR-APOE3 and TR-APOE4 groups compared to saline controls, with TR-APOE4 groups showing the highest synuclein levels. Similar results were also observed in the striatum. In saline controls, TR-APOE4 mice showed increased α -synuclein immunofluorescence compared to TR-APOE3 mice. Additionally, PQ exposure increased striatal α -synuclein immunofluorescence in both TR-APOE3 and TR-APOE4 mice, with the TR-APOE4 mice presenting the highest levels of α -synuclein immunofluorescence. In addition to elevated synuclein deposition, clinical cases of PDD/DLB often present increased lateral ventricle volume. We first examined the effects of APOE4 genotype on lateral ventricle volume in saline-administered controls. In saline-administered controls, TR-APOE4 mice presented 106% increase in ventricle volume compared to TR-APOE3 mice. Next, we evaluated the effects of PQ on ventricle volume. Compared to saline controls, PQ exposure increased ventricle volume by 57% and 69% in TR-APOE3 mice and TR-APOE4 mice, respectively. **Conclusions:** Overall, our results demonstrate that the combination of PQ exposure and APOE4 genotype affect multiple pathologies associated with PDD/DLB. These findings suggest a potential link between APOE4 genotype and PQ exposure with PDD/DLB pathology.

PS 3601 Humanized APOE4 genotype exacerbates paraquat-induced tau pathology and NLRP3 inflammasome activation

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Background and Purpose: Neurodegenerative diseases involve neuronal loss and dysfunction caused by proteotoxic and oxidative stress and neuroinflammation, leading to progressive deficits in memory, movement, cognition, and sensory functions. Their main pathological features include misfolded protein aggregates, such as α -synuclein in Parkinson's disease (PD) and amyloid- β and hyperphosphorylated Tau in Alzheimer's disease (AD). PD is marked by dopaminergic neuron loss in the substantia nigra and Lewy bodies composed of α -synuclein, which are also observed in PD with dementia (PDD) and dementia with Lewy bodies (DLB). Tau pathology is a hallmark of AD, but can be observed in PD and DLB and PDD. APOE4 is the strongest genetic risk factor for late-onset AD, promoting A β and Tau accumulation more than other APOE isoforms and is also associated with Lewy body pathology in DLB and PDD. Studies using P301S tau mice with humanized APOE knock-in alleles

showed that APOE4 significantly increases tau accumulation and phosphorylation in the hippocampus compared to APOE2 or APOE3. However, the mechanism has not been well established. Paraquat (PQ), a herbicide linked to PD, induces dopaminergic neuron loss, α -synuclein aggregation, and neuroinflammation, while also activating the NLRP3 inflammasome. Recently, we have found that APOE4 genotype results in increased NLRP3 inflammasome activation. However, the interaction between PQ exposure and APOE4 genotype in tau pathology and inflammasome activation remains unclear. Here, we examined whether exposure of humanized APOE mice to PQ affected Tau pathology and the NLRP3 inflammasome. **Methods:** Humanized APOE3 or APOE4 (TR-APOE3/4) mice from Dr. Patrick Sullivan (Duke University) were bred in-house. Male and female mice (22-25 weeks old and 12-14 months old) received intraperitoneal injections of paraquat (10 mg/kg) or saline once weekly for two weeks and were sacrificed seven days later. After isoflurane anesthesia, blood was collected, and brains were perfused with PBS, fixed in 4% paraformaldehyde, and cryoprotected in 30% sucrose. Coronal brain sections (30 μ m) were immunostained for total Tau, phospho-Tau (AT8 (Ser202/Thr205), AT180(Thr231)), NeuN, and tyrosine hydroxylase (TH), imaged with a BZ-X810 microscope, and quantified using a BZ-X800 analyzer. **Results:** We examined total Tau levels in the cortex and striatum of TR-APOE4 and APOE3 mice after paraquat (PQ) treatment. Total Tau was markedly increased in both regions of TR-ApoE4 mice compared to TR-APOE4/Saline, TR-APOE3/Saline, or TR-APOE3/PQ groups, whereas TR-APOE3 mice showed only mild to moderate increases after PQ exposure. Tau hyperphosphorylation (p-Tau AT8, AT180) was significantly elevated in the cortex of TR-ApoE4 mice after PQ treatment, with slight increases in TR-APOE3 mice. In the substantia nigra (SN), p-Tau (AT8) levels were higher in TR-APOE4/PQ mice than in other groups and moderately increased even in TR-APOE4/Saline mice relative to TR-ApoE3/Saline. PQ treatment also modestly elevated p-Tau (AT8) in TR-APOE3 mice. Notably, p-Tau (AT8) levels in the cortex and SN were significantly higher in male than in female TR-APOE4 mice after PQ treatment. NLRP3 expression colocalized with Iba-1 was significantly elevated in the striatum of TR-APOE4/PQ group and moderately increased in TR-APOE3/PQ group, indicating enhanced inflammasome activation in APOE4 backgrounds. **Conclusions:** Taken together, these findings demonstrate that PQ exposure increases total Tau protein levels and Tau hyperphosphorylation, specifically p-Tau AT8 and p-Tau AT180, in neurons of the cortex, striatum, and substantia nigra. These effects are further exacerbated by the APOE4 genotype, and NLRP3 appears to play a key role in mediating Tau alterations in PQ-treated mice. Overall, this study highlights a critical gene-environment interaction that may potentiate Tau pathology and neuroinflammation, key features of multiple neurodegenerative diseases. This work was supported by National Institutes of Health grant R01ES033892.

PS 3602 Paraquat Exposure Increases Alpha-Synuclein and Phosphorylated Tau Levels in Rat Dopaminergic Neuron Cells

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Background and Purpose: Exposure to environmental toxins, such as pesticides, has been implicated as a risk factor for Parkinson's disease (PD) in many epidemiological studies. Paraquat (PQ), a widely used herbicide, is the best-known environmental risk factor for PD, due to its role in promoting oxidative damage, neuroinflammation, and triggering the accumulation of pathological proteins associated with neuronal dysfunction. PD-pathology is characterized by the accumulation of misfolded and aggregated proteins, particularly α -synuclein (α -syn) and phosphorylated-tau (pTau). Given PQ's ability to drive oxidative damage and protein aggregation, its exposure may contribute to PD pathogenesis by exacerbating neuroinflammation and neuronal degeneration through the upregulation of these pathogenic proteins. Here, we investigated the effects of PQ-exposure on α -syn and pTau protein expression levels in N27 dopaminergic cells to further elucidate PQ's role in protein aggregation and neuronal toxicity. **Methods:** N27 rat dopaminergic neuron cells were treated with PQ at varying concentrations (10 μ M, 20 μ M, 50 μ M, and 100 μ M) for 24 hours (n=6). Immunofluorescent staining was used to visualize and measure α -syn [MJFR1], phospho-synuclein (pS129), pTau Ser202/Thr205 (AT8), and pTau Thr231 (AT180) proteins in the treated cells. Fluorescent images were captured using a BZ-X fluorescence microscope. Quantification of fluorescence intensity was completed on the BZ-X800 Analysis software. Results were analyzed via one-way ANOVA followed by Tukey's post-hoc test, with p<0.05 considered statistically significant. **Results:** PQ exposure induced dose-dependent increases in PD-related pathological proteins in N27 cells. Data analyses revealed a 57% increase in total α -syn expression following 100 μ M PQ treatment compared to controls (p < 0.0001). pS129 demonstrated similar concentration-dependent results, showing significant increases of 62% and 57% at 50 μ M (p = 0.005) and 100 μ M (p = 0.0003) PQ indicating enhanced α -synuclein aggregation following PQ exposure. Tau phosphorylation was also significantly upregulated with increasing PQ concentrations. pTau (Ser202/Thr205) was increased, indicated by 70% increased AT8 fluorescence intensity at 100 μ M PQ compared to controls (p = 0.003). Likewise, pTau (Thr231) was similarly elevated as observed by a 72% increase in AT180 fluorescence at 100 μ M PQ relative to untreated cells (p = 0.005). **Conclusions:** These findings suggest that PQ not only exacerbates α -syn aggregation but also promotes tau phosphorylation, which has recently been shown to occur before α -syn pathology in PD brains. Additionally, these results further elucidate PQ's role as a neurotoxicant capable of inducing multiple hallmark features of PD pathology. Collectively, these findings demonstrate that

paraquat induces a dose dependent accumulation of pathological α -syn, p-syn, and pTau species in dopaminergic neurons, consistent with neurotoxic mechanisms implicated in Parkinson's Disease pathology.

PS 3603 Developmental PFOS exposure increases pancreatic β -cell sensitivity to secondary ROS-driven stress in zebrafish (*Danio rerio*) embryos

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Background and Purpose: Perfluorooctane sulfonate (PFOS), a ubiquitous and persistent environmental pollutant, has been detected in human tissues, including the pancreas, and cord blood, evidencing the ability of PFOS to expose developing fetuses during gestation. PFOS exposure has been shown to disrupt pancreatic function, resulting in dysregulated insulin signaling pathways and impaired glucose homeostasis, and induce oxidative stress via accumulation of reactive oxygen species (ROS)-driven damage, a critical factor in pancreatic β -cell dysfunction. We have previously demonstrated in zebrafish that developmental PFOS exposure negatively impacts pancreatic development and islet morphology. Whether these early structural alterations make pancreatic β -cells more susceptible to secondary insults remains unknown. With the rise of chronic diseases in the U.S., understanding how PFOS exposure, particularly early in life, contributes to long-term health outcomes, such as diabetes, is needed. Therefore, the primary goal of this investigation is to test the hypothesis that developmental exposures to PFOS alter pancreatic β -cell capacity to counter secondary redox stressors. **Methods:** We employed a transgenic zebrafish line with the bacterial nitroreductase (NTR) enzyme expressed under the insulin promoter (*Tg(Ins:NTR-mCherry)*), enabling us to selectively induce redox-active byproducts in pancreatic β -cells upon activation with the pro-drug metronidazole (MTZ). At high concentrations, MTZ is used to selectively ablate cells carrying the NTR enzyme, but at low concentrations, has been shown to selectively induce ROS. To confirm MTZ generates ROS and to optimize detection, *Tg(Ins:NTR-mCherry)* zebrafish embryos were challenged at 72 hours post-fertilization (hpf) with 0 (0.1% DMSO), 2.5, 5, 7.5, or 10 mM MTZ for 6 hrs, then incubated with 10 μ M CellROX Green for 15 or 60 mins. Samples were fixed with 4% paraformaldehyde (PFA) and principal islets imaged via confocal microscopy for β -cell mass volume (i.e. ablation) and CellROX fluorescence intensity (i.e. ROS). To examine β -cell resilience in PFOS-exposed embryos to a secondary redox stressor, *Tg(Ins:NTR-mCherry, Ins:GFP)* embryos were exposed to 0 (0.01% DMSO), 1, or 2 μ M PFOS, concentrations below the LOAEL found to impact islet size and morphology, from 3 to 72 hpf. At 72 hpf, larvae were washed, then challenged with either 0.1% DMSO or 5 mM MTZ for 6 or 18 hrs. Half of each treatment group was either fixed in 4% PFA or raised to 9 dpf in clean conditions. In fixed larvae, pancreatic islets were imaged via confocal microscopy, with images blinded prior to analysis. In samples challenged with MTZ, β -cell damage was scored as mild, moderate, or severe ablation, and islet volume was measured. To assess later-life health outcomes, fish were live-imaged at 9 dpf using brightfield and fluorescence microscopy to assess primary islet morphology, number of secondary islets, and overall growth (i.e. body length). **Results:** Findings following an MTZ challenge and a 60 minute CellROX incubation revealed a dose-dependent increase in CellROX fluorescence intensity, indicative of ROS generation in β -cells. Here, 5 mM MTZ was selected as an optimal concentration that generated significant ROS detection without leading to significant cell ablation. Exposure to 1 or 2 μ M PFOS or 5 mM MTZ alone did not significantly alter islet volume; however, developmental PFOS exposure followed by a 6 hr MTZ challenge yielded a dose-dependent increase in ablation severity, reflected by 6% and 22% decreases in islet volume in the 1 μ M and 2 μ M PFOS groups, respectively. At 9 dpf, preliminary results show no significant changes in growth across PFOS or MTZ groups; however, a significant difference in positional distribution of insulin-expressing cells along the whole pancreas was observed in the 1 μ M PFOS group challenged with MTZ compared to the control. **Conclusions:** These findings support the hypothesis and demonstrate that developmental PFOS exposure impairs β -cell capacity to counter ROS-driven secondary stressors. Future work will interrogate PFOS impacts on β -cell recovery and function at later-life stages, potentially informing pharmaceutical interventions. Overall, this research highlights how developmental contaminant exposure can impair cellular resilience, contributing to metabolic disorders later in life.

PS 3604 Individual phthalate or phthalate mixture alters metabolic health and microbiome via Sirtuin regulation

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Background and Purpose: Phthalates exposure has been linked to an increased risk of diabetes. Our previous studies have shown that individual phthalates are capable of disrupting metabolic health. While human exposure involves a mixture of phthalates (MIX), and not one specific, the effect of individual phthalate or MIX in adipose tissues has not yet been explored. Specifically, regulation of sirtuins under these exposures are virtually unknown. Here, we investigated the effect of Benzyl butyl phthalate (BBP) individually and in combination with other environmentally relevant phthalates namely diisobutyl phthalate, diisononyl phthalate, dibutyl phthalate,

di(2-ethylhexyl) phthalate, and diethyl phthalate on metabolic dysregulation via sirtuin-mediated pathways along with alterations in gut microbiome composition.

Methods: To investigate the effect of individual phthalates, we exposed 8-week-old male and female mice to BBP with and without High fat diet (HFD) for 16 weeks. Sirtuin3 (Sirt3) gene expression analysis was performed in subcutaneous (SAT), visceral (VAT), and brown (BAT) adipose tissue. Additionally, to understand the mechanistic connection, 3T3L1 cells were treated with 100 μ M palmitic acid (PA) and BBP ranging from 0.1-50 μ M differentiated until Day 8. Adipogenic, Sirt3, and mitochondrial gene expression analysis as well as Oil Red O staining was performed on Day 8 of differentiation. Furthermore, to understand the chronic effect of MIX, mice were exposed to either MIX (200 μ g/kg, 5mg/kg, 200mg/kg) or VC on CD (4 kcal% fat, set1) or HFD (60 kcal% fat, set2) for 14 weeks. We examined weekly body weight, fasting glucose, glucose tolerance, insulin resistance (IR), serum insulin, triglyceride, and cholesterol levels in both sets of mice. Additionally, composition and diversity of the gut microbiome in the mice exposed to HFD and 200mg/kg of MIX using MiSeq platform were analyzed. We also carried out Ingenuity Pathway Analysis (IPA) to understand the effect of phthalates on the downstream targets specifically Sirt3 contributing to disease condition. *In silico* analysis are being carried out to connect microbiome regulation with sirtuin pathways. **Results:** Male mice exposed to BBP showed significant downregulation ($p < 0.05$) of Sirt3 in SAT along with decreasing trend in VAT and BAT. In contrast, female mice showed significant downregulation ($p < 0.05$) of Sirt3 in BAT. In our mechanistic study, BBP treatment with PA showed a dose dependent increase in adipogenic genes, PPAR γ and aP2 expression and Oil Red O staining with a concomitant decrease in Sirt3 expression and glucose uptake. Moreover, both the treatment groups showed a significant decrease in mitochondrial markers; NRF1, NRF2, and PGC-1 α . Furthermore, in set 1 mice, chronic exposure at 5mg/kg showed an increasing trend in body weight compared to VC. At week 8, 5mg/kg showed increasing trend in fasting glucose and 200mg/kg group showed significant increase in fasting blood glucose and IR. Additionally, in set 2 mice, 200 μ g/kg and 200mg/kg doses showed significant increases in body weight compared to VC. Both 200 μ g/kg ($p < 0.05$) and 5mg/kg ($p < 0.05$) showed increase in fasting blood glucose at week 10. Additionally, at week 14, an increase in fasting glucose level was observed at 5mg/kg ($p < 0.001$). Glucose intolerance was evident at all doses, with IR at only 5mg/kg ($p < 0.01$). Interestingly, 5mg/kg MIX displayed more inflamed, hypertrophied adipocytes in both SAT and VAT compared to VC or the 200mg/kg dose. Microbiome analysis revealed that the MIX significantly altered the alpha and beta diversity and reduced the abundance of beneficial bacterial species. The alpha diversity was increased in the MIX treated group at both the phylum ($p < 0.05$) and genus level ($p < 0.05$). The beta diversity exhibited non-significant differences. However, at the phylum level, Firmicutes increased and Bacteroidetes decreased after the treatment. Our IPA analysis showed that all six phthalates can directly and indirectly modulate Sirt3 and contribute to mitochondrial dysfunction. Finally, our *in-silico* analysis reveals that sirtuin regulates the microbiome. **Conclusions:** Phthalates may disrupt metabolic dysfunction via sirtuin 3 pathway. Therefore, we predict that an early epigenetic therapeutic strategy to treat phthalate induced metabolic disruption will significantly advance the field of environmental health.

PS 3605 Regulatory Effects of ZCE on 3T3-L1 Adipocytes differentiation and High-Fat Diet induced Obesity in Mice

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Background and Purpose: With the global rise in obesity prevalence, various anti-obesity drugs are being developed. However, many currently available treatments are expensive and associated with significant side effects. *Zephyranthes carinata* is a naturalized plant species native to South America. It is primarily cultivated as an ornamental plant in gardens and pots. In South Korea, it has been observed in limited areas, particularly on Jeju Island. We will investigate the anti-obesity effects of extracts from *Z. carinata* leaves (ZCE). This study aimed to investigate the anti-obesity effects and pharmaceutical potential of ZCE, a natural extract, using 3T3-L1 cells and high-fat diet induced obesity mice. **Methods:** Differentiation of 3T3-L1 preadipocytes was induced using an IBMX, Dexamethasone, Insulin (MDI cocktail) in the presence of ZCE. Cell viability was evaluated via MTT assay. Intracellular lipid accumulation was assessed using Oil Red O staining. Expression levels of proteins and genes related with lipid metabolism were analyzed by Western blotting and RT-PCR, respectively. ICR mice were fed with high-fat-diet for 6 weeks to induce obesity. Serum and epididymal adipose tissues were collected for further analysis. The effects of ZCE administration were evaluated by measuring body weight and serum biochemical parameters. Epididymal adipose tissues were analyzed by Western blotting and RT-PCR to examine the expression of lipid metabolism-related markers. **Results:** ZCE inhibited the differentiation of 3T3-L1 cells as the concentration increased. Analysis of gene and protein expression revealed a significant reduction in the expression levels of lipid metabolism-related markers. Consistently, quantitative analysis of Oil Red O staining demonstrated that intracellular lipid accumulation was also suppressed in a dose-dependent manner following ZCE treatment. In the *in vivo* study, ZCE administration reduced the body weight of obese mice compared to the untreated obese group. ZCE exerts anti-obesity effects by regulating adipogenic and lipogenic signaling path-

ways. **Conclusions:** These findings suggest that ZCE has potential as a natural therapeutic candidate for obesity treatment. Further studies will be conducted to explore other potential mechanisms involved in its anti-obesity activity.

PS 3606 Perfluorooctanesulfonic acid (PFOS) and glucose exposures alter pancreatic islet vascularization during development in zebrafish (*Danio rerio*)

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Background and Purpose: Vascularization of the islets of Langerhans provides essential contact for pancreatic β -cells to monitor blood glucose and secrete insulin to maintain normal glucose levels. However, this vascularization also renders islets vulnerable to toxicants that can enter systemic circulation. One such toxicant is perfluorooctanesulfonic acid (PFOS), a ubiquitous environmental contaminant found in >98% of human serum samples tested. Previous studies have shown that exposure of zebrafish embryos to PFOS increased aberrant islet morphology and disrupted pancreatic organogenesis, although the mechanisms that underlie these changes are not understood. In our recent study using single-cell RNA-Seq to measure gene expression changes in PFOS-exposed zebrafish eleutheroembryos, we found reduced expression of cell-cell adhesion genes including integrins that are essential cell surface proteins connecting cells to the extracellular matrix. In islets, activation of integrins at contact points between vascular endothelial cells and β -cells can drive both β -cell proliferation and polarization, with critical implications for the proper release of insulin to the vasculature. Thus, we hypothesized that the abnormal arrangements of β -cells in PFOS-exposed embryos co-occur with alterations in islet vascularization. **Methods:** To establish the pliability of islet vasculature in zebrafish embryos and larvae, we used a prolonged nutrient challenge and confocal microscopy to map and measure changes in islet size, structure, and vascularization. To examine the vasculature in combination with pancreatic β -cells, *Tg(fli1:GFP)* x *Tg(ins:mCherry)* fish were used allowing for visualization of the vasculature endothelial cells and pancreatic β -cells respectively. Embryos were exposed to glucose (0, 15, 30, or 50 mM), or PFOS (4, 8, and 16 μ M) or both starting at 24 hpf, fixed and imaged at 96 hpf. Fish were imaged using confocal microscopy to visualize contact between vasculature and Nikon NIS Elements software was used to measure the surface area of contact points between vasculature and β -cells in embryos exposed to glucose, PFOS or co-treatment. **Results:** Results showed no significant changes in β -cell volume at 15, 30, and 50 mM glucose concentrations at 96 hpf. However, β -cell volume was significantly reduced compared to controls with exposure to 4 and 8 μ M PFOS. In zebrafish that were exposed to PFOS and glucose, there were concentration dependent increases in volume compared to PFOS only groups with no significant differences compared to controls. Embryos exposed to glucose only had no significant changes in the total contact point surface area between β -cells and blood vessels although there was a trend of increase in total contact point surface area in the 15 and 50 mM groups compared to the controls. In contrast, embryos exposed to 16 μ M PFOS had a reduction in the total contact surface area between β -cells and blood vessels at 96 hpf. Results also showed a trend of reduction in total contact surface area for both PFOS and glucose exposed groups although not significant. **Conclusions:** These results show that a glucose enriched environment increases β -cell mass and the contact between vasculature and β -cells, which agrees with evidence that vascular endothelial cells and β -cells coordinate growth and interact to respond to an increased need for insulin. Exposure to PFOS during development seems to disrupt this interaction raising concerns for how fetal islet growth may occur in cases of gestation diabetes. Ongoing studies are looking at how exposure of embryos to integrin inhibitors will affect vascularization of the islet and insulin production as well as the potential of offspring developing diabetes in later life time-points. Comparative RNA-sequencing analyses from prior PFOS exposure studies across species will also be performed to assess the concordance of potential mechanisms through which PFOS may influence islet vascularization.

PS 3607 Concurrent Iron Supplementation Prevents Cardiomegaly and Metabolic Dysfunction-Associated Steatotic Liver Disease in Mice with Perinatal Cadmium Exposure

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Background and Purpose: Despite regulations and guidelines designed to minimize public contact with heavy metals, cadmium exposure through food, water, or tobacco remains prevalent during pregnancy and affects many organ systems of the developing fetus, including the nervous, cardiovascular, and hepatobiliary systems. Pregnancy is a particularly vulnerable time for cadmium exposure due to a programmed increase in uptake of divalent metals, and both pregnancy and cadmium exposure increase the risk of iron deficiency anemia. We have previously demonstrated that perinatal exposure to cadmium induces growth restriction, cardiomegaly, and juvenile metabolic dysfunction-associated steatotic liver disease (MASLD). Because iron is an essential element required for proper growth and organogenesis, we hypothesized that providing dams with supplemental iron concurrently with cadmium exposure will preclude iron deficiency and anemia onset and, in turn,

rescue the morphometric, cardiac, and hepatic outcomes in offspring. **Methods:** To test this hypothesis, virgin C57BL6/J dams were treated with cadmium in the form of 30 mg/L cadmium chloride in water. Dams were provided with treated drinking water from five weeks prior to mating and throughout gestation, until ten days post-partum. Two levels of iron supplementation, "low-dose" amounting to double the amount of ferric citrate in standard chow, or "high-dose" amounting to five-times the amount in standard chow were provided to dams. Embryos and dams were collected at embryonic day 18.5 (e18.5) or alternatively, pups were collected at postnatal day 21 (PND21), and body and organ weights were observed. Cadmium absorption and iron deficiency status were determined via inductively coupled plasma mass spectrometry (ICP-MS), and anemia status was evaluated by quantifying hemoglobin levels. Biochemical, molecular, and histological signatures of MASLD were evaluated in offspring at PND21. **Results:** The protective effects of iron supplementation depended on the subject and timepoint. In dams, the high-dose regimen effectively improved the iron deficiency and anemia outcomes that resulted from cadmium exposure, and levels of hepatic stored cadmium were reduced. However, stored iron levels remained low due to cadmium. In e18.5 embryos, low-dose iron treatment partially rescued cadmium-induced cardiomegaly, and the high-dose regimen fully returned heart size to normal. High-dose iron induced growth restriction and placental insufficiency at e18.5, signs of overt toxicity, however these outcomes disappeared by PND21. At this later timepoint, low-dose iron supplementation partially rescued cadmium-induced cardiomegaly, and high-dose iron fully ameliorated the condition, following the trend observed in embryos. Iron deficiency and anemia statuses were largely returned to normal by the low-dose regimen. Males did remain slightly anemic, but high-dose iron raised hemoglobin levels to normal. Iron supplementation was also sufficient to prevent cadmium-induced juvenile MASLD. Perinatal cadmium exposure upregulated hepatic transcription of several genes linked to steatosis and fibrosis, including genes related to fatty acid transport (*Pparg*, *Fabp4*, *Cd36*), accumulation of triacylglycerides (*Fsp27*) and collagen biosynthesis (*Tgfb1*, *Col1a1*, *Col5a2*, *Col6a1*). Low-dose iron treatment returned expression levels to control levels in males and moderated the expression profile in females. Signs of cadmium-induced hepatic steatosis disappeared with low-dose iron supplementation in both male and female offspring. **Conclusions:** The developmental toxicity of cadmium exposure can be ameliorated by dietary iron supplementation, though there are some limitations to consider. Because the high-dose iron group exhibited growth restriction and placental insufficiency in embryos near birth, a regimen containing five-times the standard iron levels in chow exceeds the appropriate level of treatment. However, these detrimental effects did not persist at PND21, and the lower iron dose was sufficient to reduce the majority of cadmium-induced effects on growth, cardiac, and hepatic presentations. Our findings highlight the potential for oral iron supplementation as a therapeutic intervention in populations with high risk for cadmium exposure.

PS 3608 Impact of Pancreatic Sweet Receptors on Beta Cell Function and Pathobiology throughout Development

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Background and Purpose: Diabetes is a public health issue affecting over 38 million Americans, with 90-95% being Type 2 Diabetes (T2D), a chronic condition marked by insulin resistance and impaired insulin secretion, causing dangerously high blood sugar levels without management. Though most often observed in adults over 45, children and teens increasingly develop both obesity and T2D. The pancreas regulates metabolic processes by maintaining glucose homeostasis through insulin and glucagon secretion. During pancreatic organogenesis, disruptions from genetic, epigenetic, or environmental factors can lead to long-term issues like T2D. Non-nutritive sweeteners (NNS) are substances that provide sweetness to foods and beverages without adding significant calories or nutrients. NNSs are extensively used in Western human diets and are environmentally prevalent due to excretion with minimal degradation. Sweet receptors (TSR) are primarily known for their role in taste perception; however, they are also expressed in pancreatic β -cells. Research has suggested that activation of the sweet receptor heterodimer, T1R2 and T1R3, can trigger insulin secretion—even in the absence of glucose. Here, we hypothesize that TSR activity is sufficient for insulin secretion and that exposure to TSR activators, such as NNS, may result in transcriptional and morphological changes in pancreatic islets during organogenesis and juvenile development. **Methods:** A well-established beta cell line, INS-1s, were exposed to biologically relevant concentrations (0.1, 1, 10, 100 μ g/mL) of glucose and several common NNSs, including Sucralose, Saccharin, and Acesulfame Potassium (AceK). Co-treatment with Lactisole (1 mM), an inhibitor of the sweet taste receptor heterodimer, determined whether actions were mediated through the sweet receptor. Cell viability and proliferation were measured using microscopy, and RNAseq was performed. *Tg(insulin::GFP)* zebrafish (*Danio rerio*) embryos were collected at 1 hour post fertilization (hpf), screened for viability, and dechorionated at 24 hpf. Embryos were exposed to NNS at either a high (1 mg/L) or a low concentration (10 μ g/L) from 24-96 hpf. Embryos were immobilized and imaged individually on a Nikon Eclipse Ti-2 microscope using NIS imaging software. Larvae that exposed from 24-216 hpf to glucose and/or Lactisole were also examined for islet size and number of secondary islets, and RNAseq was performed. For all endpoints, 1- or 2-way ANOVAs were performed after assessment of normality and variance. A confidence level of 95% ($\alpha = 0.05$) was used. **Results:** Our *in vitro*

results show that our NNSs and glucose cause concentration-dependent proliferation in INS-1 cells. Cells co-treated with Lactisole experienced lower rates of proliferation. We observed no change in cell viability due to biologically relevant concentrations of glucose or NNS. Our *in vivo* results included morphology changes in primary islets size and number of secondary islets in the presence of Glucose. However, islets sizes decrease whenever exposed to Lactisole, regardless of glucose availability. When Lactisole was co-administered with glucose, transcriptomic analyses revealed that Lactisole administration reduced the number of significantly changed metabolic pathways. Finally, embryos exposed to the NNS AceK have larger β cell areas, while sucralose and aspartame exposures did not induce islet expansion in larvae. **Conclusions:** Our *in vitro* findings suggest that NNS and glucose promote INS-1 cell proliferation in a concentration dependent manner, while co-exposure with the TSR inhibitor Lactisole suppresses this effect. Importantly, these exposures did not compromise the cells viability, indicating that the observed changes reflect altered proliferation rather than cytotoxicity. Our *in vivo* findings suggest that glucose exposure promotes pancreatic islet growth and metabolic activity, while Lactisole suppresses these effects. This indicates that TSR signaling contributes to islet development. Among the tested NNS exposures, only AceK increase β -cell area, suggesting distinct compound-specific impacts on pancreatic function. Further work is necessary to examine the TSR as a potential mechanism of insulin secretion through pancreatic β cells and to determine if NNS use during development may impact metabolic health.

PS 3609 Developmental exposure to polychlorinated biphenyls (PCBs) impacts expression of potassium ion channels and steroid hormone receptors in the bladder and brain of adult mice

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Background and Purpose: Polychlorinated biphenyls (PCBs) are anthropogenic, ubiquitous, persistent organic pollutants produced over the last century. Mice with *in utero* and lactational exposure to human-relevant PCBs develop signs of lower urinary tract dysfunction with weaker bladder muscle contraction as adults. PCB effects in this exposure model are sex- and dose-dependent, therefore, we hypothesize PCBs effects arise through increasing expression of the BK channel (large-conductance Ca^{2+} -activated K^{+} channel) which regulates bladder relaxation and is important in neuronal excitability, and/or endocrine disruption through steroid hormone signaling. PCBs are also known neurotoxicants, given that central nervous system control of the bladder is essential for proper function it is important to understand effects of PCBs at the level of the bladder and the brain. Therefore, we also test whether changes in BK channel and hormone receptors occur in the bladder, brain or both and whether these changes occur embryonically or only as adults. **Methods:** PCB exposure of mice mimicked human-relevant developmental exposure where PCBs from the maternal source were transferred *in utero* and through lactation. Female nulliparous mice (C57BL/6) were dosed with MARBLES PCBs (serum PCB profile of pregnant women enrolled in Markers of Autism Risk in Babies - Learning Early Signs) at 0, 0.1, 1, or 6 mg/kg/d 2 week prior to mating. Dam dosing continued until fetal tissue collection (E18) or until weaning (P21). Aged offspring (~P42) were euthanized with bladders and brains immediately collected. qPCR was performed to quantify RNA abundance of BK channel subunits (*Kcna1*, *Kcnmb1-Kcnmb4*), estrogen receptors (*Esr1*, *Esr2*), and progesterone receptor (*Ppr*) in the different tissues and time points. Blood was collected at P42 for a serum steroid hormone panel (progesterone, testosterone, estradiol, and estrone). **Results:** PCB exposed mice had sex- and dose-dependent impacts on transcriptional expression of genes related to bladder smooth muscle relaxation and steroid hormone signaling. At P42, PCB effects in the bladder were primarily observed in the 1 mg/kg/d PCB males with increased RNA abundance of BK channel subunits and trending increases in estrogen and progesterone receptors compared to the vehicle control. In the brain, these PCB effects were not observed, and PCBs decreased estrogen receptor alpha expression in male mice. Conversely, female mice did not display any PCB effects on expression of these genes in the bladder, however with 1 and 6 mg/kg/d PCB exposure had decreased RNA abundances of BK channel subunits, estrogen receptors, and the progesterone receptor compared to the vehicle control in the brain. Quantification of the urogenital sinus at E18 was limited to markers observed changed at P42, with no observed PCB effects. No PCB effect was observed on serum levels of testosterone or estradiol in the P42 male mice with results pending in the female mice. **Conclusions:** Human-relevant PCBs impact murine urinary voiding physiology and bladder contractility. These sex- and dose-dependent adverse outcomes may in part be influenced through PCB effects on expression of ion channels important to intracellular calcium dynamics in nerves and bladder smooth muscle, and impacts on steroid hormone receptor expression. Our results highlight that PCBs uniquely impact transcript levels of genes important for contractility/nerve activity and hormone signaling in the bladder versus the brain and do so in a sex-dependent manner. Further, adult changes in bladder gene expression were not observed embryonically, suggesting a temporal component of these effects on bladder. These differences help to understand the complex effects of developmental PCB exposure on bladder health. Adverse health outcomes of PCB exposure and influence on neurodevelopment, urological impacts including bladder smooth muscle over/underactivity, and endocrine disruption of steroid hormone signaling are clinically relevant yet the impacts of environmental chemicals on the molecular underpinnings of these disorders require further study.

PS 3610 Persistent impacts of parental BaP-exposure on adult male behavior and brain transcriptome in Japanese medaka (*Oryzias latipes*)

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Background and Purpose: Benzo[a]pyrene (BaP) has previously been shown to affect neurobehavioral responses in *F₁* *Oryzias latipes* larvae upon parental exposure. However, little is known about the persistence of these behavioral changes throughout the life span. Therefore, the present study aims to investigate differences in behavior and gene expression in brain tissue between exposed and unexposed *O. latipes* to determine whether neurological processes could contribute to downstream behavioral effects. **Methods:** Ten pairs of adult medaka (n=5) were exposed to solvent control or 1 $\mu\text{g/L}$ BaP for 21 days. Eggs were collected after the exposures ended and offspring raised to 4 months old. Male and female fish were introduced to three different experimental set-ups for 10-minute increments and recorded for software-based behavioral analysis. Lateral and diving swimming patterns as well as light/dark preference were assessed. Subsequently, brain tissue from control and BaP-exposed group male *F₁* medaka was extracted. Gene expression was analyzed using single-nuclei RNA sequencing (3DGenomics; scRNA-seq). For both control and exposed groups, 14 cell clusters were identified. Major cell types were assigned using the most significantly expressed genes in each cluster. These cell type assignments were confirmed by the presence of their respective key marker genes in previously published literature. Differentially expressed genes (DEGs) between the control and exposed groups were identified by analyzing the fold change difference in expression. Enriched pathways were identified for each cluster using gProfiler. **Results:** The data from the behavioral experiments indicate an increased affinity for darker environments and display increased patterns of lethargy in adult male fish with a parental exposure history. This is consistent with patterns previously observed in the larvae and is indicative of an increased stress response in the parentally exposed fish. The snRNA-seq allowed for identification of glycinergic neurons, GABAergic interneurons, cortical pyramidal neurons, midbrain glutamatergic neurons, endothelial cells, pallium cells, Purkinje neurons, pineal gland cells, glial cells, endoderm cells, taste bud cells, olfactory bulb neurons, oligodendrocyte progenitor cells (OPCs), and vascular smooth muscle cells. Genes most differentially expressed between the exposed and control groups were identified. Few changes were observed in the glycinergic neurons, the endothelial cells and the olfactory bulb cell cluster (<7 DEGs). Less than 20 DEGs were measured in the cortical pyramidal neurons, the pallium cells, the glial cells, the endoderm cells and the taste bud cells. Major expression changes were identified in the GABAergic interneuron cluster (16 downregulated, 4 upregulated), the midbrain glutamatergic neuron cluster (3 downregulated, 53 upregulated), the Purkinje neuron cluster (4 gene downregulated, 26 upregulated) and the pineal gland cell cluster (30 downregulated, 7 upregulated). Thus, the data indicate cell population-specific impacts of parental BaP exposure on the adult male brain. **Conclusions:** The findings of this study demonstrate that parental BaP exposure causes persistent and cross-generational neurobehavioral alterations that remain detectable in adulthood and are accompanied by cell type-specific transcriptional disruptions in the *F₁* brain. The lethargy and dark-preference behaviors in exposed males align with DEG patterns involved in inhibitory-excitatory balance, sensorimotor processing, and stress-axis regulation. Downregulation in GABAergic interneurons and cortical pyramidal neurons, coupled with large clusters of upregulated genes in midbrain glutamatergic and Purkinje neurons suggest that BaP may imbalance inhibitory and excitatory signaling pathways. Altered expression in pineal and glial cell clusters suggests impacts on circadian regulation and neuroinflammation, processes known to modulate stress responsiveness and activity levels. Since many neurological processes are conserved among vertebrates, this study may predict effects of BaP on ecosystem and human health and can inform environmental risk assessments.

PS 3611 Maternal Metabolic Health Alters Infant Adipocyte Differentiation and Inflammatory Signaling via Human Milk Lipids

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Background and Purpose: Despite extensive research on the influence of *in utero* exposures on offspring metabolic health, there remains a gap in understanding the influence of lactation on metabolic programming, particularly among women living with obesity or diagnosed with gestational diabetes (GDM). As infant metabolic programming extends beyond parturition, exposures during lactation likely contribute to downstream metabolic dysfunction; however, little is known about molecular mechanisms driving any such changes in offspring. The rates of pediatric and adult obesity and metabolic disease continue to rise worldwide, highlighting the need to investigate early-life intervention points. Identifying the impact of maternal health-status on early childhood metabolic risk parameters provides a unique opportunity to evaluate potential windows of intervention whereby changes in maternal diet may successfully reduce the burden of disease in future generations. Our proposed research aims to better understand how variation in human milk (HM) composition impacts offspring metabolism using a model of infant preadipocytes. Based on our previous work linking HM fatty acid profiles and infant adiposity, we hypothesize that infant adipocyte maturation and inflammatory states differ following HM lipid exposure from moms with varying health statuses. **Methods:** A non-immortalized,

diploid human infant preadipocyte strain derived from subcutaneous adipose tissue of a 3-month-old male donor with Simpson-Golabi-Behmel Syndrome (SGBS) was differentiated. HM underwent an *in vitro* digestion and lipid extraction protocol after which conjugated HM lipids from mothers with varying health-statuses, defined as healthy, obese, and gestational diabetes (GDM), were used to treat the SGBS cells. A lipid depleted bovine serum albumin (BSA) at 0.67% was used as the control. Differentiation was initiated on day 0 (D0) when the cells reached 80-90% confluence. To mimic circulating levels of serum free fatty acids (FFAs) in infants, SGBS preadipocytes were treated with 200µM of lipids isolated from either the healthy, obese, or GDM group on D6-8, D6-10, D6-12, or D6-14 during adipocyte maturation. After each treatment window, RNA was isolated and analyzed via RT-qPCR for PPAR γ , GLUT4, FABP4, UCP1, GATA6, METRN1, PREF1, AMPK α 1 (differentiation/browning), and CCL2, CXCL6, IL-6, IL-12 (inflammation). Statistical significance was defined as $p < 0.05$ following a two-way ANOVA with Tukey's post-hoc for multiple comparisons. **Results:** Expression of differentiation/browning (UCP1) and inflammatory markers (CCL2, CXCL6, IL-12) varied by exposure duration and maternal health status. After D6-10, UCP1 increased in GDM compared to healthy followed by a decrease in expression with extended treatment, D6-12 and D6-14 ($p = 0.006$). Following D6-8, CCL2 and CXCL6 trended towards increased expression in GDM treated groups compared to healthy ($p < 0.1$). This increase recovered during the next treatment window; however, following D6-14, CCL2 and CXCL6 significantly increased in GDM treated groups compared to healthy, aligning with a biphasic increase in inflammatory signaling ($p < 0.0001$). IL-12 increased in GDM compared to healthy from the beginning of treatment (D6), a trend which then increased in severity through the end of treatment period (D14) (p -values ranging from 0.0483 to < 0.0001). **Conclusions:** From D6-8 to D6-10, the increase in UCP1 observed in the GDM groups, together with elevations in the chemokines CCL2 and CXCL6 (innate-immune signaling), may indicate a browning phenotype: potentially a protective or compensatory response to lipid/fatty-acid exposure in which cells enhance oxidative capacity and immune-like signaling for homeostasis. As treatment continued, UCP1 drops while IL-12 increases significantly and stays elevated which suggests a shift toward chronic inflammatory signaling and possible loss of metabolic flexibility. This may reflect the breakdown of the compensatory browning program toward a dysfunctional phenotype. The second increase in CCL2 and CXCL6 and sustained increase of IL-12 reinforces a pro-inflammatory, metabolically stressed adipocyte phenotype. These preliminary findings suggest that maternal health status may significantly influence infant adipocyte development and downstream metabolic risk via exposures occurring during lactation.

PS 3612 Green-engineered montmorillonite clays for the adsorption and detoxification of polychlorinated biphenyls

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Background and Purpose: The prevalence of polychlorinated biphenyls (PCBs) in the environment has been linked to a broad range of adverse toxicological effects in both animals and humans. These include carcinogenicity, immunosuppression, neurotoxicity, obesity, diabetes, and endocrine disruption. In this study, *in vitro*, *in silico*, and *in vivo* models were used to investigate the surface interactions of PCBs with green-engineered hydrophobic clays. In earlier work, we showed that these green clays tightly adsorbed benzene and significantly reduced its toxicity. Our objective for this study was to show that green clays could also sorb PCBs and decrease the toxicity of a commercial PCB mixture. **Methods:** Green-engineered clays (GECs) were synthesized by amending montmorillonite clays with chlorophyll and chlorophyllin to increase their binding proficiency. Gastrointestinal pH and temperature were simulated *in vitro*, and the clay surface binding interactions of six PCB congeners (PCB 77, PCB 126, PCB 153, PCB 154, PCB 155, and PCB 157) were characterized using isothermal analyses. Binding parameters and thermodynamics, including sorption capacity, enthalpy of sorption, free energy of sorption, affinity, and capacity of the GECs for the six PCB congeners, were investigated. The *in vitro* data was validated while *in silico*, and molecular dynamics simulations were used to explore the amendments of the clay and provide atomistic insights into PCB congener binding to both the parent and green-engineered clays. To confirm the ability of the GECs to protect a living organism, a commercial mixture of PCBs, Aroclor 1260, was investigated using a well-established hydra assay. **Results:** The results for both the parent clays and GECs demonstrated that all the PCB congeners fit the Langmuir model, indicating saturable binding sites with high capacities and affinities at both acidic and neutral conditions. The GECs had increased capacities for the PCBs at acidic and neutral conditions. For instance, chlorophyll amendment markedly increased the clay's binding capacity for PCB 153 ($0.19 \rightarrow 0.46 \text{ mol kg}^{-1}$), PCB 154 ($0.26 \rightarrow 0.54 \text{ mol kg}^{-1}$), PCB 155 ($0.15 \rightarrow 0.27 \text{ mol kg}^{-1}$), PCB 157 ($0.12 \rightarrow 0.22 \text{ mol kg}^{-1}$) and PCB 77 ($0.36 \rightarrow 0.70 \text{ mol kg}^{-1}$). Similarly, chlorophyllin amendment enhanced the GEC's adsorption capacity for PCB 153 ($0.19 \rightarrow 0.54 \text{ mol kg}^{-1}$), PCB 154 ($0.26 \rightarrow 0.55 \text{ mol kg}^{-1}$), PCB 155 ($0.15 \rightarrow 0.25 \text{ mol kg}^{-1}$), PCB 157 ($0.12 \rightarrow 0.22 \text{ mol kg}^{-1}$) and PCB 77 (from 0.36 to 0.66 mol kg^{-1}). This finding was corroborated by independent molecular dynamics (MD) simulation, which revealed that PCB molecules capable of adapting co-planar configurations (PCB77, PCB126, and partly PCB157) were able to align both rings in the same plane, enabling the molecules to bind to clay surfaces and maximizing interactions across both aromatic rings and the clay surface. PCB molecules interacted mainly with both the head (chlorin ring) and tail (aliphatic) groups of chlorophyll simultaneously or only the tail of chlorophyll, and to a lesser extent, the head

of the chlorophyll only. The MD simulation showed that chlorophyll amendments enhanced the binding of PCBs. Moreover, isotherms of adsorption for all the PCBs were thermodynamically favorable with ΔG values ranging from -19.00 to -32 kJ/mol . **Conclusions:** The findings of this study indicate that edible green organoclays adsorb PCBs with the highest sorption associated with co-planar congeners. The mechanism for this adsorption to chlorophyll-amended surfaces may involve van der Waals attraction of PCBs to the hydrophobic tail on chlorophyll and/or pi-to-pi and cation to pi interactions from benzene rings in PCBs to aromatic chlorin rings in chlorophyll. Further studies are warranted to study the application of these "edible" clays as potential supplements in the diet to reduce the bioavailability of PCBs from contaminated food and water, especially following disasters and emergencies.

PS 3613 Integrating Behavioral, Morphological, and Transcriptomic Endpoints to Define PCB Developmental Toxicity in Zebrafish

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Background and Purpose: Polychlorinated biphenyls (PCBs) are neurotoxic, persistent organic pollutants; however, the mechanisms underlying their developmental neurotoxicity remain incompletely defined. Zebrafish (*Danio rerio*) provide a high-throughput, mechanistic model to assess chemical bioactivity across behavioral, morphological, and molecular endpoints. This study aimed to systematically evaluate the developmental effects of 28 PCB congeners, metabolites, and mixtures in zebrafish, identify morphological and behavioral phenotypes associated with exposure, and characterize transcriptional pathways responsive to representative PCBs. **Methods:** Wild-type (Tropical 5D) and transgenic *cyp1a* reporter zebrafish were bred and maintained at the Sinnhuber Aquatic Research Laboratory (Oregon State University). At 6 hpf, enzymatically dechlorinated embryos were placed into 96-well plates and exposed to PCBs ($0-100 \text{ }\mu\text{M}$) in DMSO. Developmental (neuro)toxicity was assessed using a suite of high-throughput assays: embryonic photomotor response (EPR, 24 hpf), larval photomotor response (LPR, 120 hpf), and morphological scoring at 24 and 120 hpf. A benchmark dose (BMD10) was calculated for behavioral effects. A set of 10 PCBs that spanned diverse activity profiles in EPR, LPR, and morphology were selected for further testing in *cyp1a* reporter zebrafish. Another set of 10 PCBs that included parent compounds and their metabolites, as well as 2 mixtures, was selected for transcriptomic profiling in wild-type larvae at 120 hpf to identify KEGG pathways altered by PCB exposure using gene set enrichment analysis (GSEA). **Results:** Among the 28 PCB congeners, metabolites, and mixtures tested, distinct patterns of (neuro)developmental toxicity were observed across behavioral and morphological endpoints. Eight compounds produced effects in morphology, EPR, and LPR. Fourteen compounds elicited responses in morphology and EPR only. One compound affected both EPR and LPR without morphological change. The remaining five compounds altered either morphology or EPR alone. In *cyp1a* reporter zebrafish, only PCB126 induced measurable *cyp1a* activation, consistent with its known aryl hydrocarbon receptor agonist activity. Principal component analysis (PCA) of transcriptomic data revealed that 5-OH-PCB11 elicited a transcriptional profile distinct from both controls and other test compounds, indicating a unique mechanism of action. The two PCB mixtures tested also produced transcriptomic signatures distinct from those of individual congeners or metabolites. GSEA identified enrichment of multiple signaling and metabolic pathways across treatments. Eight compounds or mixtures significantly altered cytokine-cytokine receptor interaction pathways. Another eight PCBs affected neuroactive ligand-receptor interaction pathways, consistent with the observed behavioral disruption. Six PCBs showed mixed activation of ATP-dependent chromatin remodeling and ribosome biogenesis pathways, suggesting potential impacts on transcriptional regulation and protein synthesis. **Conclusions:** This integrative analysis reveals that specific PCB congeners, metabolites, and mixtures elicit distinct patterns of (neuro)developmental toxicity across behavioral and morphological endpoints. These patterns are associated with distinct transcriptomic effects. These results offer novel insights into how PCBs or their metabolites disrupt neurodevelopmental processes in a translational zebrafish model.

PS 3614 Acute PCB126 exposure alters cell type specific transcriptional profiles in the adult zebrafish brain

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Background and Purpose: Neuropsychiatric disorders such as anxiety, depression, and schizophrenia are major public health concerns, and growing evidence suggests that environmental chemical exposures contribute to their etiology by altering neuro-immune signaling. The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that mediates responses to environmental contaminants and also plays essential roles in neurodevelopment and immune regulation. Our previous work demonstrated that developmental exposure to the dioxin-like polychlorinated biphenyl congener PCB126 disrupted microglial abundance and morphology in zebrafish, implicating AHR-mediated interference with primitive macrophage development. The present study examined whether acute AHR activation in the adult brain induces cell type-specific transcriptional alterations that could contribute to neuroimmune dysregulation. **Methods:** Adult male zebrafish (9 month old) were exposed to either

control (DMSO; solvent control) or 10 nM PCB126, a potent AHR agonist, for 24 hours. Brains were collected immediately following exposure for single-nuclei RNA sequencing (snRNA-seq) using the 10x Genomics Chromium platform to characterize acute, cell type-specific transcriptional responses. Approximately 10,000 nuclei per sample were sequenced at an average depth of 20,000 reads per nucleus, with four biological replicates per treatment. Cell clustering, annotation, and differential expression analyses will be performed to identify transcriptional changes across neuronal and glial populations. **Results:** Previous work from our group demonstrated that early-life exposure to PCB126 alters microglial development in zebrafish. Specifically, exposure at 4 hours post-fertilization (hpf) caused a reduction in microglial abundance without affecting morphology, suggesting impaired proliferation of primitive macrophages originating from the rostral blood island. In contrast, exposures at 2 and 3 days post-fertilization (dpf) did not change microglial numbers but produced pronounced morphological alterations consistent with microglial activation and a potential response to PCB-induced neuronal apoptosis. These findings indicate that the timing of AHR activation during development determines distinct neuroimmune outcomes. Building on these observations, we are now characterizing the transcriptional consequences of acute AHR activation in the adult zebrafish brain. snRNA-seq data generation and quality assessment are complete, encompassing approximately 10,000 nuclei per sample at high sequencing depth. Ongoing analyses will define major neuronal and glial cell clusters and evaluate transcriptional responses to 24-hour PCB126 exposure. We will present detailed cell type-specific gene expression profiles and identify molecular pathways responsive to AHR activation in the adult brain. Together, these developmental and adult datasets will provide a comprehensive framework for understanding how PCB126 perturbs neuro-immune function and neuronal signaling across life stages. **Conclusions:** This study is designed to determine how acute AHR activation in the adult brain influences cell type-specific transcriptional regulation. The results will provide insight into how environmentally relevant contaminants rapidly alter molecular processes governing brain homeostasis and immune function. This work extends previous findings on developmental neurotoxicity to the adult stage and contributes to understanding AHR-mediated mechanisms underlying the neurobiological effects of environmental exposures linked to neuropsychiatric outcomes. This research is supported by the National Institute of Environmental Health Sciences (1R21ES034839-01) and Woods Hole Center for Oceans and Human Health (NIEHS P01 ES028938; NSF OCE-2418297)

PS 3615 The bladder as a target for PCB toxicity: Evidence from PCB levels, phase I metabolite levels, and cytochrome P450 expression following developmental exposure to a human-relevant PCB mixture in mice

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Background and Purpose: Lower urinary tract dysfunction is multifactorial, yet the role of environmental exposures is not well understood. Developmental exposure to polychlorinated biphenyls (PCBs) has been linked to altered voiding in mice; however, the disposition of PCBs in the bladder, their bioactivation, and the effects on cytochrome P450 (CYP) expression remain unclear. **Methods:** We exposed mice to an environmentally relevant PCB mixture via maternal diet during gestation and lactation (vehicle, 0.1, 1, or 6 mg/kg/day). Offspring were euthanized at 6 to 7 weeks of age. PCBs and hydroxylated PCBs (OH-PCBs) levels were quantified in bladder, liver, blood, and urine. CYP expression was measured in bladder and liver. **Results:** PCBs and OH-PCBs accumulated in all tissues in a dose- and sex-dependent manner, with higher-chlorinated congeners (e.g., PCB118, PCB138, PCB153, and PCB180) preferentially retained. Females exhibited greater hepatic accumulation, reduced urinary elimination, and distinct CYP regulation, characterized by increased hepatic and decreased bladder expression. **Conclusions:** These findings, for the first time, define the signature of PCBs and OH-PCBs in the bladder and reveal sex-specific PCB disposition and CYP responses. Our results provide new mechanistic insight into developmental PCB exposure and its potential contribution to voiding dysfunction relevant to wildlife, domestic animals, and humans.

PS 3616 Sex-dependent Hepatotoxicity of an Environmentally Relevant Dioxin Mixture in a Humanized AHR Mouse Model

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Background and Purpose: Legacy persistent organic pollutants like dioxins and dioxin-like compounds (DLCs) remain present in our environment in relatively low yet significant levels and are associated with metabolic disruptions that initiate or drive the progression of steatotic liver disease. Dioxins and DLCs are potent aryl hydrocarbon receptor (AHR) activators and have been reported to induce hepatotoxicity

via this pathway. However, the majority of studies utilize murine models (mAHR), which have higher affinity and responsiveness to these pollutants compared to the human AHR (hAHR). Knowledge on how dioxins act through the hAHR to promote liver and metabolic dysregulation is still limited. Thus, this study aims to investigate the translational relevance of acute exposure to an environmentally relevant dioxin mixture namely 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), 2,3,4,7,8-Pentachlorodibenzofuran (PeCDF) and Polychlorinated biphenyl 126 (PCB 126), in the context of liver disease and underlying sex differences, using a humanized AHR mouse model. **Methods:** 6-8-week-old male and female hAHR-C57BL/6 mice were fed a low-fat diet and administered a weekly dose of either TCDD (10ng/kg) + PeCDF (80ng/kg) + PCB 126 (140ng/kg) or vehicle control (corn oil) over a 2-week period. Body weight and food consumption were recorded weekly, and body composition measured using Echo-MRI. At euthanasia, plasma and tissues were collected for downstream analyses. Statistical tests were performed using two-way ANOVA or Kruskal Wallis followed by post-hoc tests with correction for multiple testing ($p \leq 0.05$). **Results:** Body composition analyses revealed that compared to males, females had higher fat mass but lower lean mass. Females also exhibited elevated hepatic triglyceride and cholesterol levels than males. Plasma glucose and glucose uptake levels were increased by dioxin exposure in males but decreased in females. This was accompanied by a dioxin-driven reduction in insulin resistance and pancreatic insulin production in the male group, while exposed females displayed increased levels of these measurements. Furthermore, the dioxin mixture did not appear to activate hepatic hAHR in this model as canonical target genes (*Cyp1a1* and *Cyp1a2*) were not induced with exposure in both sexes. This lack of classic AHR target gene induction with exposure was also observed in extra-hepatic tissue including the ileum. Notably, transcription of the hepatic *hAhr* gene which had higher expression in females was reduced by dioxin exposure in this group alone. Additionally, hepatic gene expression of xenobiotic receptors *Car* and *Pxr*, as well as endobiotic receptors *Lxr* and *Ppara* were also selectively decreased with dioxin exposure in females only. Further, exposure of primary hAHR-C57BL/6 hepatocytes to individual components of the dioxin mixture at relatively higher concentrations (0.5nM TCDD, 10nM PeCDF, 100nM PCB 126) led to induction of AHR targets, *Cyp1a1* and *Cyp1a2*, with the magnitude of induction differing by sex and chemical. Importantly, for males, induction of AHR targets was more robust in wildtype C57BL6 mouse hepatocytes expressing mAHR, compared with hAHR-C57BL/6 hepatocytes. **Conclusions:** Phenotypic changes including body composition, glucose and lipid disruption patterns were consistent with our previous findings in the mAHR model, yet classic AHR activation was absent *in vivo*, suggesting off-target effects and mechanisms. While primary hepatocyte exposures confirmed that the hAHR is activated by these dioxin chemicals, this was only observed at relatively higher concentrations and is sex dependent. The current data also demonstrated sex-specific dioxin alterations of liver transcriptional machinery, highlighting sex-specific mechanistic differences between mAHR and hAHR in its role in mediating dioxin-induced hepatotoxicity and metabolic dysregulation. Comprehensive evaluation of canonical and non-canonical AHR mechanisms, extrahepatic responses, and chronic outcomes in this human-relevant model, will better assess how dioxins, particularly at current environmental levels, drive sex-specific liver and metabolic toxicity in humans.

PS 3617 Sex- and Diet-Specific Disruption of the Adipose-Liver Axis with Subchronic Exposure to a Dioxin Mixture

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Background and Purpose: Persistent organic pollutants (POPs), including "dioxin and dioxin-like" compounds, are linked to metabolic disorders such as diabetes, obesity and steatotic liver disease. However, the combined effects of mixtures of dioxins and dioxin-like compounds on the adipose-liver axis, and how these effects contribute to metabolic outcomes, remain unclear. Studies investigating the longer-term effects from exposures to these environmentally relevant mixtures are also currently lacking. Thus, the objective of this study is to examine the impact of subchronic exposures to a dioxin mixture in male and female mice in the context of a hypercaloric diet, with focus on adipose, liver and systemic metabolic outcomes. **Methods:** Male and female C57BL/6 mice were fed a low-fat diet (LFD) or a high-fat diet (HFD) and administered a vehicle control (corn oil) or defined mixture of dioxin and dioxin-like compounds, namely TCDD (10ng/kg) + PeCDF (80ng/kg) + PCB 126 (140 ng/kg) weekly over a 12-week period. Body weight was recorded weekly. Body composition was assessed using Echo-MRI and glucose tolerance tests were performed at the mid-study period and prior to study termination. At study completion, liver, adipose tissue, and plasma were collected. White adipose tissue (WAT) morphology and liver sections were assessed histologically, while hepatic and circulating lipids were quantified by biochemical assays. Hepatic gene expression was measured by RT-PCR. Statistical analysis was performed using Three-Way ANOVA followed by post-hoc test with Holm-Sidak correction ($p < 0.05$). **Results:** Males generally exhibited higher body weight and total fat mass compared to females. Male mice also had greater adipose tissue weights relative to body weight. In terms of adipose morphology, males displayed larger adipocyte area than females, while crown-like structure (CLS) burden showed modest sex differences. Diet exerted strong effects on metabolic endpoints. HFD significantly increased body weight, adiposity, adipocyte hypertrophy, and CLS burden compared to low-fat diet (LFD), regardless of sex. HFD also elevated plasma triglycerides, hepatic lipid accumulation, and markers of liver injury

(ALT and AST), confirming its role as a primary driver of metabolic disruption. Dioxin exposure did not exacerbate these HFD-driven adipose parameters but led to altered adipocyte morphology only in the LFD-fed mice. This was mirrored by increased body fat composition (Echo-MRI at 12 weeks) with dioxin exposure in LFD-fed females. Moreover, LFD-fed females exposed to the dioxin mixture displayed elevated hepatic triglyceride levels, alongside increased circulating ALT levels, and upregulation of the hepatic fatty acid transport gene *Cd36*. This observation was absent in diet-matched males. Instead, dioxin exposed LFD-fed males showed suppressed liver *de novo* lipogenic gene expression (*Fasn*, *Scd1*) coupled with resistance to glucose uptake. Activation of hepatic xenobiotic receptors differed by context: AHR was activated across all exposed groups as seen by *Cyp1a1* and *Cyp1a2* induction, although HFD feeding decreased AHR target gene induction in male livers. Notably, exposure to the dioxin mixture also resulted in CAR activation (*iCyp2b10*, *Cyp2c29*), specifically in LFD-fed males but not females. **Conclusions:** Taken together, the results suggest that exposure to the dioxin mixture produced sex- and diet-specific metabolic effects, along the adipose-liver axis. Sex-specific toxicant responses observed included dioxin-mediated steatotic liver phenotype and altered fat composition in females and glucose perturbation in males, highlighting the negative impact of these chemicals on liver and metabolic outcomes even at low doses. Intriguingly, the dioxin mixture did not exacerbate HFD effects with regards to liver and adipose endpoints. Overall, these findings demonstrate that both biological sex and dietary context critically shape toxicant responses and may better inform risk assessment of these chemical mixtures.

PS 3618 Exposure to polychlorinated biphenyls (PCBs) alters endothelial-pericyte crosstalk at the blood brain barrier

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Background and Purpose: Polychlorinated biphenyls (PCBs) are stable, environmental toxicants that are widely found in the environment. Once used for industrial purposes, PCBs have since been banned but are still found in the environment today. PCBs have been associated with neurotoxicity, with one potential mechanism being disruption of the blood brain barrier (BBB). The BBB is a semi-permeable barrier that separates the central nervous system from the cerebral vasculature. The BBB's semi-permeable properties are maintained by tight junctions between endothelial cells that form the main vascular barrier, in concert with pericytes and astrocytes that regulate its integrity and function. PCBs have been shown to dysregulate tight junction integrity, but little is known about how PCBs affect endothelial cell-pericyte intercellular communication, which is essential for proper functioning of the BBB. **Methods:** Primary human BBB pericytes were cultured with or without human brain microvascular endothelial cells. For pericyte monocultures, pericyte medium was treated with 5µM of PCB mix, reflecting the PCB concentration found in postmortem human brain samples or with a vehicle (0.1% DMSO). To evaluate endothelial-pericyte communication, a co-culture model was established using transwell inserts, with pericytes seeded on the basolateral surface of the membrane and endothelial cells on the apical surface. To mimic *in vivo* conditions in co-culture, only endothelial cell medium was treated with PCBs, as described above. RT-qPCR was used to assess the mRNA expression of several signaling molecules, while Western Blotting was used to assess the protein expression of gap junction proteins. **Results:** In pericyte monocultures, there was a decrease in the mRNA expression of the signaling molecule Angiopoietin 1 (*Angpt1*) 6 hours after treatment, and an increase in the expression of matrix metalloproteinase 9 (*MMP9*) and vascular endothelial growth factor A (*VEGFA*) at 6 hours after treatment. In endothelial cells cultured with pericytes, there was an increase in the mRNA expression of the inflammatory chemokine *CCL2* and *VEGFA*, and a decrease in *Angpt1* at 6 hours after treatment. Also, there was a decrease in the protein expression of Cx43 after 12 hours of treatment. Finally, there was an increase in the mRNA expression of *Angpt1* in co-cultured pericytes. **Conclusions:** Our results indicate that PCBs alter the expression of signaling molecules and inflammatory cytokines in both pericyte monocultures and endothelial-pericyte co-cultures. Thus, our findings suggest that exposure to PCBs may disrupt endothelial-pericyte crosstalk which is essential for BBB maintenance. Additionally, we show that direct intercellular communication between endothelial cells and pericytes may be compromised due to decreased expression of Cx43 in co-cultured endothelial cells. Taken together, our findings provide unique implications into the mechanisms by which PCBs may lead to BBB dysfunction, which may inform strategies to mediate the effects PCB-induced neurotoxicity.

PS 3619 Developmental risks of PBDE exposure through altered miRNA signaling in human placenta and breastmilk

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Background and Purpose: Polybrominated diphenyl ethers are persistent organic pollutants linked to preterm birth and adverse neurodevelopmental outcomes. Preterm infants may face compounded risks due to dual PBDE exposure via the placenta and breast milk. While *in vitro* studies suggest that PBDE disrupts extracellular vesicle (EV) miRNA packaging, its effects on placental immune tolerance (mediated by EV-miR-519c) or its impact on the composition of neuroprotective milk EV-miRNAs remain unknown. We hypothesize that elevated PBDE levels reduce protective placental EV-miR-519c, increasing the risk of preterm birth, and that higher PBDE concentrations in breast milk will negatively correlate with neuroprotective miRNAs in milk EVs, potentially impacting preterm infant neurodevelopmental outcomes. **Methods:** Human placental explants (n=11) were exposed to repeated doses of LPS to model infection-mediated preterm birth, with or without PBDE-47. EVs were isolated from culture media to assess whether PBDE-47 alters EV-miR-519c secretion following repeated LPS exposure. Additionally, breast milk PBDE levels from preterm (n=55), full-term (n=15), and donor (n=12) milk were quantified via gas chromatography-mass spectrometry (GC-MS). EVs were also isolated from breast milk samples and neuroprotective miRNAs (miR-22-3p, miR-30b-5p, and miR-146a-5p) were quantified using the LNA miRCURY miRNA assay on droplet digital PCR (ddPCR). Placental miR-519c levels were compared between treatment groups and controls by two-way ANOVA. PBDE concentrations in preterm and full-term milk were compared by Mann-Whitney U-test. Milk samples were also sorted into "high" (n=14) and "low" (n=68) PBDE categories with high samples defined as those with cumulative PBDE levels greater than 4 standard deviations above the average of healthy milk donors in the NY area. Correlation between PBDE-47, -99, and -153 concentrations and protective miRNAs was assessed by Spearman correlation and simple linear regression. Data were analyzed in GraphPad Prism v10.4.1 **Results:** PBDE-47 co-treatment in placental explants decreases protective EV-miR-519c expression in response to LPS (p<0.0001). GC-MS demonstrates that ~20% of all samples have high cumulative PBDE level, with 93% of high PBDE samples collected from preterm delivery mothers. Significant inverse correlations are evident between BDE-99 concentration and miR-22-3p (p<0.0001), miR-30b-5p (p<0.001), and miR-146a-5p (p<0.001). Cumulative PBDE burden is also inversely associated with miR-146a-5p (p<0.01) and miR-22-3p (p<0.05). **Conclusions:** These data suggest that PBDEs may alter EV-miRNA content in both human placenta and breast milk with serious implications for preterm infant health. Maternal PBDE exposure may impact infant neurodevelopment through multiple pathways. First, alterations in placental immune tolerance EV-miR-519c could increase the risk of preterm birth, which is independently linked to poor neurodevelopmental outcomes. Second, there may be direct PBDE ingestion through breast milk by preterm infants. Third, disruptions in neuroprotective milk EV-miRNAs may adversely affect infant neurodevelopment. This highlights a novel mechanism by which PBDEs may contribute to both preterm birth and neurodevelopmental delays in preterm infants.

PS 3620 In vitro mixtures toxicity testing to address wildfire smoke exposures and individual chemical components contributing to human health risk

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Background and Purpose: Wildfire incidence and severity have steadily increased in recent decades, leading to increases in smoke emissions that pose risks to public health. Due to the wide variety of chemical mixtures in smoke from wildfires, there is a need to couple high-throughput toxicity screening approaches with computational methods to assess wildfire-relevant mixtures toxicity. This study sought to evaluate transcriptomic responses of respiratory epithelial cells to individual components, defined mixtures, and complex woodsmoke mixtures with two goals: (1) identifying potential modes of action that are shared versus distinct between wildfire components, and (2) quantifying relative impacts across these various mixtures and mixtures components to inform human health risk estimates. **Methods:** The six chemicals prioritized for this study frequently co-occur across ten different wildfire-relevant burn scenarios (eucalyptus, pine, pine needles, peat, and red oak burned under flaming or smoldering conditions) and were previously positively or negatively correlated with toxicity endpoints in exposed mice through mixtures modeling. Spanning polyaromatic hydrocarbon, methoxyphenol, and metal chemical classes, they included: benzo(a)pyrene, benz(a)anthracene, coniferyl aldehyde, vanillin, sodium dichromate, and copper sulfate. Human bronchial epithelial (16HBE) cells in 12-well plates were exposed at 5 different exposure-relevant concentrations to these single chemicals, equimolar binary mixtures, an equimolar defined mixture of all 6 candidate chemicals, and 4 complex biomass mixtures (flaming or smoldering eucalyptus and flaming or smoldering pine). Bulk RNA sequencing was performed in samples collected at 4 hours post-exposure. Differentially expressed genes (DEGs), gene set enrichment, benchmark concentration (BMC) modeling, transcriptomic point of departure (tPOD) derivation, and concentration addition mixtures modeling (CAM)

analyses were performed to evaluate transcriptomic response. **Results:** Patterns in DEGs, significantly enriched pathways, BMCs, and tPODs were shared between the complex biomass mixtures, defined mixture, sodium dichromate alone, and sodium dichromate in binary mixture with copper sulfate. Ras/ERK and Ras/PI3K signaling were identified as top ranking enriched pathways highly shared across these exposure conditions, suggesting a shared mode of action involved in response to these wildfire-relevant mixtures perhaps driven by sodium dichromate. Comparisons of observed versus CAM-predicted tPODs revealed that the metals binary mixture and defined mixture exhibited synergistic effects (e.g. 0.120 μ M observed versus 0.265 μ M predicted for metals, and 0.006 μ M observed versus 0.05 μ M predicted for the defined mixture). Notably, relative potencies of the complex biomass mixtures mirrored results obtained from mice exposed to those same mixtures, providing rare *in vivo* anchoring towards this *in vitro*-based mixtures testing paradigm. **Conclusions:** Altogether, these results suggest that metals and metal mixtures may be a particular chemical class and/or mixture of public health concern when considering the respiratory health effects of wildfire smoke exposure. However, the nearly 10-fold difference in observed versus predicted tPODs in the defined mixture indicates that interactions with other co-occurring chemical classes must also be considered when determining wildfire smoke exposure risk. These results highlight the importance of variable mixtures-based approaches to assess wildfire smoke toxicity and contribute a novel dataset of risk assessment relevance.

PS 3621 Developmental Toxicity of Metal Mixture Exposures in Zebrafish Embryos

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Background and Purpose: HF operations rely on the injection of large volumes of water, sand, and chemicals under high pressure to extract oil and natural gas. Hydraulic fracturing (HF) releases heavy metals into the environment through leaks, spills, and incomplete fluid recovery, raising concerns for communities near drilling sites. Metals such as barium (Ba), strontium (Sr), aluminum (Al), manganese (Mn), cobalt (Co), arsenic (As), and iron (Fe) are often enriched in affected water sources and have been associated with developmental toxicity. Populations living near HF sites may therefore face repeated or chronic exposures through drinking water. Epidemiology studies suggest relationships between increased birth defect risks in children born to mothers living near HF operations, raising concerns about the developmental effects of such exposures. Given these findings, it remains unclear whether metal exposures contribute directly to the birth outcomes reported near hydraulic fracturing sites. To address this, we are modeling environmentally relevant metal mixtures in the laboratory using zebrafish embryos to assess their potential to disrupt early development. **Methods:** Metals were obtained as inorganic salts, prepared in ultrapure water, and formulated into three equimolar mixtures to reflect regional differences in North American geology (5MMAs: As+Al+Ba+Mn+Sr; 5MMCo: Co+Al+Ba+Mn+Sr; 5MMFe: Fe+Al+Ba+Mn+Sr). Adult wild-type AB zebrafish were set up in breeding tanks (3 females: 2 males), and spawning occurred the following morning after gate pulls. Embryos were collected, sorted, and cleaned. At 24 hours post-fertilization (hpf), embryos were staged and seeded into 6-well tissue culture plates, with 20 embryos in each well. Treatments included embryo media (negative control), dimethyl sulfoxide (DMSO) control, 10 μ M β -naphthoflavone (BNF), and exposures to our mixtures at concentrations of 1 nM, 10 nM, 100 nM, and 1 μ M. Embryos were exposed starting at 24 hpf. At 48 hpf, survival was assessed, any dead animals were removed, and treatments were renewed after embryo media change. At 72 hpf, survival was recorded, and embryos were switched into fresh media without test chemicals prior to developmental measurements. The larvae were anesthetized and imaged, and video recorded using a Leica S91 microscope. Images were analyzed for differences in body length, yolk sac size, and pericardial edema size. Videos were analyzed to measure heart rate (HR) in each treatment group. Heart rate was determined from 5-second intervals. **Results:** Neither metal mixtures nor positive controls significantly decreased survival at 48 hpf or 72 hpf compared to negative controls. As expected, 10 μ M BNF significantly increased yolk sac and pericardial edema size (both +26% compared to negative control), decreasing overall body length (-26%), and reducing heart rate (-15%), confirming effective positive control activity. Only 500 nM 5MMAs slightly, but significantly, decreased larvae body length (-3%). Significant decreases, but not increases, in yolk sac size were observed with 5 μ M 5MMAs (-5%) and 5 nM 5MMFe (-6%) treatment. No significant changes in pericardial edema size were noted following mixture exposure. HR analysis showed that 100 nM (+5.5%) and 1 μ M (+6.0%) 5MMAs exposure significantly increased HR. **Conclusions:** The results indicate that environmentally relevant metal mixtures cause only subtle developmental effects in zebrafish embryos under the tested conditions, including small changes in body length and yolk sac size. While survival was similar between treated and untreated animals, these findings suggest that early-life exposures may still contribute to developmental disruption. The HR data indicate that the As-containing exposure increased HR at 72 hpf, suggesting potential functional changes. Future work will assess different exposure windows (earlier and later than 24 hpf) and extend observations beyond 72 hpf to allow us to observe any delayed or progressive effects. Future work will use video analysis to assess other hemodynamic endpoints, focusing on As-containing mixtures to understand their effects on cardiac function and development.

PS 3622 From Pixels to Potency: Image-Based Modeling of Component Contributions to PFAS Mixture Effects

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are widespread environmental contaminants with diverse chemical structures and biological activities. Since humans are typically exposed to PFAS mixtures rather than individual compounds, quantitative approaches that integrate mechanistic bioassays with mixture modeling are needed to improve risk assessment of PFAS mixtures. This study applied a component-based concentration-addition framework to predict the biological activity of PFAS mixtures using high-content image data, commonly referred to as Cell Painting, linking morphological bioactivity profiles to mixture potency and mechanistic interpretation. **Methods:** Seven PFAS (perfluorobutanesulfonic acid (PFBS), perfluorodecanoic acid (PFDA), perfluorooctanesulfonic acid (PFOS), perfluorohexanoic acid (PFHxA), perfluorononanoic acid (PFNA), perfluorooctanoic acid (PFOA), and perfluorohexanesulfonic acid (PFHxS)) were evaluated in human U2-OS cells using the Cell Painting assay. Cells were cultured in 96-well plates and exposed for 24 hours to each compound individually or to two defined mixtures: an equimolar mixture and an environmentally relevant mixture reflecting plasma concentrations from the Canadian Health Measures Survey (CHMS). Following exposure, cells were stained with six fluorescent dyes to visualize diverse organelles and morphological features and imaged using the Opera Phenix high-content screening platform. Images were processed through the Columbus image analysis software and a validated Cell Painting analysis pipeline in R to extract quantitative morphological features and aggregate cell-level values to normalized well-level medians for each treatment condition. Benchmark concentration (BMC) values and their lower (BMCL) and upper (BMCU) confidence limits were derived from concentration-response modeling for each feature and chemical. Mixture potencies were predicted using a concentration-addition model that integrated individual-chemical BMCs according to their respective proportions in each mixture (equimolar or environmental). Predictions were generated using both feature-median and feature-weighted median (1/BMC-weighted) approaches to account for differences in feature sensitivity across chemicals. Predicted mixture BMCs and confidence intervals were compared with experimentally measured mixture responses to evaluate additivity and identify dominant chemical contributors to overall mixture potency. **Results:** Predicted mixture potencies spanned several orders of magnitude across morphological features. The environmentally weighted mixture exhibited greater overall potency (lower BMCs) than the equimolar mixture, primarily driven by the high-potency and high-abundance constituents PFOS and PFOA. PFOS accounted for up to 60% of total mixture activity in the most sensitive morphological features, particularly those describing cytoplasmic texture, nuclear intensity, and granularity. Both the feature-median and feature-weighted median mixture predictions aligned closely with the measured mixture potencies, supporting additive effects among the PFAS components at this level of biological organization. Overall, predicted mixture potencies mirrored single-chemical potency rankings, underscoring consistent feature-level sensitivities and dominant chemical drivers. **Conclusions:** Component-based mixture modeling effectively captured additive PFAS responses in high-content imaging data and identified key contributors under realistic exposure scenarios. Integrating Cell Painting bioactivity with concentration-addition modeling provides a mechanistic, quantitative framework for evaluating PFAS mixture toxicity. By linking cellular phenotypes to potential modes of action, this approach advances mechanistic-based risk assessment and supports data-driven prioritization of PFAS and other complex environmental mixtures.

PS 3623 In vitro endocrine and adipogenic effects of oil and gas-associated metal mixtures: intersections with glucocorticoid signaling

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Background and Purpose: Hydraulic fracturing (HF) operations inject complex chemical mixtures underground to retrieve previously inaccessible energy reserves. Metals such as barium (Ba), strontium (Sr), aluminum (Al), and cobalt (Co) are common components of HF fluid used to keep fractures open ("proppants"). HF sites can increase environmental concentrations of proppant metals, and endemic metals such as arsenic (As), iron (Fe), and manganese (Mn), via incomplete fluid recovery, wastewater mishandling, and metal aerosolization. Pregnant women residing closer to HF sites have higher hair and urinary metal levels than those living further away, constituting potential early life exposures. Metal exposure can result in metabolic dysfunction, including increased adiposity, through activation of key nuclear receptors (NRs). Epidemiologic and *in vivo* relationships between single metals and metabolism are complex, particularly those driven by NRs. Interplay between environmentally relevant HF metal mixtures, NR signaling, and adipogenesis remains poorly characterized and requires empirical assessment. **Methods:** Inorganic metals salts were prepared in ultrapure water individually and formulated into 3 equimolar mixtures to model regional differences in North American HF site geology (5MMAs: Al+Ba+Mn+Sr+As; 5MCo: Al+Ba+Mn+Sr+Co; and 5MFe: Al+Ba+Mn+Sr+Fe). Mixtures and individual metals were tested in transiently transfected luminescence reporter gene bioassays for human retinoid X receptor α (RXR α), peroxisome proliferator activated receptor γ (PPAR γ), and glucocorticoid receptor (GR). Transfected cells

were exposed to increasing, environmentally-relevant concentrations of metals (5 nM - 5 μ M for mixtures, 1 nM - 1 μ M for individual metals) or positive control chemicals for NR activation (only test chemicals) or modulation (EC₅₀ agonist co-exposure). Mixture-GR interactions were assessed by 5 μ M mixture co-exposure with mifepristone (GR competitive antagonist), Dexamethasone (DEX; GR agonist), or dithiothreitol (DTT; thiol-sparing reductant). Adipogenic effects were measured by triglyceride accumulation (lipogenesis) and DNA levels using a standardized human mesenchymal stem cell (hMSC; 6 year old female donor) differentiation protocol following exposure to metals or positive controls, with or without EC₅₀ DEX for GR-mediated lipogenesis modulation assessment. **Results:** None of the mixtures activated RXR α or PPAR γ . 5 μ M mixtures weakly activated GR (5MAs: 10% relative to positive control; 5MCo: 7%; 5MFe: 16%). Individual 1 μ M Ba significantly activated GR at greater-than-mixtures levels (24%), potentially driving mixture effects. Individual Fe activated (8-13%) and Mn inhibited (-11-6%) GR across tested concentrations. Individual Al, As, and Co did not activate GR. 5 μ M 5MCo and 5MFe enhanced EC50 DEX-mediated GR activity (+350% and +30%, respectively), as did individual 1 μ M Ba (+340%) and Sr (+240%). Nanomolar concentrations of Co, Al, and Mn inhibited GR (-100% - -71%). As and Fe did not affect DEX-mediated GR activity. Mifepristone blocked GR activity for each 5 μ M mixture. DTT increased only 5MAs-induced activity (+30%), suggesting mixture-specific interactions with GR thiol groups that could limit mixture efficacy. This was corroborated by varying magnitudes of full DEX concentration-response curves with 5 μ M mixture co-exposures (As < Co < Fe), suggesting mixture-specific non-competitive regulation of GR. 5 μ M mixtures increased hMSC lipogenesis (5MAs: 31%; 5MCo: 20%; 5MFe: 29%). DEX-mediated lipogenesis was enhanced by 5 μ M 5MAs (+117%) and 5MFe (+25%), but not 5MMCo. Individual 1 μ M Sr, As, and Ba increased lipogenesis (17%, 22%, and 16%, respectively), indicating these metals might drive mixture effects. The remaining metals did not affect lipogenesis. 1 μ M Al and Mn inhibited DEX-mediated lipogenesis (-76% and -78%, respectively) while 1 μ M As strongly enhanced (+170%). Individual Co, Sr, and Ba did not affect DEX-induced lipogenesis. **Conclusions:** Our results suggest that HF-related metal mixtures specifically activated and enhanced GR activity, potentially driven by Ba and through non-competitive receptor regulation. Some metal mixtures induced and enhanced hMSC lipogenesis. On-going mixture-exposed hMSC transcriptome analyses will help determine how mixtures mechanistically intersect with GR signaling and spur lipid deposition.

PS 3624 Assessment of Bisphenol Mixture Toxicity Using Zebrafish Larvae and HepG2 Cells

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Background and Purpose: Bisphenol analogues (BPs), extensively used in polycarbonate plastics and epoxy resins, are ubiquitous in consumer products and the environment, raising concerns regarding human and ecological health. While most toxicological studies address single compounds, real-world exposures involve complex mixtures, and the cumulative toxicity and mechanisms of BPs remain poorly understood. This study examined the toxicological effects and interactions of four bisphenol analogues—BPA, BPE, BPF, and BPS—using zebrafish larvae and HepG2 cells. **Methods:** Acute toxicity was assessed in zebrafish larvae over 48 h for individual compounds and binary, ternary, and quaternary mixtures, with mortality as the endpoint. Cytotoxicity in HepG2 cells was determined via MTT assays after 4 h and 24 h exposures, and genotoxicity of single compounds was evaluated using the comet assay. Mixture toxicity was predicted using the concentration addition (CA) and independent action (IA) models and verified by comparing experimental data with model predictions through the model deviation ratio (MDR). **Results:** All four BPs induced concentration-dependent mortality and cytotoxicity, with potency ranked as BPA > BPE > BPF > BPS. Mixture exposures in zebrafish showed composition-dependent effects, where certain binary and ternary combinations (e.g., BPF + BPS, BPA + BPF + BPS) enhanced mortality. In HepG2 cells, extended exposure (24 h vs. 4 h) significantly increased cytotoxicity. Mixtures containing BPA were generally more toxic, whereas BPE- or BPS-dominant mixtures exhibited lower toxicity. **Conclusions:** Bisphenol analogues differ markedly in potency, and their mixtures display complex, composition-dependent interactions deviating from simple dose-response behavior. Statistical analyses based on the IA and CA models revealed additive, synergistic, and antagonistic effects. These results underscore the importance of incorporating mixture toxicity into consumer product risk assessments, which may otherwise underestimate potential hazards.

PS 3625 Inhalation exposure risks from resin 3D printing used in dental fields

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Background and Purpose: Vat photopolymerization 3D printing cures a photopolymer resin with a light source selectively to form an object from a digital model. Due to its cost-effectiveness, high accuracy and the smooth surface finish of printed objects, resin 3D printing is widely used in dental and medical fields, such as dental schools and clinics. However, liquid resins typically contain ingredients that are irri-

tating and/or sensitizing, which could off gas during normal use and pose inhalation risks to the users and occupants. Therefore, it is important to understand the potential exposure risks associated with resin 3D printing. **Methods:** We developed and validated methods to evaluate volatile organic compound (VOC) emissions from resin 3D printing using specialized exposure chamber setups. A full-scale chamber was used for evaluating emissions from the printing and post-printing processes; a micro-scale chamber was used for characterizing off gas from resin materials without operations. VOCs were collected on Tenax® TA tubes and analyzed by gas chromatography-mass spectrometry; low-molecular-weight aldehydes were collected on 2,4-dinitrophenylhydrazine cartridges and analyzed by high performance liquid chromatography, both followed EPA methods. Based on exposure chamber conditions, emission factors were calculated following standard methods and were applied to an indoor exposure model to estimate exposure potentials in different environments. **Results:** Resin 3D printing showed a total VOC emission rate of over 4 mg/h. Even without operation, total VOC level was over 10 times higher than the empty chamber background, indicating off gassing from resins happened in normal room conditions. The most abundantly emitted VOCs during printing included 2-hydroxypropyl methacrylate, propylene glycol, and 2-hydroxyethyl methacrylate, which were associated with the resin compositions. The printing process emitted much higher VOCs than the post-printing wash and cure processes, except for the wash solvent. Various esters, hydrocarbons, alcohols, aldehydes and ketones were detected during the operation processes. Resin off gas tests showed complex mixtures of VOCs were released at room temperature from various resin types commonly used in the dental fields. In addition to the specified ingredients listed on safety data sheets, typically acrylates and methacrylates, more compounds were released from resin off gas, which could be associated with undisclosed additives, polymer degradation, or chemical reactions. Our emission tests identified some hazardous VOCs with potential health concerns, such as 2-methoxyethyl acetate, formaldehyde, naphthalene, acetaldehyde, and toluene. These included carcinogens, reproductive and developmental toxicants, irritants and sensitizers, exposure to which could result in adverse health effects, especially at non-industrial indoor environments such as dental offices. For example, estimated personal exposure to some VOCs with health concern, such as toluene and formaldehyde, may exceed the recommended indoor levels. **Conclusions:** Resin 3D printing emitted a complex mixture of acrylates, aldehydes, alcohols, ketones, and hydrocarbons during normal use or even without operation. The emissions contain irritating, carcinogenic, and/or toxic compounds. Users and occupants are potentially exposed to hazardous mixtures when resins are present indoors, which may result in acute irritation or long-term effects. In addition, manufacturer safety data sheets may not be sufficient for assessing exposure hazards, especially inhalation risks that can be associated with byproducts from materials and processing.

PS 3626 Modeling Female Reproductive Toxicity of Endocrine Disruptor Mixtures: Insights from Targeted Chemicals

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Background and Purpose: The ovaries are central to female reproduction as sites of hormone synthesis and maturation and ovulation of oocytes within follicles, the functional unit of the ovary. Follicle development, steroidogenesis, and ovulation are highly susceptible to endocrine disruptors (EDCs). Most of previous research focuses on a single compound; however, humans are exposed to complex EDC mixtures which may target multiple signaling transduction pathways and their interactions. This study applied novel machine learning frameworks to evaluate the effects of real-world waterborne EDC mixtures on ovarian follicular development and ovulation, and to identify potential driving chemicals and toxicity mechanisms. **Methods:** Eight water samples were collected from the Delaware, Passaic, and Raritan Rivers in New Jersey and tested for their effects on ovarian follicular outcomes. Concentrations of over 70 organic chemicals—including PFAS, herbicides, pharmaceuticals, phthalates, and other contaminants, were quantified. Biological activity data were retrieved from PubChem, and assays representing molecular initiating events (MIEs) and key events (KEs) relevant to ovarian follicular outcomes were selected. Machine learning classification models were trained to predict untested chemical activities, and regression models were developed to predict mixture-level ovarian disruption, defined as the ratio of mixture-treated to control values, by integrating bioactivity profiles, exposure concentrations, and chemical descriptors. Model interpretation was used to identify mixture-specific toxicity-driving chemicals and mechanisms. **Results:** Chemical composition and concentrations varied across rivers, with Passaic River mixtures showing the greatest chemical diversity and strongest inhibitory effects on hormone secretion, follicle growth and ovulation. Machine learning models were developed for assays including nuclear receptor signaling (e.g., ER, AR, PPAR), mitochondrial dysfunction, p53 activation, and oxidative stress, representing plausible MIEs and KEs, with most models achieving balanced accuracy above 0.7. Regression models to predict different ovarian follicular outcomes achieved R² of 0.6-0.8. Key driving chemicals such as dibutyl phthalate, bisphenol S, and triclosan were found at elevated concentrations in Passaic River samples and predicted to modulate multiple MIEs and KEs. **Conclusions:** This study demonstrates the utility of machine learning-based mixture modeling for assessing female reproductive toxicity of complex environmental EDC mixtures. The integration of multimodal data, including exposure

levels, chemical descriptors, and bioactivity information, enables accurate prediction and mechanistic interpretation of how co-occurring environmental chemicals may jointly contribute to adverse ovarian outcomes.

PS 3627 Mixtures at the intersection of toxicology and epidemiology: Single index models and Relative Potency Factors

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Background and Purpose: Single index models express the outcome as a function of weighted sums of exposures. Several such models have been proposed for analyzing mixtures in epidemiology, including weighted quantile sum regression (wqs), quantile g-computation (qgcomp) and the Bayesian multiple index model (BMIM). The single index model is related to the relative potency factor (RPF) model used in toxicology. However, it is not clear how well the epidemiologic models reflect toxicologic principles. The objective of this paper is to compare and apply ideas from toxicology to epidemiology. **Methods:** We review the assumptions and generalizations of the RPF model. We mathematically examine the implications of these toxicologic ideas for the three mixtures epidemiology models. **Results:** The RPF model is perhaps best known for its application to dioxin-like compounds and saxitoxins. It assumes that all included compounds have the same dose-response curve (same shape and efficacy), differing only in potency. RPFs are computed using a reference compound, e.g., 2,3,7,8-TCDD for dioxin-like compounds. The RPFs are multiplied by the concentrations or doses (which may alter RPFs) and summed; the dose-response curve of the reference compound is applied to the sum. The RPFs can be mathematically converted into positive weights that sum to one, converting it to a typical single index model. BMIM is consistent with this toxicologic RPF model; wqs and qgcomp differ by using quantiles, which can distort dose-response curves. The RPF model is a special case of concentration addition, one of the standard toxicologic definitions of non-interaction. Generalized concentration addition (GCA) extends this concept to compounds with different efficacies, including competitive antagonists. GCA has a firm foundation in the kinetics of receptor activation by ligands. Application of a dose-response function to the result of GCA is not a single index model over the entire dose range. However, we show that at low doses, GCA is closely approximated by a single index model, allowing application to a broader range of compounds than those differing only in potency. When compounds differ in maximal effects, the weights depend on both the relative potencies and the relative efficacies. Competitive antagonists have negative weights, providing a rationale for weights of different sign in both qgcomp and BMIM. The toxicologic and epidemiologic definitions of interaction may not agree when dose-response curves are non-linear. **Conclusions:** Single index models applied to concentrations or doses have a biological basis in the toxicologic relative potency model. Related toxicology mixtures models such as GCA generalize these ideas at low concentrations, allowing for negative weights and application to compounds differing in efficacy. Appropriate single index models can be used to estimate RPFs using epidemiologic or toxicologic data.

PS 3628 Interpretable Dose-Response Modeling for Chemical Mixtures Using Symbolic Regression: A Case Study with Wildfire Smoke In Vitro Testing

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Background and Purpose: Characterizing chemical exposures is complicated by the fact that individuals are rarely exposed to a single chemical at a time, but rather to mixtures that can interact in synergistic, antagonistic, or additive ways. To address this challenge, computational mixture modeling has focused on characterizing individual dose-response relationships and then combining them to predict mixture effects under frameworks such as concentration addition (CA) and independent action (IA). Traditionally, dose-response curves use predefined models (Hill, log-logistic, etc.), but real chemical responses often deviate from the suite of specified models. Model averaging can improve fits by blending models, yet reduces interpretability and complicates mechanistic insight and integration into mixture-prediction workflows. Here, we examine symbolic regression, which generates equations from input data using combinations of mathematical operators, as an alternative approach to model fitting. This method returns interpretable equations that can be directly used in CA and IA frameworks, offering a scalable, mechanistically transparent strategy for mixture toxicology. **Methods:** We evaluated dose-response modeling approaches using transcriptomic profiles from human bronchial epithelial (16HBE) cells exposed to wildfire-relevant chemicals and mixtures. Cells were treated with five concentrations of six chemicals across three classes: polycyclic aromatic hydrocarbons (benzo(a)pyrene, benz(a)anthracene), methoxyphenols (coniferyl aldehyde, vanillin), and metals (sodium dichromate, copper sulfate). In addition to single-chemical exposures, we included equimolar binary mixtures within classes, a six-chemical mixture, and four complex biomass mixtures from flaming and smoldering eucalyptus and pine combustion. Dose-response curves for each gene-exposure pair were modeled using: (i) Hill, exponential, polynomial, and power functions via *tcplfit2*; (ii) Bayesian model averaging of the same model families via *ToxicR*; and (iii) symbolic regression via *pysr*, allowing arithmetic, logarithmic, exponential, and power operations. Model performance was compared using Akaike Information Criterion (AIC),

Bayesian Information Criterion (BIC), and root mean squared error (RMSE) to assess fit quality. Fitted models were also used to predict mixture responses under CA and IA assumptions, and predictions were compared to observed mixture transcriptomic responses. **Results:** Across all genes and exposure conditions, single-model parametric fits (Hill, exponential, polynomial, power) performed poorest, particularly for non-monotonic responses or subtle inflections where rigid curves could not capture complex biology. Bayesian model averaging and symbolic regression demonstrated/exhibited similar overall fit quality, with comparable distributions of goodness-of-fit metrics. However, symbolic regression offered a key advantage by yielding compact, closed-form equations rather than weighted ensembles, thereby improving interpretability and enabling more direct integration into mixture-evaluation and risk-assessment workflows. Performance trends were consistent when models were used to predict mixture responses. Results also indicated that the optimal mixture-prediction framework varies by chemical class and mixture composition, highlighting the complexity of chemical interactions and the need for more refined predictive approaches. **Conclusions:** Together, these findings highlight symbolic regression as a promising approach for mechanistically informed dose-response modeling in the context of multi-stressor toxicity, including complex chemical mixtures.

PS 3629 Combined Arsenic and Uranium Exposure Drives Tissue-Specific Molecular Responses

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Background and Purpose: Legacy mining operations have left uranium (U) and arsenic (As) as persistent co-contaminants in groundwater and soils across the Southwestern United States. These metals, though naturally present in the environment, continue to expose rural and tribal populations. Chronic exposure has been linked to renal, pulmonary, and gastrointestinal (GI) injury, yet the mechanisms driving their individual and combined toxic actions are still not well understood. We hypothesize that differences in metal accumulation and activity across tissues give rise to unique transcriptional signatures that reveal pathways underlying tissue-selective toxicity. **Methods:** To test this, female C57BL/6J mice were exposed to 50 ppm uranyl acetate (UA), 500 ppb arsenite (AsIII), or both in drinking water for 60 days. Tissues critical for xenobiotic absorption and elimination, including kidney, lung, and GI tract, were analyzed for metal accumulation using inductively coupled plasma mass spectrometry (ICP-MS). Transcriptional responses were characterized by RNA sequencing (RNA-seq) to identify differentially expressed genes (DEGs) and enriched biological pathways. **Results:** ICP-MS revealed that the GI tract is the primary site of U accumulation, with levels reaching or exceeding administered doses. U levels were lower in kidney and lung compared to the GI tract, while As accumulated most prominently in kidney and lung following AsIII exposure. In kidney, UA induced 36 DEGs and UA + AsIII co-exposure elicited 539 DEGs, with minimal overlap in differentially expressed genes between UA and UA + AsIII exposure groups. Pathway analysis of kidney treated with UA revealed enriched metabolic and mitochondrial function pathways. In lung tissue, AsIII, UA, and UA + AsIII co-exposure produced 70, 244, and 194 DEGs. Enrichment of angiogenesis and cell junction pathways was observed in UA-treated lung tissue, whereas transcriptional and immune-related pathways were predominant in As-exposed lung tissue, reflecting distinct tissue-specific mechanisms of response and susceptibility. **Conclusions:** These findings suggest that patterns of metal distribution shape organ-selective transcriptomic responses that may underlie tissue-specific sensitivity under co-exposure conditions. Ongoing RNA-seq of intestinal tissues, combined with spatial metal imaging and speciation analyses, will further define spatial and molecular determinants of co-exposure toxicity.

PS 3630 Combined Effects of Metal Mixtures Toxicity in C. elegans

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Background and Purpose: Exposure to non-essential heavy metals such as cadmium (Cd) and lead (Pb) is a continued public health concern in the United States as heavy metals are naturally occurring elements found ubiquitously in the environment and are used in many parts of manufacturing and consumer products. Both elements produce pronounced toxic effects on multiple organ systems and in different windows of susceptibility and are listed as top ten agents of concern on the ATSDR. Given the heterogeneity of exposures that humans experience, it is crucial to understand how these exposures to mixtures differ from single exposures to better understand the complex interactions of the body and the environment. Thus, it is critical to explore the interactive effects of these heavy metals on the body. **Methods:** Using *C. elegans*, we investigated the acute and chronic impact of individual and/or combined effects of Cd and Pb on worm pharyngeal pumping rate (Feeding rate), metal body burden, brood size, and mitochondrial enzyme activity. We carried out an acute (24 h) dose-response for Cd (0, 0.1, 1, 10, 50 and 100 μ M) and Pb (0, 0.1, 1, 10, 50, and 100 μ M) on worm pumping rate. We then selected a No Observable Adverse Effect Level (NOAEL) dose (1 μ M) and a Lowest Observable Adverse Effect Level (LOAEL) dose (10 μ M) for both Cd and Pb, we used these conditions in our subsequent studies. A chronic(5-day) study of NOAEL, LOAEL, and mixtures was carried out to determine the long-term toxicity profile. Coincubation with either Ethylenediaminetetraacetic acid

(EDTA), a broad metal chelator, Ferristatin II, a Divalent metal transporter (DMT)-1 inhibitor, DMT 1 KO, or N-acetylcysteine (NAC), an antioxidant and glutathione precursor, was performed to investigate mechanisms of intervention. Worm body burden of heavy metals was measured to analyze metal homeostasis dysregulation after acute exposure. In addition to these readouts, we examined the brood size of worms after acute exposure as well as the enzyme activity of TCA and electron transport chain enzymes of isolated mitochondria. **Results:** We observed that acute exposure of NOAEL of Pb or Cd had no effect on the pumping rate. However, when combined, the mixtures reduced worm pumping rate. Chronic (5-days) exposure of NOAEL to Cd or Pb reduced the pumping rate, with the mixtures potentiating their individual effect on worm pumping rate. Both acute and chronic exposure to LOAEL of Pb or Cd impacted worm pumping rate. We noted that the removal of the metals after initial impact did not reverse the reduced pumping rate. We also observed reduced brood size for Pb or Cd exposure to LOAEL for individual metals or NOAEL for mixtures. The use of the broad metal chelator Ethylenediaminetetraacetic acid (EDTA) abolished both Pb or Cd induced toxicity. Like EDTA, N-acetylcysteine (NAC), an antioxidant and glutathione precursor and Ferristatin II, a Divalent metal transporter (DMT)-1 inhibitor, mitigated against Pb and/or Cd toxicity in both NOAEL and LOAEL. Genetic ablation of DMT1 proffered similar protection as those observed with pharmacologic inhibition of DMT1. Worm tissue burden revealed an increase in Pb or Cd accumulation with no impact on other resident metal homeostasis. Further, co-incubation of the metals with EDTA, NAC or DMT1 inhibitor or genetic ablation of DMT1 reduced Pb or Cd tissue burden. We observed reduced mitochondrial citrate synthase enzyme activity following exposure to Pb and Cd. **Conclusions:** Overall, our data demonstrates the critical nature of mixtures studies because we observed a synergistic interactive effect of physiologically relevant doses of Cd and Pb exposure, which can persist chronically after the removal of the exposure. We showed DMT-1 is an entry mechanism for Pb and Cd and NOAEL of the metal mixtures impacted brood size, pumping rate and mitochondrial function and could be mediated through oxidative damage.

PS 3631 Strain-Dependent Effects of Trichloroethylene Exposure and Western Diet in Wild Type and Humanized PPAR α Mice

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Background and Purpose: Metabolic dysfunction-associated steatotic liver disease (MASLD) affects nearly one-third of US adults. MASLD diagnosis requires the presence of excessive hepatic fat and at least one component of metabolic syndrome of which consumption of a western diet (WD) is a major risk factor. Environmental toxicants can also cause liver injury, as seen in toxicant-associated steatohepatitis (TASH). Our research combines the concepts of MASLD and TASH focusing on an environmentally relevant, hepatotoxic chemical: trichloroethylene (TCE). TCE was the main contaminant in the water at U.S.M.C. Base Camp Lejeune, North Carolina, where as many as 1,000,000 individuals were exposed to contaminated drinking water. The TCE metabolites trichloroacetic acid (TCA) and dichloroacetic acid (DCA) are agonists of peroxisome proliferator-activated receptor α (PPAR α), a nuclear receptor with a critical role in maintaining lipid homeostasis and thus steatotic development and overall MASLD progression. PPAR α displays notable species differences; therefore, mice may not be good models to study human health effects of PPAR α agonists. **Methods:** The guiding hypothesis is that TCE exposure alters the expression of PPAR α dependent genes and predisposes the liver to fat accumulation in a sex and strain dependent manner. To elucidate these potential differences, male and female wildtype (WT) and transgenic humanized PPAR α mice (hPPAR α) were subjected to a 12-week exposure to TCE (0.5 mg/mL), WD, or their combination (n=10-12). Two-way ANOVA with the Tukey's post hoc test was performed to determine statistical significance with a predetermined p value of 0.05. **Results:** Regardless of strain, male mice displayed little response to TCE exposure but were susceptible to WD-induced liver injury as evidenced by elevated serum enzymes, increased liver weight, and decreased glucose tolerance. Both WT and hPPAR α female mice responded similarly to WD but displayed differential response to TCE exposure. Like in the males, TCE exposure did not affect WT female mice; however, in the female hPPAR α mice TCE protected against WD-induced injury as evidenced by a return to control values of white adipose tissue weight, relative fat mass, true lean mass, and percentage body weight change. **Conclusions:** These results demonstrate a sex and strain specific response to TCE exposure in which TCE exposure did not affect the male mice of either strain or the female WT mice but protected against the injury of WD in the female hPPAR α mice. This effect may involve differential PPAR α activation thereby altering the hepatic response to WD and inducing protection from WD-induced injury. These results highlight the importance of considering sex and strain specific factors when evaluating environmental exposures alongside dietary factors.

PS 3632 Liver Injury in the U.S. Population: The Combined Impact of PFAS and VOC Mixtures

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Background and Purpose: Adults in the United States face a growing burden of liver disease, with an estimated one in four adults affected by metabolic dysfunction-associated steatotic liver disease (MASLD). Exposure to environmental chemicals is one contributor to MASLD, with industrial emissions, consumer product degradation, and contaminated drinking water supplies leading to joint contamination of per- and polyfluoroalkyl substances (PFAS) and volatile organic compounds (VOCs). Because these chemicals frequently co-occur across air, water, and household environments, exposure is ubiquitous. Given this widespread co-exposure, this study examined combined associations between PFAS and VOC mixtures and markers of liver injury. **Methods:** We analyzed nationally representative dataset of adults aged ≥ 20 years participating in the National Health and Nutrition Examination Survey (NHANES) cycles 2003-2012. Exposures included four serum PFAS (PFOA, PFOS, PFHxS, PFNA) and three VOCs (bromomethane, xylene, chloroform) with detection frequencies $\geq 70\%$. We utilized covariate-adjusted multiple linear regression with the R Survey Package to assess associations between PFAS (log_e-transformed), VOCs (ln-transformed), and alanine-aminotransferase (ALT; ln-transformed), a biomarker of hepatocellular injury. Liver injury was defined using established clinical thresholds of ALT ≥ 33 U/L in males and ≥ 25 U/L in females for both single-pollutant and mixture analyses. Quantile g-computation models were then applied to evaluate the joint associations of PFAS and VOC mixtures, modeled on their original (untransformed) scale following quantile g-computation recommendations, with ALT (ln-transformed) and liver injury prevalence. **Results:** Across individual PFAS, higher serum concentrations were consistently associated with elevated ALT. Perfluorononanoic acid (PFNA) showed the strongest association, with each doubling in PFNA concentration corresponding to a 3.0% increase in ALT levels (95% CI: 1.4%-4.6%) and a 20.5% higher odds of liver injury (95% CI: 7.3%-35.4%). Each tertile increase in the PFAS mixture was associated with a 6.8% higher ALT (95% CI: 2.7%-11.0%) and a 34.9% higher odds of liver injury (95% CI: 13.8%-60.1%). In contrast, the VOC mixture was not significantly associated with ALT (1.7%; 95% CI: -2.7%-6.2%) or liver injury (15%; 95% CI: -9.1%-45.0%). The combined PFAS+VOC mixture showed the strongest association, with each tertile increase corresponding to a 7.1% higher ALT (95% CI: 0.7%-13.9%) and a 42.5% higher odds of liver injury (95% CI: 7.3%-89.3%). Within the combined mixture, chloroform contributed most inversely, while bromomethane, PFNA, and PFOA contributed most positively to both outcomes. **Conclusions:** Combined PFAS and VOC mixtures were more strongly associated with elevated ALT and increased risk of liver injury than PFAS mixtures alone. These findings suggest that co-exposure to PFAS and VOCs may amplify liver injury risk beyond that observed for PFAS exposure alone.

PS 3633 Increased levels of interindividual variation in dose reduces population risks from synergistic interactions

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Background and Purpose: Synergistic interactions between chemicals are a concern in mixture risk assessments since they can generate responses that would not occur if the chemicals were administered separately. Synergistic interactions are also a concern since they may produce risks that exceed the risks predicted by the dose-addition models that are typically used to assess combined exposures. In this project, we analyzed the impact of different levels of interindividual variation of doses on mixture risks in a population that received doses of two chemicals that have a synergistic interaction and determined if higher levels of variation resulted in reduced risks. Higher levels of interindividual variation in dose have previously been shown to reduce risks from combined exposures that follow dose addition models but the impact of increases in variation on chemicals that have synergistic interactions has not been investigated. **Methods:** The goal of the assessment is to measure mixture risk which is here defined as the increase in the fraction of the population that receive unsafe combined exposures that is caused by a synergistic interaction. The model used in this analysis is based on an interaction between two chemicals. The analysis assumes that both chemicals are regulated and the fraction of the population receiving doses of concern are limited to a small fraction of the population (e.g., 0.01 or 0.001). The impact of the synergistic interaction is assumed to result in a reduction of the safe dose of both chemicals by either a factor of ten or three (co-synergy). The synergistic interaction is also assumed to be dose dependent and to occur only when the dose of the other chemical exceeds 0.1 of the chemical's original permitted dose (e.g., synergy is assumed to occur at doses ten-fold below doses that cause adverse effects). The interindividual variations in the doses of the two chemicals received by the population are assumed to be independent and to follow log normal distributions. For simplicity, the doses of the chemicals are assumed to have the same distribution. The model does not consider temporal variation in doses and can be viewed as a model of acute toxicity with exposures occurring on a single day. A probabilistic model is created that assigns each individual a dose of the two chemicals by randomly sampling the dose distributions. The parameters for the distributions were derived based on the geometric standard deviation (GSD) and the level of residual

risk permitted under the regulations. Decision rules are created for determining if the combined exposures are safe based on the assumptions described above. Separate analyses were performed for 1) log-normal distributions with different GSDs (1.77, 3.16, and 5.6), 2) different levels of synergy (three-fold and ten-fold reduction in safe levels), and 3) different levels of residual risk (0.01 and 0.001). The three GSD values used correspond to ratios of 10, 100, and 1,000 for the doses received by the 98th and 2nd percentiles of the population. **Results:** The results of the analysis found that increases in interindividual variation in dose reduced the mixture risks for all of the combinations of synergy and residual risk assessed. At a residual risk of 0.01 and a synergy based reduction of ten, the mixture risks declined from 0.91 to 0.026. At a residual risk of 0.001 and a synergy based reduction of three, the mixture risks declined from 0.18 to 0.0005. These reductions occurred because individuals received lower doses of one or both chemicals when the upper bound is fixed by the permitted doses of the individual chemicals and the interindividual variation in dose is increased. **Conclusions:** The implication of this work is that when a population has high levels of interindividual variation in dose and the upper bounds of the dose distributions are protective, then the potential for synergistic interactions to cause population risks is small. This suggests that synergy may be a greater concern for sources of exposure that produce similar doses across large portions of the exposed population.

PS 3634 Western Diet and Environmental Chemical Exposures: A Transcriptomic Approach

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Background and Purpose: Populations living in areas of the Rust Belt in the north-eastern US may face unique environmental chemical exposures due to a history of industrial activities, followed by economic decline, then reinvestment. Prior work suggests that there may be increased risk of exposure to environmental chemical mixtures such as metals and per-/poly-fluoroalkyl substances (PFAS). Alongside chemical mixture exposures, a Western diet (WD) consisting of excess dietary fat is associated with a substantial burden of major cardiometabolic and chronic diseases. While adverse cardiac effects of Western diet consumption are understood, specific cardiotoxic pathways that may be altered due to co-exposure with real-world environmental chemical mixtures are not well studied. In this study, we examined the transcriptomic effects of chronic Western diet and environmental chemical mixture co-exposure in heart tissue. **Methods:** We previously defined the 'R2R' chemical mixture derived from biomonitoring metal and PFAS exposures among pregnant women in southwestern Pennsylvania. The mixture comprises 20.1% cadmium, 10.5% arsenic(III), 15.1% lead, 19.5% perfluorooctanesulfonic acid (PFOS), and 34.8% perfluorooctanoic acid (PFOA). Groups of 6-8 week-old C57Bl/6J mice received either a 13% fat control diet (CD) or a 43% fat Western diet (WD). Mice were then exposed to vehicle control, R2R mixture at 1x (R2R₁) and 100x (R2R₁₀₀) concentrations, or arsenic positive control via drinking water for 12 weeks. Hearts were collected and total RNA was extracted. Bulk RNAseq was performed followed by differential gene expression and pathway enrichment analyses. Differentially expressed genes (DEGs) were identified using a log2fold-change threshold of ± 0.5 and Benjamini-Hochberg adjusted p-value of < 0.05 . Pathway enrichment analysis was performed with a z-score threshold of $|z| > 2$. **Results:** We identified a greater number of significant DEGs in cardiac tissue associated with co-exposure to WD and R2R mixture than with individual diet or mixture exposure alone. We identified 2107 DEGs among co-exposed hearts (WD + R2R₁₀₀) compared to 1769 for high fat diet (WD) exposure alone or 107 for mixture (R2R₁₀₀) exposure alone. Further, we observed a trend for downregulated DEGs which represented 71% of co-exposure (WD+R2R₁₀₀) DEGs, 82% of Western diet DEGs, and 98% of R2R₁₀₀-associated DEGs. Pathway enrichment analysis of high fat diet (WD) identified activation of cardiotoxicity pathways involved in heart failure, congestive heart failure, dilated cardiomyopathy, and arrhythmia. Under mixture stress alone (R2R₁₀₀), pathways identified include activation of genes enriched for cardiomyopathy and cardiac enlargement. Consistent with individual high fat diet (WD) and mixture (R2R₁₀₀) exposure samples, co-exposure (WD+R2R₁₀₀) showed pathway activation of genes involved congestive heart failure, necrosis of cardiac muscle, and cell death. **Conclusions:** We observed evidence that Western diet and population-relevant co-exposure to common metal and PFAS toxicants resulted in dysregulation of cardiac gene expression. Pathway analysis suggests that co-exposure to a high fat diet and environmental chemical mixtures affects genes critical for heart health with pathways distinctly enriched for cardiac cell death that were not associated with either diet or mixture exposure alone. Our findings suggest that chronic exposure to low-level environmental chemical mixtures, combined with a typical Western diet, likely compound and contribute to poorer heart health.

PS 3635 Mitochondria-enriched extracellular vesicles as mediators and biomarkers of endothelial stress induced by polycyclic aromatic hydrocarbon mixtures

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Background and Purpose: Chronic exposure to polycyclic aromatic hydrocarbons (PAHs), persistent environmental pollutants commonly found in food, is associated with mitochondrial dysfunction, leading to oxidative stress, impaired energy metabolism, and activation of apoptotic pathways. Mitochondria are thus emerging as key targets of PAH toxicity. Endothelial cells, located at the blood-tissue interface, are also sensitive to circulating PAHs and respond by increasing the production of extracellular vesicles (EVs). These EVs may carry mitochondrial components (Mito-EVs), which could reflect mitochondrial status and mediate intercellular communication. This study aimed to (i) assess how PAH mixtures influence the release of EVs and their mitochondrial cargo by human endothelial cells, (ii) characterize EV subpopulations and their biochemical signatures using an integrated workflow, and (iii) investigate the biological effects of EVs derived from endothelial cells exposed to PAHs on immune recipient cells. **Methods:** Human microvascular endothelial cells (HMEC-1) were exposed for 24 h to 100nM of benzo[a]pyrene, of a 4-PAH regulatory mixture, or of a complex 18-PAH mixture representative of human dietary exposure. EVs were isolated by ultracentrifugation and fractionated by density-gradient centrifugation. This fractionation, combined with a multi-approach characterization pipeline - nanoparticle tracking analysis, transmission electron microscopy, flow cytometry, Western blot, Raman spectroscopy, Surface Plasmon Resonance imaging (SPRi), lipidomics, proteomics, and mitochondrial DNA (mtDNA) quantification by digital PCR - allows identification of biochemical fingerprints of EVs derived from HMEC-1 cells exposed to PAHs alone or in mixture. **Results:** All PAH exposures significantly increased the total release of EVs and Mito-EVs, with the strongest effects observed with the 18-PAH mixture. Density-gradient fractionation revealed the presence of distinct EV subpopulations, including Mito-EVs and mitochondria-depleted EV fractions. The intravesicular presence of intact mitochondria - either polarized or depolarized - as well as their mitochondrial protein, lipid, and mtDNA content, was subsequently evaluated after PAH exposure. Raman spectroscopy analyses highlighted modifications in both quantity and secondary structure of vesicular proteins, allowing discrimination between the PAH exposure conditions. The interaction of EVs from crude-conditioned media with multiple ligands was then monitored using a SPRi approach. These studies revealed distinct response patterns for potential EV surface markers, including CD36 and ATP synthase. The biological impact of Mito-EVs and mitochondria-depleted EV subpopulations was further investigated on the immune recipient cells, using primary human peripheral blood mononuclear cells. Cell viability, oxidative stress, mitochondrial transfer, and bioenergetic alterations were assessed to characterize the functional consequences of exposure to the different EV fractions. **Conclusions:** This integrated workflow, combining density-gradient fractionation with multi-omics and biophysical profiling, enables detailed characterization of EV subpopulations and analysis of the influence of PAH exposure under realistic conditions. Overall, this approach could reveal EV exposure fingerprints associated with environmental pollutants, including mitochondrial signatures, and thus offer a non-invasive tool for monitoring environmental exposure.

PS 3636 Metal Mixtures and Parkinsonism: From Weighted Quantile Sum Regression to Exposure Risk Score-Guided Autoantibody Profiling

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Background and Purpose: Chronic manganese (Mn) exposure from industrial activities, particularly ferroalloy production in Brescia, Italy, has long been linked to parkinsonian motor symptoms. Yet Mn rarely acts alone. Individuals in industrial regions are often co-exposed to other metals, such as copper, aluminum, chromium, lead and zinc, that may amplify or counteract Mn's neurotoxic effects, complicating single-metal inference. Because these metals are highly correlated and elicit diverse biological responses, mixture-based approaches are needed to capture their joint contribution to Parkinsonism (PD). This question carries growing public health importance as global demand for Mn and other industrial metals used in electric vehicle lithium-ion batteries and alternative green technologies increases the likelihood of chronic environmental exposure. Our previous metabolomic and lipidomic findings suggested that oxidative and immune pathways underlie Mn-associated neurotoxicity (Lewis *et al.*, 2025). To test this further, we performed multiplexed neural autoantibody profiling to examine whether immune reactivity to neurological

antigens mediate metal mixture toxicity. **Methods:** A matched case-control study (N = 97) quantified whole-blood metals by inductively coupled plasma mass spectrometry and neural autoantibodies (IgG/IgA/IgM) by multiplexed chemiluminescent immunoassay. Metals were log-transformed, winsorized (1st-99th percentile), and z-standardized. Conditional logistic regression (CLR) matched on age and sex estimated odds ratios (ORs) and 95% confidence intervals (CIs). Mixture effects were evaluated using two-index weighted quantile sum regression (2iWQS; $q = 3$, 100 bootstraps, 100 repeated holdouts) and adaptive elastic net (AENET) modeling with 10-fold pair-aware cross-validation. The AENET-derived exposure risk score (ERS), computed as the weighted sum of standardized metal concentrations, was assessed using conditional and standard logistic models to estimate odds ratios per SD and evaluate discrimination by ROC area (AUC). ERS-antibody associations were tested using robust linear models adjusted for age and sex with FDR correction. **Results:** Elevated Mn (OR = 2.20, 95% CI 1.27-3.83, $q = 0.039$) and Cu (OR = 2.05, 95% CI 1.15-3.64, $q = 0.039$) were associated with higher odds of PD in univariate CLR. In multivariate models adjusting for co-exposures, Mn remained significant (OR = 3.05, 95% CI 1.12-8.27, $q = 0.087$) and Al showed a positive trend (OR = 2.75, 95% CI 0.89-8.49). The positive WQS mixture (PWQS per 0.5 unit = 3.14, 95% CI 1.47-6.70) was driven by Mn (weight = 0.33), Al (0.28), and Cu (0.25), while the negative index was nonsignificant. The ERS, representing a standardized composite of weighted metals, showed strong associations with PD: conditional logit OR = 8.44 per SD (95% CI 2.19-32.45) and logistic OR = 7.12 (95% CI 3.13-16.18). These larger estimates are appropriate for an index summarizing multiple correlated exposures. The ERS model demonstrated excellent classification (AUC = 0.878, 95% CI 0.80-0.95). Higher ERS values were linked to increased autoantibody reactivity toward dopaminergic and neuronal proteins, including α -synuclein ($\beta = 0.40$, FDR = 0.001), D2 receptor ($\beta = 0.63$, FDR < 0.001), GAD65 ($\beta = 0.41$, FDR = 0.001), Amphiphysin ($\beta = 0.61$, FDR < 0.001), and dopamine β -hydroxylase ($\beta = 0.37$, FDR = 0.003), consistent with immune activation and dopaminergic injury. **Conclusions:** Across conditional and mixture models, Mn and Cu consistently emerged as key contributors to Parkinsonism risk, with Al also playing a role. Both WQS and ERS approaches demonstrated that higher metal co-exposure burdens markedly increased disease odds. While ERS odds ratios appear large, they appropriately reflect cumulative, standardized mixture effects rather than single-metal changes. The accompanying elevation in neural autoantibodies linked to dopaminergic and astroglial pathways suggests that Mn-Cu-Al mixtures may promote neuroimmune activation and early parkinsonian processes.

PS 3637 *In vitro* to *in vivo* Translation of Developmental and Adult Models to assess Neurotoxic Mechanisms of Metal Mixtures

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Background and Purpose: Heavy metals such as lead (Pb), arsenic (As), cadmium (Cd), and hexavalent chromium (Cr(VI)) frequently co-occur in contaminated environments, such as Superfund sites, yet their combined neurotoxic effects remain poorly understood. The Neurotoxicity due to Environmental Metal Mixture Exposure (NEMMEX) study investigates developmental and adult exposure to this "PACC" metal mixture, representing U.S. EPA drinking water standards, to uncover shared and age-specific mechanisms of neurotoxicity. Our findings inform risk assessment for how metal mixtures contribute to neurodevelopmental disorders such as Autism, and neurodegenerative diseases such as Alzheimer's Disease and Alzheimer's related Dementia (AD/ADRD). **Methods:** C57BL/6J mice exposed perinatally (2 weeks preconception through weaning), or chronically in adulthood (6wks to 6.5 months of age) to environmentally relevant PACC concentrations or individual metals [at the maximum contaminant levels (MCL) for As (10 ppb), Cd (5 ppb), and Cr(VI) (100 ppb), or maximum action level (ACL) for Pb (15 ppb)]. Behavioral tests assessed memory, anxiety, and locomotion, while electrophysiology (mPFC and CA1) characterized neuronal excitability and synaptic function. Complementary *in vitro* human neural organoids were exposed to the same conditions to examine neuronal differentiation, oxidative stress and synaptic functions. Molecular correlates were further assessed via transcriptomic profiling. We also quantified biomarkers of neurotoxicity and neuroinflammation. **Results:** Perinatal PACC metal mixture exposure caused impaired short-term memory and increased anxiety-like behavior in both sexes without affecting locomotion or working memory. Patch-clamp recordings revealed increased intrinsic excitability and larger spontaneous EPSC amplitudes in mPFC neurons, indicating enhanced postsynaptic responsiveness. Principal component analysis integrating behavioral and electrophysiological features identified distinct neurobehavioral signatures separating mixture-exposed and control mice. Chronic adult exposure similarly impaired memory and anxiety behavior, coupled with increased neuronal injury markers. Human organoids recapitulated key findings, showing oxidative stress, reduced synaptic and progenitor markers, and diminished neural differentiation. Ongoing RNAseq data analyses will identify molecular pathways driving the observed neurobehavioral phenotypes and cross-validate conserved mechanisms between animal and organoid systems. **Conclusions:** Our comparative findings demonstrate that environmentally relevant metal mixtures elicit persistent, sex-dependent neurobehavioral and physiological alterations across life stages. The convergent results across models highlight oxidative stress, neuroinflammation, and

disrupted synaptic signaling as shared adverse outcome pathways (AOPs). These insights support mixture-based risk assessment frameworks and regulatory efforts to protect neurodevelopmental and cognitive health from cumulative metal exposures.

PS 3638 Behavioral Effects and Bioaccumulation of Tire-Derived Compounds in Zebrafish: Unraveling the Chemical Drivers of Acute Sensorimotor Disruption

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Background and Purpose: Tire wear particles (TWP) are an emerging class of environmental contaminants released into surface waters via roadway runoff. Particles leach numerous organic and inorganic constituents, including 6PPD-quinone, a compound linked to acute mortality in Coho salmon. The nervous system has been implicated as a potential target of TWP exposure, though the underlying mechanisms remain poorly understood. This study aimed to identify the chemical components and mechanistic pathways responsible for acute sensorimotor disruption induced by tire tread leachates in zebrafish larvae. **Methods:** Five-day-post-fertilization zebrafish larvae were acutely exposed (1 h) to cryo-milled tire tread (CMTT) leachates (0.1-31.6 g/L, leached for 3 d at 20 °C). Motor behavior was assessed using an automated, video-based assay battery that sequentially evaluated responses to infrared, visible-light, and acoustic stimuli. Organic compounds in leachates and larvae were analyzed by LC-MS, while metals were quantified by ICP-MS. Bioconcentration factors (BCFs) were derived from measured internal and external concentrations. *In-silico* target predictions were performed to identify molecular targets of detected compounds. **Results:** CMTT leachate exposure induced a biphasic increase in total swimming activity (EC50 = 1.2 g/L), peaking at 3.2-10 g/L where larvae were ~2-fold more active than the control ($n = 64$). The effect persisted for ≥ 5 h, with partial recovery 3 h after leachate removal. Behavioral changes were stimulus-specific, affecting responses to infrared and acoustic cues but not visible light responses, suggesting selective disruption of sensorimotor processing. LC-MS analysis of 25 organic compounds revealed a mixture dominated by benzothiazoles (43%), para-phenylenediamines (PPDs; 25%), and phenylguanidines (21%). Several PPDs accumulated in larvae with BCFs up to ~1000 and correlated with compound hydrophobicity (LogD). A synthetic mixture comprising all 25 detected organic compounds failed to reproduce the overall hyperactivity phenotype observed with CMTT leachate, although it specifically altered habituation behavior to repeated acoustic stimuli. ICP-MS revealed zinc (Zn; 0.3-8.6 μ M) as the predominant metal in CMTT leachates. Acute Zn exposure largely recapitulated hyperactivity (EC50 = 10.2 μ M) observed with CMTT-exposed zebrafish, consistent with reported Zn-mediated inhibition of GABA_A receptors. Co-exposure with the GABA_A agonist propofol rescued the CMTT-induced behavioral phenotype, implicating Zn-driven inhibition of GABAergic signaling. However, Zn alone did not account for the complete spectrum of behavioral alterations. Given the distinct, yet notable effect of the synthetic mixture on sensorimotor processing, *in-silico* predictions identified Kv12.2 potassium channels (kcnh3) as a shared target for all eleven detected PPDs. The Kv12.2 inhibitor ASP2905 phenocopied the habituation deficit induced by the synthetic mixture. High phenotypic similarity with both the mixture and ASP2905 was also found with 6PPD, a representative PPD from the mixture. These findings support the contribution of PPD-driven Kv12.2 modulation to the observed behavioral effects. **Conclusions:** These findings demonstrate that acute exposure to tire-derived mixtures rapidly disrupts zebrafish sensorimotor behavior through multi-mechanistic interactions involving both inorganic and organic constituents. Zn and PPDs emerge as co-drivers of GABAergic and potassium channel modulation, likely converging on the observed behavioral phenotype. The identification of Kv12.2 as a novel, previously unrecognized PPD target expands mechanistic understanding of TWP toxicity and may aid in elucidating the mode of action of related compounds such as 6PPD-quinone. This mechanistic insight underscores the need for mixture-specific, mechanistically informed approaches in the environmental toxicology and risk assessment of tire wear emissions to accurately capture the real-world implications of mixture toxicity.

PS 3639 Exploring Synergistic Toxicity Between Açai Extracts and Methotrexate in Human Breast and Vascular Cell Lines

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Background and Purpose: Breast cancer is the most common and deadly cancer in women worldwide, leading many survivors to use botanical dietary supplements (BDS) like açai to improve quality of life and ease treatment symptoms. Açai has antioxidant, anti-inflammatory, and antiproliferative effects, but its use alongside anticancer drugs raises toxicological concerns. FDA reports show increased cardiovascular events, such as chest pain and pulmonary thrombosis, linked to açai combined with certain chemotherapies not metabolized by CYP3A4. These suggest possible drug-supplement interactions that may worsen toxicities or affect drug metabolism, yet no mechanistic studies exist. Therefore, thorough toxicological research is urgently needed to understand how açai impacts the safety and effectiveness of these anticancer drugs. **Methods:** Açai fruit powder extracts from a trusted

Brazilian supplier, along with two commercial açai BDS brands, were standardized for cyanidin-3-O-glucoside (C3G) content. These extracts were tested on three breast cell lines: MCF-7 (ER-positive breast cancer), MDA-MB-231 (triple-negative breast cancer), and non-tumorigenic MCF-10A cells. Cell viability was measured by MTT assay after exposure to açai extracts alone and combined with methotrexate (MTX). SynergyFinder Plus visualized cell viability to identify synergistic, antagonistic, or additive effects. Apoptosis from the most synergistic combinations was assessed using Annexin V and NucFix viability dyes with fluorescence microscopy to explore toxicity mechanisms. Açai extracts were fractionated by liquid-liquid extraction with solvents of varying polarity. These fractions were tested on the breast cell lines and analyzed via biochemometrics to pinpoint toxic compounds. Human umbilical vein endothelial cells (HUVECs) will be tested with extracts and fractions to assess the direct impact of açai-methotrexate interaction on cardiovascular health. **Results:** The single treatments of açai fruit powder and açai BDS extracts showed no significant toxicity when administered alone for the two breast cancer cell lines, MCF-7 and MDA-MB-231. The açai BDS extract showed slight toxicity towards the non-tumorigenic breast cell line, MCF-10A. When combined with MTX, all açai extracts produced at least an additive response, as reported via SynergyFinder Plus for the two breast cancer cells. For MCF-10A cells, there was reported synergistic activity from MTX with açai BDS extracts and the açai fruit powder extract. The combinations with MTX were the highest synergy reported across all cell lines, indicating specific toxicity towards non-cancerous cells with non-CYP3A4 interactive anticancer drugs and açai. No antagonistic interactions were observed. The preliminary fluorescence apoptosis analysis showed a high signal of necrosis from the combination for MCF-10A cells. Subsequently, the three most synergistic açai extracts were fractionated and tested on all 3 cell lines, with the hexane and ethyl acetate fractions showing the most toxicity toward all cells. The fractions were analyzed via LC-MS to produce the top 10 compounds specific to those fractions that may contribute to the toxicity of the whole extract. HUVECs will be subjected to the same analysis of the breast cells to determine whether the toxicity translates to the cardiovascular system, confirming the reports of the adverse events. **Conclusions:** Açai extracts enhance MTX toxicity in both cancerous and normal breast epithelial cells. The increased sensitivity of normal cells raises concerns about potential side effects during combined treatments. Including endothelial cell data will improve understanding of vascular effects, such as impacts on endothelial viability and function, which are vital for drug delivery and tumor microenvironment interactions. This study lays the groundwork for mechanistic research to identify the active compounds in açai responsible for these effects and their influence on endothelial cells, ultimately supporting safer combination cancer therapies.

PS 3640 The therapeutic effects of nobiletin treatment on hypertensive nephropathy via ferroptosis-associated axis

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Background and Purpose: Taiwan is rapidly transitioning into a super-aged society, accompanied by a marked increase in chronic diseases such as hypertension and kidney disease. According to the 2022 report from Taiwan's Ministry of Health and Welfare, mortality related to hypertension—particularly hypertensive nephropathy—has risen from ninth to seventh place over the past decade. *Citrus depressa*, a member of the Rutaceae family, possesses a peel rich in bioactive compounds such as nobiletin, which exhibit antioxidative, anti-inflammatory, and antitumor activities. However, its potential role in attenuating hypertension-induced chronic kidney injury remains insufficiently explored. **Methods:** In this study, molecular docking and a dipeptidyl-peptidase-4 (DPP-4) enzymatic activity assay were performed to evaluate the binding affinity and specificity of nobiletin in renal proximal tubular cells. A uninephrectomized, deoxycorticosterone acetate (DOCA)-salt-induced hypertensive nephropathy (1K/DOCA/salt) mouse model was employed to assess the renoprotective effects of nobiletin and to delineate the associated signaling mechanisms. **Results:** Our findings demonstrate that nobiletin isolated from *Citrus depressa* peel effectively ameliorates renal damage and modulates the ferroptosis signaling network. Molecular docking analysis revealed that nobiletin exhibits a strong binding affinity for the catalytic subunit of dipeptidyl peptidase-4 (DPP-4; PDB ID: 4FFW). Subsequent enzymatic assays confirmed that nobiletin inhibits DPP-4 activity, suggesting its potential to mitigate hypertension-induced renal injury in proximal tubular cells. Moreover, treatment with *Citrus depressa* peel extract or purified nobiletin suppressed ferroptosis-related signaling, as evidenced by reduced expression of DPP4, GPX4, and XCT in a mouse model of hypertensive nephropathy induced by uninephrectomy, deoxycorticosterone acetate (DOCA), and salt loading (1K/DOCA/salt model). **Conclusions:** Collectively, these results indicate that nobiletin exerts renoprotective effects against hypertension-induced kidney injury, potentially through modulation of the DPP-4-ferroptosis axis. This pathway may represent a promising therapeutic target for the prevention and treatment of hypertensive nephropathy.

PS 3641 The study on the safety of cannabidiol isolated from *Cannabis sativa* cultivated in Korea

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Background and Purpose: Cannabidiol (CBD) is a major compound derived from *Cannabis sativa* (*C. sativa*), known as a hemp. CBD has been known for its anti-inflammatory, antioxidant and anti-acne effects, and has been utilized and permitted research for medical purposes in hemp regulation-free zone in Korea. However, the adverse effects and safety of CBD associated with its topical exposure are not fully established, highlighting the need for further research. This study aims to evaluate the acute and local toxicity of CBD and its topical formulations, an ointment. **Methods:** An active pharmaceutical ingredient (API) grade cannabidiol (CBD) powder with a purity of ≥99.5% was manufactured from *Cannabis sativa* cultivated in a smart farm. For acute oral and dermal toxicity tests, CBD isolates were administered orally or topically to Sprague-Dawley (SD) rats at doses up to 2,000 mg/kg. To evaluate the skin and eye irritancy of CBD and its topical formulations, CBD isolate and various concentrations of CBD ointments were applied to New Zealand white (NZW) rabbits. Additionally, *in vitro* reconstructed human epidermis (RhE) test method (OECD TG 439) and cornea-like epithelium (RhCE) test method (OECD TG 492) were conducted. Finally, skin absorption tests for CBD isolate and CBD ointments were performed using a skin-like synthetic membrane, Strat-M, with Franz diffusion cell system. Quantification of CBD in collected samples was performed using high-performance liquid chromatography (HPLC). **Results:** The oral and dermal lethal dose (LD50) was determined to be greater than 2,000 mg/kg body weight for both males and females. In the skin irritation test, the CBD ointment was classified as non-irritating *in vitro* but slightly irritating *in vivo*. All test materials were classified as non-irritating to the eyes or not affecting serious eye damage *in vitro*. Additionally, no eye irritation was observed in any of the animals during the observation period (M.I.O.I and I.A.O.I scores were both 0). In the skin absorption test, CBD exhibited moderate percutaneous penetration in a dose-dependent manner. The absorbed amount of CBD was 9,981.24±147.00 µg/cm² for the 1% CBD solution, and 8,256.86±588.07 µg/cm² (1%), 15,788.58±270.06 µg/cm² (2%) to 56,536.43±8,111.47 µg/cm² (4%) for the CBD ointments. **Conclusions:** These results suggest that CBD is considered a topically safe active ingredient in its raw form and topical formulations.

PS 3642 PIPERINE ATTENUATES CISPLATIN-INDUCED TOXICITIES IN RATS: IN VIVO STUDY

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Background and Purpose: Piperine, a natural alkaloid compound, was shown to have anti-cancer, anti-depression and anti-diarrhea effects along with its known hepatoprotective and nephroprotective activities. On the other hand, chemotherapeutic agents, such as cisplatin, are known to be associated with various side effects including depression, anxiety, anorexia, congestive heart failure and nephrotoxicity. Moreover, cisplatin and Piperine co-treatment has been showed a synergetic effect against multiple cancer types. Thus, this study was conducted to investigate the ability of Piperine co-treatment to alleviate cisplatin-induced toxicities. **Methods:** Albino male rats (average weight = 250.54 ± 25.3 g) were randomly divided into four treatment groups (n= 5). These groups were vehicle (control), cisplatin (CDDP), piperine (PIP), and cisplatin with piperine co-treatment (CDDP + PIP). All treatments were administered as intraperitoneal injection. PIP (10 mg/ kg) was given for five consecutive days, whereas a single dose of CDDP (6 mg/ kg) was given on the 3rd day of PIP treatment. Splash test was conducted to evaluate depression effects, also body and organs weight were recorded. Moreover, blood brain tissue samples were collected for further biochemical and cellular assessments such as Serotonergic, CREB, BDNF and MAPK pathways. **Results:** Results showed that CDDP treatment significantly increased the grooming latency time (250 ± 38.02 sec.), decreased grooming frequency (1.4 ± 1.02 times/ 5 min.) and grooming duration (13.4 ± 13.47 sec./ 5 min.) compared to control treatment. However, PIP significantly reversed cisplatin-induced depression in CDDP + PIP treatment group (120.25 ± 105.76 sec., 3.25 ± 1.79 times/ 5 min., 34.4 ± 17.11 sec./ 5 min. for grooming latency time, frequency, and grooming duration, respectively with p < 0.05). In addition, blind observational assessment showed that PIP significantly reduced cisplatin-induced diarrhea in CDDP + PIP group by 70% (p < 0.05). **Conclusions:** In conclusion, these findings suggest that adding PIP + CDDP co-treatment may not only increase cisplatin potency as a chemotherapy, but also reduce cisplatin toxicities.

PS 3643 Safety findings for *Cordyceps militaris* fruiting body through USP Admission Evaluation process

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Background and Purpose: *Cordyceps militaris* and *Cordyceps sinensis*, are considered valuable edible fungi; they are widely used as functional foods and for medicinal properties in East Asia and, increasingly, in Western countries. *C. militaris* is

the main source of cultivated *Cordyceps* fruiting bodies in international trade. This material is cultivated successfully and available as an ingredient of dietary supplements in the US and Europe. *C. sinensis*, is a TCM made from caterpillar fungus that is used to treat chronic kidney disease. However, due to market unavailability and concerns about heavy metal contamination, *C. sinensis* has been substituted by *C. militaris* and is now incorporated into dietary supplements. Admission evaluations for *C. militaris* and *C. sinensis* were conducted in May 2025 by the United States Pharmacopeia (USP) Dietary Supplement Admission Evaluation and Labeling Expert Committee (DSAE EC). The primary objective of USP admission evaluations is to assess whether ingredients proposed for monograph development present serious risk to human health. Since neither applicable NDIN nor GRAS notices to the FDA were identified at the time of review for either species, comprehensive reviews were conducted. The primary focus of the work presented is on *C. militaris*, with reference to *C. sinensis* for comparative purposes. **Methods:** The admission evaluation was conducted according to USP Guidelines. This process includes a thorough review of clinical and toxicological studies, clinical case reports, and adverse event data. Other important components of an admission evaluation include other pharmacopeial monographs, regulatory standards, chemistry, adulterants and contaminants, intended uses, intake levels, and interactions. The admission evaluation process concludes with ingredients admitted (Class A) or not admitted (Class B) to the USP compendial monograph development process. The DSAEL EC can recommend that articles admitted under Class A include cautionary labeling. Articles are determined to be Class B if the available evidence indicates they present a serious risk to health, or public health concern, that precludes development of a monograph that could be approved for inclusion in the compendia. **Results:** *C. militaris* has not been the subject of GRAS or NDI notifications and is not listed in the US CFR for any food uses, and its use as a dietary supplement under DSHEA has not been confirmed. Results from *in vitro* and *in vivo* genotoxicity studies are negative for *C. militaris*. Although several sub-chronic, repeat-dose oral toxicology studies in rodents did not indicate concerns about *C. militaris*, other rodent toxicology studies indicated potential toxicities, particularly to the kidney. Furthermore, 11 documented cases of acute kidney injury associated with the consumption of legal products containing *C. militaris* in Thailand were identified. Several reports of suspected kidney and liver toxicity were identified among several national pharmacovigilance databases, albeit for '*Cordyceps*' (not further specified). *C. militaris* has been studied in several clinical trials involving healthy and diseased participants, including individuals with chronic kidney disease. These trials included monitoring of basic safety parameters, and no adverse events were reported. However, many of these clinical trials may not have been sufficiently powered to identify specific organ risks, were short in duration to detect adverse events, or relied on self-reporting of adverse effects. **Conclusions:** Clinical trials conducted with *C. militaris* at dosages similar to those recommended by marketed products did not show adverse effects across diverse study populations and treatment durations. However, several animal toxicology studies revealed significant kidney effects, including histopathological alterations at higher doses. Furthermore, documented acute kidney injury in elderly individuals with prolonged use (3 months) of *C. militaris* has been reported. After reviewing these data, along with potential kidney-related signals, the USP DSAEL EC determined that high doses and prolonged exposure to *C. militaris* may pose risks to kidney health, warranting further research to confirm safety. Thus, *C. militaris* was determined to be Class B and not admitted for monograph development at this time. Concerns were not raised for *C. sinensis*, which was admitted as Class A.

PS 3644 Defining the typical composition of spearmint (*Mentha spicata*) aerial parts essential oil: A GC/MS and controlled vocabulary-based approach

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Background and Purpose: Essential oils (EOs) are natural complex substances typically composed of dozens of chemically distinct constituents, each with a unique molecular structure. Constituents are commonly characterized and quantified by gas chromatography mass spectrometry (GC/MS). However, the naming consistency of constituents has often lacked reproducibility across literature and within the essential oil industry. This inconsistency complicates rapid data integration and reduces ease of comparison across studies. Establishing unified nomenclature through development of a controlled vocabulary (CV) for essential oil constituents, together with standardized reporting of their relative abundances, would define a typical composition for an essential oil. This typical composition could then be linked with constituent and whole-oil toxicological data and inform essential oil safety assessments. GC/MS data on spearmint (*Mentha spicata*) aerial parts EO was collected, and a typical composition was generated. **Methods:** 21 GC/MS reports, analyzed from 2018-2025, on spearmint EO were gathered and integrated into a database. The EO data analyzed was on Indian sourced, steam distilled, and re-distilled spearmint (*Mentha spicata*) aerial parts EO. Compound names were unified into a CV by evaluation of the chemical structure of constituents, and sorted by criteria such as stereochemistry, bond constitution, known synonyms, shared CAS number, and other metrics. Descriptive statistics were derived by evaluating each chromatographic peak in the chromatogram, summing the corresponding values for the constituents in each compound group, and calculating the mean, median, minimum, maximum, and standard deviation values for each. Frequency of constituent appearance was

also evaluated, and histograms were generated for each compound to assess distribution. The 25 most abundant constituents in the material, as detected by GC/MS, are included in the typical composition. **Results:** In the 21 GC/MS reports analyzing spearmint EO, carvone was found to be the primary constituent, with a median abundance of 58.03%. Other constituents $\geq 1\%$ include limonene (21.28%), dihydrocarvone (2.45%), myrcene (2.17%), 1,8-cineole (2.14%), bourbonene (1.49%), β -caryophyllene (1.09%), menthol (1.06%), and β -pinene (1.06%). The constituents with the most variation in nomenclature in this GC/MS dataset were found to be β -caryophyllene and carveol, each with 7 different variations of their respective names. In the top 25 compounds reported, an average of 3.32 name variations per constituent were found. **Conclusions:** Developing typical compositions based on standardized nomenclature and quantitative profiling can inform the safety assessment of essential oils. Having the expected range and average relative abundance of key constituents also allows for the compositional comparison of an EO standard to compositions in whole-oil toxicology studies in order to weigh relevance. In cases where compositions vary greatly, the established CV enables direct linkage between compositional data and constituent-specific toxicological information, and a constituent-based assessment approach can be taken. The spearmint EO typical composition illustrates how defining compositional baselines allows constituent variability to be systematically unified and can be used to inform its safety assessment. This approach facilitates cross-study comparability, providing a robust foundation for establishing relevance and informing safety assessment. Additionally, typical compositions may increase interoperability, influencing and supporting other important toxicological approaches and industry demands. These may include quantitative comparison for quality evaluation, efficacy studies including clinical trials and mechanistic *in silico* or *in vitro* studies, exposure surveys, regulatory reviews, and many other applications.

PS 3645 Chemical Profile and In Vitro Evaluation of Hydroalcoholic Extracts from *Hyptis lantanifolia* and *Ageratina popayanensis* Obtained from Plants Grown in Colombia

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Background and Purpose: The use of medicinal plants is a deeply rooted component of traditional knowledge in many human communities. For centuries, diverse plant species have been employed to address a wide range of health conditions. In many developing countries, phytomedicine remains the first line of treatment for various signs and symptoms. However, numerous plant species have not yet been scientifically investigated, and much of the ancestral knowledge preserved by traditional healers continues to be largely unexplored. In this context, the present study aims to evaluate the chemical composition, and flavonoid, phenolic, and tannin contents, as well as the *in vitro* UV-protective, antioxidant, and cytotoxic activities of *Hyptis lantanifolia* and *Ageratina popayanensis* obtained from plants grown in Colombia. **Methods:** The chemical composition of two extracts was analyzed using UHPLC/ESI-Q-Orbitrap-HRMS, the total phenolic, flavonoid, and condensed tannins contents were determined using the AlCl₃, Folin-Ciocalteu, and vanillin-H₂SO₄ methods, respectively. The antioxidant activity and photoprotective capacity were evaluated by spectrophotometry. The cytotoxicity of the extracts was measured over a concentration range of 4-250 µg/mL in Calu-1 and HepG2 cells using the MTT assay, and intracellular ROS production was monitored using the H₂-DCFDA probe. **Results:** UHPLC/ESI-Q-Orbitrap-HRMS analysis revealed that the hydroalcoholic extract of *H. lantanifolia* is characterized by methoxylated and glycosylated flavonoids, together with phenolic acids such as caffeic and rosmarinic acids. In contrast, *A. popayanensis* is dominated by hydroxycinnamic acids, particularly caffeoylquinic and dicaffeoylquinic acids, as well as glycosylated flavonoids including rutin and kaempferol-3-rutinoside. The hydroalcoholic extract of *H. lantanifolia* exhibited the highest flavonoid content (>170 mg RE/g DT), whereas *A. popayanensis* had the highest phenolic (>190 mg GAE/g DT) and condensed tannins (>40 mg CE/g DT) contents. *A. popayanensis* showed good DPPH radical scavenging activity with a concentration-dependent relationship. Both extracts showed photoprotective properties (SPF_{in vitro}: >30) and long-wavelength UVA protection (λ_c > 370 nm). In the cytotoxicity assays, *H. lantanifolia* showed a dose-dependent reduction in cell viability, with IC₅₀ values of 116 µg/mL in Calu-1 cells and 216 µg/mL in HepG2 cells. In contrast, *A. popayanensis* exhibited minimal cytotoxicity, with markedly higher IC₅₀ values (>250 µg/mL for both cell lines), maintaining viability close to control levels across most concentrations. At 15.63 µg/mL, neither *H. lantanifolia* nor *A. popayanensis* induced increases in intracellular ROS levels in either cell line. **Conclusions:** The distinct phytochemical signatures and bioactivities of these two Colombian species reveal their potential as valuable sources of bioactive metabolites. Their strong *in vitro* photoprotective and antioxidant effects, together with moderate-to-low cytotoxicity and absence of ROS induction, support further bioprospecting and functional characterization for pharmaceutical and cosmeceutical applications. Minciencias - University of Cartagena (Grant: 116688, call 969-2025).

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Background and Purpose: The development of therapeutics for the prevention of life-threatening diseases such as neurological disease, heart disease, and cancer is becoming more urgent every day. Currently, over one in three people are affected by neurological conditions which are the leading cause of human illness and disability. Natural remedies to cure illness have been utilized throughout history due to their abundance of polyphenols. Polyphenols are a diverse group of natural compounds found exclusively in plants that are studied for their antioxidant effects. The polyphenols found in the seaweed *Fucus vesiculosus* (bladderwrack) could contribute to bioactivity and have therapeutic effects. Research suggests that polyphenols can help combat oxidative stress, which is linked to various chronic diseases, including neurodegenerative conditions, cardiovascular disorders, and cancers. MnCl₂ is known to cause neurodegenerative diseases including Parkinson's disease and Alzheimer's disease. *Caenorhabditis elegans* has been used to examine the neurotoxicity of MnCl₂ due to its conserved pathways related to oxidative stress, making it a suitable model for human research. We hypothesize that polyphenols extracted from bladderwrack algae extract (BAE) will exhibit antioxidant properties in the nematode worm, *C. elegans*, when exposed to the oxidant manganese (II) chloride (MnCl₂). **Methods:** Total phenolic content was determined using the Folin-Ciocalteu method using gallic acid as the standard and reading the absorbance at 750 nm. Dose response-survival curves were created using L1 stage worm pretreated with increasing concentrations of BAE for 30 minutes in survival assays to determine the lethal dose in *C. elegans*. For survival curves with MnCl₂, worms were pre-treated with BAE for 30 minutes followed by treatment with varying MnCl₂ concentrations for 30 minutes. The lifespan of *C. elegans* when pre-treated with 7.5% BAE +/- 5 mM MnCl₂ was determined. DCFDA fluorescence was used to quantify reactive oxygen species (ROS) after exposure to MnCl₂ in presence or absence of BAE. Activation of the oxidative stress response genes was measured using the fluorescent reporter worm strain VP596. **Results:** Three separate extracts of the bladderwrack algae were created, and the total phenolic content of the BAE was determined to be 360 +/- 85 mg gallic acid equivalents per mg of dry material. The dose-response survival curve determined the LD₅₀, LD₂₀, and LD₁₀ values of BAE to be 40%, 7.5%, and 1%, respectively. Since oxidative stress decreases lifespan of *C. elegans*, worms were pre-treated with BAE prior to MnCl₂. Treated worms had a 2-day increase in median survival increased in the presence of BAE. Total reactive oxygen species (ROS) were measured in the presence of BAE and MnCl₂, revealing a two-fold, dose-dependent decrease in ROS with BAE pre-treatment. The oxidative stress reporter assay showed a two-fold decrease in MnCl₂-treated *C. elegans* pre-treated with 7.5%, and 1% BAE. **Conclusions:** Taken together BAE contains polyphenols which can effectively prevented oxidative damage as seen by a decrease in ROS, increase in lifespan, and decrease in activation of the antioxidant stress pathway. These results suggest that BAE reduces oxidative stress through multiple mechanisms, supporting its potential as a natural therapeutic agent for promoting cellular health and stress resistance.

PS 3647 Mechanisms of action of four major constituent cannabinoids in hemp extract

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Background and Purpose: Hemp and hemp extracts may contain a variety of different cannabinoids, such as cannabidiol (CBD) cannabichromene (CBC), cannabigerol (CBG), and cannabinol (CBN). CBD has been extensively studied and reported to cause hepatotoxicity in clinical trials and research studies, little is known about the safety of other cannabinoids. In the current study, we set out to understand the mechanism(s) of action of these cannabinoids, beneficial or adverse, through a comprehensive functional analysis of a recently published transcriptomic dataset. **Methods:** iPSC-derived hepatocytes were exposed to 40 µM CBC, 20 µM CBD, 40 µM CBG, and 10 µM CBN, respectively, for 24 h. Gene expression changes were profiled using Affymetrix GeneChip PrimeView Human Gene Expression Arrays. Functional analysis was conducted using Ingenuity Pathway Analysis (IPA). **Results:** Each compound impacted a unique list of canonical pathways, upstream regulators, diseases and biological functions, toxicity functions, and gene interaction networks with distinctive activation/inhibition patterns. Based on the overall IPA outcome, the cannabinoids were predicted to affect metabolism and to possibly have some beneficial effects on cardiovascular disease but adverse effects on the neural system. In addition, CBC and CBN, similar to but more potently than CBD, displayed liver toxicity and cancer-inducing potential while CBG protected from these adverse effects. **Conclusions:** This study provides a comprehensive comparison across the four cannabinoids on the health effects and potential toxicities associated with their use. Further studies are needed to clarify the effects on cardiovascular disease and the oncogenic potential of these compounds.

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Background and Purpose: The global use of botanical dietary supplements highlights the need for cost-effective and mechanistically informative approaches to ensure consumer safety. To address these gaps, the Botanical Safety Consortium was established to advance botanical safety assessment by integrating new approach methodologies (NAMs) into the evaluation of botanicals. Thirteen botanicals: aristolochia, ashwagandha, Asian ginseng, blue cohosh, comfrey, ephedra, goldenseal, green tea extract, kava, kratom, milk thistle, usnea lichen, and yohimbe were selected to represent cases with either known hepatotoxicity or established safety data from the literature. This study aims to 1) develop and apply a multi-tiered *in silico* screening approach to assess potential hepatotoxicity of botanicals, and 2) evaluate the performance of available *in silico* tools for predicting hepatotoxicity in botanicals. **Methods:** In this study, we developed and applied a three-tiered *in silico* screening approach. In Tier 1, several quantitative structure-activity relationship (QSAR) models, including ones in ADMET Predictor, Case Ultra, and Leadscape, were used to predict hepatotoxicity endpoints for 103 constituents in thirteen botanicals, categorized as (1) hepatocellular, (2) biliary, and (3) general liver toxicity. Predictions within the applicability domain were included. Consensus predictions were defined as those with ≥ 50% agreement, representing the majority-vote predictions among the QSAR models for each category. In Tier 2, physiologically based pharmacokinetic (PBPK) models, ADMET Predictor v10.4, were used to estimate maximum plasma concentration (C_{max}) of each constituent. The estimated oral dose for each constituent was derived from the maximum recommended consumer daily intake of the botanical, combined with quantitative chemical analysis of constituent concentrations. A threshold of 1 µM for predicted C_{max} was applied to identify potential hepatotoxic botanical constituents. Constituents with both positive QSAR predictions and C_{max} ≥ 1 µM were classified as positive for human hepatotoxicity. In Tier 3, a botanical was classified as potentially hepatotoxic if at least one of its constituents had a positive prediction in Tier 2. Finally, concordance between the model predictions and the established human hepatotoxicity of the botanicals was used to evaluate the model's predictive performance. **Results:** Of the 13 botanicals evaluated, seven have reported evidence of human liver toxicity: ashwagandha, comfrey, ephedra, green tea extract, kava, kratom, and usnea, whereas the rest have not. In Tier 1, QSAR models predicted that at least one constituent in all 13 botanicals was positive for hepatocellular endpoints, 9 botanicals were positive for biliary endpoints, and 10 botanicals were positive for general liver toxicity. In Tier 2, predicted C_{max} values of all constituents in six botanicals (including ashwagandha, Asian ginseng, and yohimbe) were below 1 µM, indicating that these constituents are either present at low concentrations in the botanicals or have limited bioavailability to reach systemic circulation, making them unlikely to cause liver toxicity. In Tier 3, the hepatocellular endpoint performed best among the three categories, yielding 5 true positives, 4 true negatives, 2 false positives, and 2 false negatives. **Conclusions:** The developed tiered *in silico* approach provides valuable insights for initial screening of liver toxicity of botanicals and their constituents. **Disclaimer:** The findings & conclusions in this presentation have not been formally disseminated by the Centers for Disease Control and Prevention/ Agency for Toxic Substances and Disease Registry and should not be construed to represent any agency determination or policy.

PS 3649 A User-Friendly iPBPK Platform for Predicting Florfenicol and Florfenicol Amine Residues in Sheep and Goats

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Background and Purpose: Florfenicol (FF) is an antimicrobial approved for use in cattle and swine to treat respiratory infections, but it is not approved for use in small ruminants, such as sheep and goats. Extralabel use of FF in these species can lead to violative residues of FF and its metabolite, florfenicol amine (FFA), in edible tissues, posing potential risks to human health. Scientifically based extralabel withdrawal intervals (WDIs) are essential to ensure safety of meat products derived from sheep and goats treated with FF. This study aimed to develop physiologically based pharmacokinetic (PBPK) models for FF and FFA in sheep and goats to predict tissue

concentrations and determine safe WDLs following extralabel FF use. **Methods:** Two flow-limited submodels were constructed for FF and FFA, each consisting of seven compartments (plasma, liver, kidney, lung, muscle, fat, and the rest of the body). The sheep model was calibrated using plasma FF data from intravenous (IV), intramuscular (IM), and subcutaneous (SC) administrations, along with tissue FFA data (liver, muscle, kidney, and fat) from SC exposure. Model evaluation was performed using independent datasets from the Food Animal Residue Avoidance Databank (FARAD). The goat model was calibrated separately using FF and FFA plasma and tissue data from multiple routes of administration and evaluated via independent plasma datasets. Monte Carlo simulations were conducted to characterize parameter uncertainty and population variability. The final model was implemented as a web-based interactive PBPK tool using the R Shiny package to estimate WDLs under various regulatory standards. **Results:** Model validation using independent datasets demonstrated strong predictive performance, with R^2 values of 0.94 and 0.99 for sheep and goats during calibration, and 0.97 and 0.98 during evaluation. Population-based simulations predicted the longest WDL of 42.71 days for a single 20 mg/kg IM dose in sheep based on an operating tolerance of 0.3 ppm in kidney. The predicted WDL for two repeated 40 mg/kg SC doses in goats based on an operating tolerance of 3.7 ppm in liver is 91.21 days. **Conclusions:** The developed PBPK models provide a robust, quantitative framework for predicting FF and FFA residues in sheep and goats. This work advances efforts to establish science-based WDLs, enhances veterinary and regulatory decision-making, and offers a practical, user-friendly web-based tool for estimating WDLs across diverse exposure scenarios.

PS 3650 Cannabinoid and Sensory Evaluation of Cannabidiol and Baby Leaf Hemp in Yuma, Arizona

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Background and Purpose: Hemp (*Cannabis sativa*) has been legalized for production and sale in the U.S. since the passage of the Farm Bill in 2018. However, there is a legal cutoff of 0.3% delta-9 tetrahydrocannabinol (THC, by weight) for hemp and hemp-derived products, including cannabidiol (CBD). Additionally, only hulled hemp seed, hemp seed protein powder, and hemp seed oil are considered Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration. In this study, we aim to examine the cannabinoid content and sensory properties of CBD hemp and baby leaf hemp (a novel use of the crop) in Yuma, Arizona. **Methods:** In the first part of this study, we conducted a field trial to investigate flowering dates, CBD concentration, and crop limitations across different grow environments. In the second part, we examined growing baby leaf hemp, a novel commodity that was harvested earlier than CBD hemp and unlikely to exceed the legal cutoff of 0.3% THC. **Results:** A total of 17 varieties of CBD hemp (3 auto-flower and 14 day length-dependent) were cultivated at the Yuma Valley and Mesa Stations. The first site represented the alluvial valley soil (Holtville Clay: 18% sand, 46% silt, 36% clay), and the second site represented the sandy mesa soils typical of the region (82% sand, 6% silt, 12% clay). At the Yuma Valley Station, CBD levels tended to be lower. The maximum observed CBD levels per variety ranged from 2.1% to 14.4% with an overall average of 6.1%. The average maximum THC concentration was 0.38%, also lower than in Mesa. At the Yuma Mesa Station, CBD levels were higher, ranging from 4.9% to 14.5% across varieties. The average maximum CBD content per variety was 10.4%, while the average maximum THC content was 0.60%. Notably, some varieties such as Early Spectrum and Z-5 reached CBD levels above 14%. Certain varieties such as Early Spectrum and Z-5 exceeded 14% CBD under Mesa conditions, highlighting the influence of the environment on cannabinoid content. The THC to CBD ratio remained constant over time, suggesting that this measure may serve as a reliable indicator of identifying high-risk candidates with elevated THC levels. Next, two 'drive-thru tasting' events were conducted 18 and 25 days after the planting of baby leaf hemp, and survey data were collected from 86 respondents. Hemp varieties were consistently rated higher in fuzziness compared with traditional leafy greens. The means of hemp varieties ranged between 3.0-3.3 on the scale of 1 (weakest) to 5 (strongest). These were compared to leafy greens such as romaine, kale, and spinach, with the means ranging between 2.3-2.5. Among the hemp samples, Santhica 70 received the highest fuzziness score (3.3), suggesting that textural perception differed between hemp and other leafy greens. Hemp varieties were rated lower in crispiness (means = 2.4-2.7) than leafy greens (means = 2.8-4.0). Both romaine and hemp were perceived as more floral (means = 2.5-3.2), which may be related to romaine consistently being the first sample presented to participants. Additionally, leafy greens are often bred for neutral flavor, which may have accentuated the relative perception of floral notes. Hemp samples were generally considered fruitier (means = 2.3-2.8) than kale (1.6) and more minty (means = 2.3-3.0) than other leafy greens (means = 1.7-1.8). **Conclusions:** Results from the field trial demonstrated clear differences in cannabinoid accumulation between sites. It also demonstrated the influence of environmental factors on cannabinoid content. Grown as baby leaf, hemp varieties were perceived as less bitter and with higher likability than kale. Further studies are needed to characterize such factors and the sensory attributes of hemp varieties, thereby developing hemp as an alternative leafy greens with distinct sensory attributes.

PS 3651 Safety Evaluation of a Sugar Replacement (Honey Truffle Sweet Protein) Derived from Nature

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Background and Purpose: Identification of sweetener alternatives has been the subject of intense research to provide a lower calorie substitute for sugar in the diet. Honey truffles (*Mattirolomyces terfezioides*) contain a sweet natural protein referred to as Honey Truffle Sweet Protein (HTSP), which exhibits intense sweetness. HTSP was expressed in *Komagataella phaffii* (formerly *Pichia pastoris*) and produced via precision fermentation. A safety and risk assessment was undertaken for its use as a sweetener in food and beverages. **Methods:** Genotoxicity tests (Ames and micronucleus assays) and a 13-week oral repeated dose study in rats were conducted (according to OECD test guidelines) to assess the safety of Honey Truffle Sweetener Protein (HTSP). **Results:** The results of the *in vitro* bacterial mutagenicity and mammalian cell micronucleus tests demonstrated a lack of genotoxic effects. In the 13-week oral exposure study, male and female Wistar Han rats were dosed by gavage to provide 1800, 3600, and 5400 mg/kg/day of HTSP (equivalent to approximately 500, 1000 and 1500 mg/kg/day of the honey truffle sweet protein active component). Treatment was well tolerated with neither mortality nor HTSP-related adverse clinical pathology findings, organ weight effects, or macroscopic or microscopic observations. **Conclusions:** The no observed adverse effect level (NOAEL) was 5400 mg/kg/day (1500 mg/kg/day honey truffle sweet protein active component). The NOAEL in association with estimated daily human intake (EDI) supports the conclusion that HTSP may be considered safe for use as a dietary sweetener.

PS 3652 Sensitive Detection of Emerging *Alternaria* Mycotoxins with Novel Aptamer- and Antibody-Based Strategies

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Background and Purpose: Alternariol (AOH) and tenuazonic acid (TeA) are emerging mycotoxins produced by *Alternaria* fungal species that frequently contaminate cereals, oilseeds, spices, and various fruits and vegetables. Chronic dietary exposure to these emerging mycotoxins has been linked to hepatic and renal dysfunction, and potential carcinogenicity, highlighting their importance as food safety hazards. To enable sensitive and specific detection of AOH and TeA residues, this study aimed to develop antibody- and aptamer-based analytical tools, supported by chromatographic validation, for rapid and reliable monitoring of these toxins in food and feed matrices. **Methods:** Polyclonal antibodies specific to AOH and TeA were generated in corresponding New Zealand White rabbits and used to establish competitive direct enzyme-linked immunosorbent assays (cdELISAs). The half-maximal inhibitory concentrations (IC_{50}) were determined to be 46 ng/mL for AOH and 10 ng/mL for TeA, respectively. In parallel, eight single-stranded DNA aptamers—four for AOH and four for TeA—were selected through the SELEX process. Molecular docking simulations identified AOH Apt #4 and TeA Apt #2 as the highest-affinity sequences, which were subsequently validated by noncompetitive enzyme-linked aptamer assays (ELAAs). Complementary HPLC-FLD methods for AOH and TeA quantification were also developed to serve as reference standards. **Results:** The developed cdELISAs demonstrated high specificity and sensitivity, enabling quantitative detection of AOH and TeA within low nanogram per milliliter ranges. Aptamer-based assays constructed using AOH Apt #4 and TeA Apt #2 achieved measurable responses, although their IC_{50} values exceeded 100 ng/mL, indicating relatively lower affinity compared with antibody-based systems. The HPLC-FLD methods achieved detection limits of 0.1 ng for AOH and 20 ng for TeA, with excellent linear correlations ($R^2 = 0.999$). Collectively, these results confirm the successful generation of functional antibodies and aptamers for both mycotoxins and establish reliable immunochemical and chromatographic platforms for their detection. **Conclusions:** This study provides a comprehensive set of analytical tools for monitoring AOH and TeA contamination in food and feed. The developed antibody-based cdELISAs offer high sensitivity and specificity, while the aptamer systems present a promising alternative for future biosensor integration. Together with the validated HPLC-FLD methods, these assays support regulatory surveillance and contribute to enhanced food safety management strategies against *Alternaria* mycotoxin contamination.

PS 3653 Approach for systematically assessing study reliability and relevance in evaluations of monosodium glutamate safety

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Background and Purpose: Monosodium glutamate (MSG) is a well-studied food additive. In addition to numerous authoritative assessments of safety, a substantial volume of research is ongoing with MSG; this includes a growing volume of research assessing the ameliorating potential of various substances against purported MSG-induced toxicity. This work set out to develop a framework for evaluating the

combined reliability and relevance of these types of investigations as part of ingredient safety assessments, and subsequently, to apply such to a case study of MSG publications involving co-exposures with other substances. **Methods:** For the development of the framework, key aspects related to internal, external, construct and biological validity were considered with particular focus on the safety of food ingredients. As the evidence base for some food ingredients often contains non-guideline studies, SciRAP was chosen to assess reliability (internal and construct validity) due to its specific development to focus on such studies. To assess the relevance (external and biological validity) study attributes that are fundamental for a food safety risk assessment were considered to ensure the most relevant studies were found. The study attributes included the study design (ensuring relevant and meaningful results), route of exposure (potentially allows for more relevant food exposures), outcomes assessed (apical or complex responses often considered when setting an ADI), and dosing regimen (chronic studies and those at levels below understood NOAELs often provide more insight than high dose acute studies). A categorization scheme was developed for both the reliability and relevance evaluation as well as an aggregate study quality categorization. After development of the framework, a case study was conducted on a unique set of publications related to MSG. Specifically, these studies involved a co-exposure investigating a potential ameliorating effect for a wide range of chemicals. **Results:** The approach assesses the reliability of the studies utilizing SciRAP, and the relevance in context of study design, dose relevance, and biological validity, resulting in an overall categorization of the informativeness of an individual study, or study quality. In a case study application to 39 studies assessing MSG toxicity and ameliorative properties of a variety of substances, no publications were deemed of 'high' study quality for the purpose of assessing safety, due primarily to low relevance to human safety (e.g., use of an acute high dose during neonatal lifestages) and limited reliability in study conduct and reporting. Consideration of the biological plausibility of the reported endpoints further determined the limited informativeness of the studies in understanding MSG safety. **Conclusions:** In all, this work developed an approach for evaluating the combined reliability and relevance of these types of investigations as part of ingredient safety assessments, and subsequently, was applied to a case study of MSG publications involving co-exposures with other substances. Such a framework has applicability to a wide range of food ingredients and offers an approach to thoroughly and reproducibly evaluate the scientific literature. Additionally, the case study did not determine any studies to be of "high" quality indicating limited utility in informing MSG safety.

PS 3654 Cell-Type-Dependent Mechanisms of Patulin Toxicity: Insights from SH-SY5Y and Caco-2 Cell Models

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Background and Purpose: Patulin (PAT) is a mycotoxin produced by *Penicillium*, *Aspergillus*, and *Byssoschlamys* species, frequently found in apple-derived products. Its exposure poses a risk to human health due to its cytotoxic, genotoxic, and neurotoxic properties (Malir et al., 2023; Zhong et al., 2018). This study compared the toxic effects of PAT on human neuroblastoma (SH-SY5Y) and human colon adenocarcinoma (Caco-2) cell lines, aiming to evaluate its cytotoxicity, alterations in the cell cycle, and cell death mechanisms. **Methods:** Cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 24 and 48 h of exposure to concentrations ranging from 0 to 3 µM. In parallel, cell cycle phases and cell death were analyzed by flow cytometry. **Results:** Results revealed a higher sensitivity of SH-SY5Y cells, showing a significant reduction in viability from 0.19 µM, with IC₅₀ values of 2.5 µM (24 h) and 1.5 µM (48 h). In contrast, Caco-2 cells exhibited a more stable response and even a slight increase in viability after 48 h, with no IC₅₀ values reached at either exposure time. Cell cycle analysis showed that PAT induced marked alterations in SH-SY5Y cells, including M-phase arrest at higher exposure concentrations and a decrease in all cell cycle populations after prolonged exposure. Conversely, in Caco-2 cells, the alterations were mild and transient. Regarding cell death, PAT triggered early necrosis in both lines, followed by apoptotic activation in SH-SY5Y cells and predominant necrosis in Caco-2 cells at intermediate concentrations after prolonged exposure. **Conclusions:** In conclusion, PAT exerts time- and concentration-dependent toxic effects, with significantly greater susceptibility observed in neuronal cells. These differences reflect cell type-specific response mechanisms and highlight the need to consider cellular vulnerability when assessing the toxic risk associated with this mycotoxin. **Acknowledgments:** This work has been funded by the Ministry of Science and Innovation PID2020-115871RB-I00 and by project CIAICO2022/199 of the Ministry of Education, Universities and Employment of the Generalitat Valenciana. CML would like to thank the Generalitat Valenciana and the Conselleria d'Educació, Universitats i Ocupació for the contract associated with the CIAICO/2022/199 project. **References:** Malir, F., Pickova, D., Toman, J., et al. (2023). *Mycotoxin Research*, 39(2), 81-93. Zhong, L., Carere, J., Lu, Z., et al., (2018). *Toxins*. <https://doi.org/10.3390/toxins10110475>

PS 3655 Multiorgan Toxicity of Microbiome-Derived Delta-Valerobetaine in Aged Mice Occurs Through Disruption of Lipid and Lipid-Soluble Vitamin Metabolism

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Background and Purpose: Toxicologic mechanisms of low dose exposures that contribute to age-related disease remain poorly understood. Declining capacity to maintain homeostatic balance heightens susceptibility to both endogenous and exogenous stressors. Metabolism—an interdependent, multi-organ process—often becomes dysregulated with age, contributing to a wide-array of adverse health outcomes. Recently, the concept of microbiome-derived metabolites as modulators of host metabolism has gained much interest. We previously showed that microbiome-derived δ-valerobetaine (VB), a metabolite enriched in ruminant meat/dairy and produced by gut microbes, promotes weight gain and adiposity in young mice on Western diet. Significant alterations to carnitine shuttle pathway, critical in energy production and fatty acid metabolism, highlighted VB-induced metabolic disruption, providing evidence of a microbiome-derived effect on host metabolism. However, toxicities resulting from microbiome metabolites remain poorly established. Moreover, because such factors may rapidly enter circulation and can act across organs, there is a need to map coordinated, multiorgan metabolic responses. Here, we investigated how dietary exposure to VB affects adiposity and lipid-related metabolism in aged mice, with attention to observable sex differences. **Methods:** 21-month-old C57BL/6J mice on standard mouse diet received drinking water *ad libitum* without (vehicle control) or with 1.62mM VB infused for 12 weeks. Body weight and diet/water intakes were monitored weekly. Perigonadal white adipose tissue (WAT) and interscapular brown adipose tissue (BAT) were collected to assess adiposity. Plasma, liver, kidney, and heart tissues were extracted and analyzed by high-resolution metabolomics. MS features with significantly altered abundance (*limma* analysis, *p* < 0.05) were used for metabolic pathway enrichment analysis with *metformin* 2.2. Abundance of hepatic transcripts for cytochrome P450 enzymes relevant to lipid and vitamin metabolism (CYP2R1, CYP27B1, CYP27A1, CYP3A11) was assessed. Hepatic and plasma lipid contents were evaluated by Oil Red O staining and colorimetric assays for plasma triglycerides and cholesterol, respectively. **Results:** VB increased body weight and WAT:BAT ratio in aged female mice but not in aged male mice. Despite increased adiposity, hepatic steatosis and systemic triglycerides remained unchanged. Interestingly, VB levels were 65% (plasma), 41% (liver), 75% (kidney) higher in aged female mice than in male counterparts despite very similar weekly water intake. Untargeted metabolomics across plasma, liver, kidney, and heart revealed sex- and exposure-dependent metabolic differences. VB predominantly perturbed lipid pathways, including the carnitine shuttle, bile acid biosynthesis, glycosphingolipid metabolism, arachidonic acid metabolism, and fat-soluble vitamin (A, D, E) pathways. Aged females consistently showed perturbation of vitamin D3 metabolism across all organs examined, whereas aged males showed more consistent alteration in vitamin A metabolism. Targeted analyses showed decreases in metabolites related to vitamins A, D, and E, while levels of the parent vitamin forms were largely unchanged. Hepatic mRNA levels of CYP2R1, CYP27B1, CYP27A1, and CYP3A11 were unchanged, suggesting that VB negatively impacts vitamin bioavailability of these vitamins independent of canonical hydroxylase gene regulation. **Conclusions:** Collectively, VB exposure perturbs lipid-associated metabolic networks across multiple major organ systems in both aged male and female mice. VB differentially impacted females, however, by inducing weight gain and altering adipose tissue composition. VB also had more pronounced effects on vitamin D metabolism than observed in males. Vitamin D decrease is one of the hallmarks of aging in women with implication in loss of bone density and osteoporosis, which is far more prominent in women than in men. VB was more impactful in males on vitamin A metabolism. Vitamin A decrease is associated with decreased sperm development, which is also one of the hallmarks of aging in older men. These results identify VB as a microbiome-derived metabolite with a potential for contributing to age-related metabolic dysfunction in a sex-dependent manner.

PS 3656 Dose optimization of tilmicosin against *P. multocida* in swine by physiologically-based pharmacokinetic - pharmacodynamic Model

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Background and Purpose: Porcine respiratory disease complex causes substantial economic losses in intensive farming, with *Pasteurella multocida* as a key bacterial pathogen. While tilmicosin, a veterinary macrolide antibiotic, has been used to control these infections, its efficacy is increasingly compromised by antimicrobial resistance. This study aims to develop a physiologically based pharmacokinetic-pharmacodynamic (PBPK-PD) model to optimize tilmicosin dosing regimens against *P. multocida*, with the objective of restoring clinical efficacy and extending its clinical lifespan. **Methods:** A physiologically based pharmacokinetic (PBPK) model for tilmicosin in pigs was developed and validated using visual predictive

checks and mean absolute percentage error (MAPE). The model incorporated key tissues such as pulmonary interstitial fluid, liver, muscle, kidneys, and plasma. Using a hollow-fiber infection model, *ex vivo* PK-PD studies were conducted with a highly virulent strain of *P. multocida* selected at the MIC₉₀ level. These studies established PK-PD targets for bacteriostatic, bactericidal, and eradication effects based on drug exposure in plasma and pulmonary interstitial fluid. Corresponding dosing regimens were optimized for each therapeutic endpoint, and a Monte Carlo analysis based on the PBPK model was used to estimate the withdrawal period for the optimized regimen. **Results:** The developed PBPK model successfully characterized tilimicosin pharmacokinetics in key porcine tissues, including pulmonary interstitial fluid, liver, kidneys, and muscle. While the model showed slight overestimation of plasma terminal elimination, it demonstrated acceptable predictive performance overall. Based on hollow-fiber infection model studies, AUC/MIC was identified as the optimal PK-PD index. PK-PD targets in pulmonary interstitial fluid and plasma for bacteriostatic, bactericidal, and eradication effects were 12.13, 28.48, 51.08 h and 7.17, 46.54, 78.66 h, respectively. A 40 mg/kg oral dose administered over three days achieved favorable therapeutic outcomes, with Monte Carlo simulations predicting an 8-day withdrawal period for the optimized regimen. **Conclusions:** This study utilized an integrated PBPK/PD modeling approach to establish the PK-PD target parameters of tilimicosin against *P. multocida* in pigs. Based on these targets, an optimized clinical dosing regimen was developed and a corresponding withdrawal period was proposed, thereby providing a scientific basis for enhancing the clinical efficacy of tilimicosin and delaying the development of bacterial resistance.

PS 3657 Knowledge, Attitudes, and Practices of Pre-Service Teachers Toward Food Additives

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Background and Purpose: Food additives fall into two main groups: those intentionally added to improve the quality, taste, and appearance of food, and those unintentionally introduced during manufacturing, storage, or transport. While food additives provide clear technological and nutritional benefits, they may also cause health risks such as allergies, behavioral changes, and cancer. Previous studies examined knowledge, attitudes, and behaviors toward food additives among various populations, but little is known about pre-service teachers, who play a vital role in shaping public health awareness. This study aimed to assess the levels of knowledge, attitudes, and behaviors toward food additives among 96 pre-service teachers in a teacher-education college. **Methods:** A quantitative study was employed, using a computerized questionnaire developed specifically for the current study. The study tool was adapted from published and validated questionnaires (Osaili et al., 2023; Ingram, 2020; Bellad & Doyizode, 2017; Jagadeesan et al., 2017; Xue et al., 2021; Kna'an et al., 2022; Dongmo et al., 2023; Omatola, 2017; Oyewande et al., 2019). **Results:** Our findings indicate that females showed higher knowledge (M=0.45) and attitudes (M=0.38) than males (M=0.40; M=0.31). Singles demonstrated higher knowledge (M=0.45) but lower behavior (M=3.47) than married participants (M=0.40; M=3.64). Participants aged 36+ exhibited the highest levels (knowledge M=0.55; attitudes M=0.50; behavior M=3.69). Language majors had higher knowledge (M=0.56) and behavior (M=4.11), while science majors showed more positive attitudes (M=0.54). Fourth-year students reported higher scores across all variables. Druze participants presented higher knowledge (M=0.52) and attitudes (M=0.44), while Muslims demonstrated higher behavior (M=3.59). Positive correlations appeared between knowledge and behavior (r=0.170) and between attitudes and behavior (r=0.177). **Conclusions:** These findings highlight the importance of taking educational initiatives to enhance knowledge, attitudes, and informed behavior toward the benefits and potential harmful effects of food additives among future teachers in charge of education our future generations.

PS 3658 Risk Assessment of Mycotoxin Exposure Through Plant-Based Meat Alternatives from Spain and Portugal

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Background and Purpose: Plant-based meat alternatives (PBMA) are gaining popularity as sustainable substitutes for conventional meat products. However, their complex composition (based on cereals, protein isolates, legumes, spices) may increase the presence of fungal metabolites such as mycotoxins. This study aimed to perform a risk assessment of dietary exposure to twenty mycotoxins analyzed in PBMA from Spain and Portugal. **Methods:** A total of 100 PBMA samples (burgers, sausages, nuggets, and mince) were analyzed, and mean concentrations were used to estimate the Estimated Daily Intake (EDI) for the adult population. Exposure was calculated using two consumption scenarios: an average consumer (42.8 g/day; 70 kg b.w.) and a high consumer (100 g/day; P95). Risk characterization was performed using EFSA reference values (TDI, MOE, or TTC) depending on the toxicological profile of each mycotoxin. **Results:** Results showed frequent detection

of emerging mycotoxins such as moniliformin (MON), tentoxin (TTX) and enniatin B (ENNB), whereas regulated mycotoxins like DON, HT-2/T-2 and ZEN were not detected. For most compounds, hazard quotients (HQ) and percentages of TTC were below 1, and margins of exposure (MOE) exceeded 10,000, indicating no concern for health. For the average consumer, only aflatoxin B1 (AFB1) and aflatoxin G2 (AFG2) showed a possible risk, while AFB1, AFG2 and ocratoxin A (OTA) showed MOE values approaching the limit of potential concern in the high-intake scenario. **Conclusions:** The estimated exposure to mycotoxins through PBMA does not represent a health risk for Spanish and Portuguese adults under current consumption patterns. Continuous monitoring and extension of data to other European markets are needed. **Acknowledgements:** This work was supported by the Conselleria d'Educació, Universitat I Ocupació from Generalitat Valenciana under Grant CIAICO2022/199; and FCT/MCTES under Grant UIDB/50006/2020 DOI 10.54499/UIDB/50006/2020 through national funds. Llorens, P. would like to acknowledge the pre-PhD scholarship program of the University of Valencia "Atracció de Talent". **References:** EFSA Panel CONTAM. (2011). *EFSA Journal*, 9(12), 2481. European Commission, Health and Food Safety Directorate-General. (2024). *Guidance on the risk assessment of metabolites produced by microorganisms used as plant protection active substances*. European Food Safety Authority (EFSA). (2012). *EFSA Journal*, 10(3), 2605. European Food Safety Authority (EFSA). (2016). *EFSA Journal*, 14(4), 4425. European Food Safety Authority (EFSA). (2017). *EFSA Journal*, 15(4), 4751. European Food Safety Authority (EFSA). (2017). *EFSA Journal*, 15(9), 4718. European Food Safety Authority (EFSA). (2020). *EFSA Journal*, 18(3), 6040. European Food Safety Authority (EFSA). (2020). *EFSA Journal*, 18(5), 6113.

PS 3659 ITALIAN POPULATION UPDATED RISK ASSESSMENT FOR ACRYLAMIDE AND MYCOTOXINS FROM MARKETED BISCUITS

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Background and Purpose: Italy is one of the European countries with the highest per capita consumption of biscuits, and these foods can become contaminated throughout their production stage, either with acrylamide (AA) formed during cooking (classified as 2A by the IARC and with neurotoxic effects) and mycotoxins, fungal secondary metabolites with harmful effects on health. Knowing the occurrence of these contaminants in marketed foods is essential for assessing population exposure, and therefore risk. This study evaluated biscuits marketed in Italy to determine the occurrence of mycotoxins and AA, using data from the INRAN SCAI IV study on Italian food consumption and anthropometry, updating their risk assessment. **Methods:** A total of 34 samples of biscuits marketed in Italy were analyzed by QuEChERS and UHPLC-Q-Orbitrap HRMS to determine their AA and mycotoxin content (trichothecenes, aflatoxins, zearalenone, ochratoxin A, enniatins and beauvericin). Based on median and P₉₅ biscuit consumption and anthropometric data, the estimated daily intake (EDI) was calculated and used to calculate the margins of exposure (MoE) for AA, and the percentage of the tolerable daily intake or threshold of toxicological concern (% of TDI or TTC) for mycotoxins, in order to assess risk. Four exposure scenarios were modeled using the maximum, minimum, median, and average contaminant levels. **Results:** AA was detected in all samples, and mycotoxins co-occurred in 97%. DON appeared in 68% of samples, ZEA in 6%, BEA in 38%, and at least one ENN in all but one sample. AA levels ranged from 1, B, and B1) between consumption.. **Conclusions:** In this study, the naturally occurring AA and mycotoxins contamination was analyzed in 34 samples of biscuits sold in Italy. Although no sample exceeded the maximum or benchmark levels established by European legislation, a significant number of samples showed co-occurrence of several analyzed food contaminants. The risk assessment revealed a potential hazard for cancer-related effects (MoE <10,000) with median consumption and average levels of AA found, especially among children (aged 1 to 17) of both sexes. In addition, a 100% exceedance of TTC for BEA was observed in children of both sexes (1 to 9 years) with the highest recorded consumption. This is the first study, to the authors' knowledge, to evaluate the co-occurrence of AA and mycotoxins in biscuits samples sold in Italy, and to assess the risk using the most recent consumption data of Italian population. The potential genotoxic effects observed highlight the importance of continuous monitoring of contaminants in products marketed. **Acknowledgements:** This research was funded by MICIU/AEI/10.13039/501100011033/FEDER, UE through the project PID2024-1605270B-I00. The authors thank also the support provided by the ON Foods Project - Research and innovation network on food and nutrition Sustainability, Safety and Security - Working ON Foods, funded under the National Recovery and Resilience Plan (NRRP), PE000000003 CUP D93C22000890001, Mission 4 Component 2 Investment 1.3 - of March 15, 2022 of Italian Ministry of University and Research funded by the European Union-NextGenerationEU. V.Z. acknowledges support from the Santander Postdoctoral Research Grant, developed under the General Collaboration Agreement between the University of Valencia and Banco Santander, S.A.

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Background and Purpose: Tropane alkaloids (TAs) are naturally occurring plant toxins that can contaminate food and feed, leading to recent poisoning incidents. The European Food Safety Authority (EFSA) established a group acute reference dose (ARfD) of 0.016 µg/kg body weight for (-)-hyoscyamine and (-)-scopolamine, while the Joint FAO/WHO Expert Committee on Food Additives derived a point of departure (PoD) of 1.54 µg/kg body weight. Reported levels of TAs in food may exceed these reference values. This study aims to generate comparative data for multiple TAs to prioritise the most potent compounds and determine which should be included for future monitoring by the UK Food Standards Agency (FSA). A four-tiered framework was developed to address this need. Here we report results on Tier 1 and early Tier 2. **Methods:** Tier 1 included literature and database searches, *in silico* identification of molecular initiating events (MIEs), and potency prediction through QSAR modeling. Tier 2 focuses on *in vitro* cytotoxicity and mechanistic testing, Tier 3 on multi-omics analysis to establish molecular PoDs, and Tier 4 on quantitative *in vitro-in vivo* extrapolation (QIVIVE) to derive health-based guidance values. For Tier 1, SMILES structures of approximately 30 TAs were analyzed using DrugEngine (AIA Insights) to predict relevant MIEs and affected pathways. MIEs with credibility scores above 0.65 across all compounds were selected for further evaluation. Regression QSAR models were developed to predict potency against the most relevant MIEs using publicly available data and applied to prioritise a subset of TAs to be tested *in vitro* in tier 2. **Results:** Computational analysis identified mAChRs as the key MIEs associated with TA toxicity, consistent with their known antimuscarinic mode of action. A dataset of ~6100 chemicals with published *Ki* values for muscarinic acetylcholine receptors (mAChRs; M1-M5) from BindingDB supported development of regression QSAR models, which were generated and validated following OECD principles and applied to rank-potency across the 30 TAs. Only compounds with predicted $-\log(Ki) \geq 7.0$ for at least one mAChR were acquired to be tested. Ten TAs were prioritized for *in vitro* evaluation in Tier 2: atropine, scopolamine, 2-hydroxymethylatropine, aposcopolamine, noratropine, anisodine, convolvine, phylalbine, O-acetylscopolamine, and norscopolamine. Tier 2 testing commenced in SH-SY5Y neuronal cells, assessing cytotoxicity (AlamarBlue and SYTOX), mitochondrial membrane potential (MMP; Mitotracker), and intracellular calcium dynamics. Cytotoxicity was observed only at higher concentrations for aposcopolamine, convolvine, and O-acetylscopolamine. Significant MMP reductions occurred for most TAs at high concentrations, except scopolamine (no effect) and noratropine, anisodine, convolvine, and phylalbine (weak or non-significant effects). No increases in intracellular calcium flux were observed, aligning with their action as mAChR antagonists. Further tests and integrative analysis across Tier 2 are underway to refine relative potencies and select candidates for Tier 3 evaluation. **Conclusions:** A four-tiered framework has been developed to assess the toxicological potential of TAs. Findings from Tier 1 were consistent with the known antagonism of mAChRs. Further, predictive regression QSAR models were used to select 10 TAs to be acquired and tested *in vitro*. Early Tier 2 work results were reported, although further *in vitro* tests and an integrative analysis of all tier 2 results are still underway. The reported approach integrates computational modeling, initial mechanistic screening, and extrapolation methodologies to contribute to the UK FSA biomonitoring of the tropane alkaloids to protect the health of consumers.

PS 3661 **Apportionment of Food Safety Guidance Values: Practical Applications for Food Ingredient and Contaminant Exposures**

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Background and Purpose: The chemical safety of food impacts all consumers, and the application of toxicological concepts to determine safe exposure levels is a fundamental aspect of food chemical safety assessments. Safe exposure values for intentionally or unintentionally present chemical components in food are established with consideration of total dietary intake. A common misunderstanding in food chemical safety is the misapplication of these values to a single food rather than the whole diet. For example, aspartame, a nonnutritive sweetener used in numerous foods, has an FDA-established acceptable daily intake (ADI) of 50 mg/kg body weight/day, intended for cumulative intake from all sources. Lead (Pb), an unwanted contaminant found in many foods, provides another example. The FDA has set an interim reference level (IRL) for dietary exposure to Pb from foods at 2.2 µg/day for children, reflecting total dietary intake. **Methods:** Using the entire IRL for a single product or consumption occurrence is inappropriate; instead, the dietary contribution of the food product to the overall diet, as determined by US National Health and Examination Survey (NHANES) and What We Eat in America (WWEIA) data, guides the portion the ADI for aspartame and IRL for Pb to be used in safety assessments, ingredient use levels, or for setting science-based specifications in food products

or ingredients. **Results:** A case study is presented as an example associated with the misapplication of the ADI for aspartame in food safety assessment and demonstrates the appropriate approach to apportioning the ADI for beverages to determine appropriate use levels for ingredients such as aspartame. A second case study is presented as an example for the occurrence of Pb in a food ingredient intended for use in food for young children and demonstrates the appropriate approach to apportioning the IRL for Pb. **Conclusions:** Case studies for aspartame and Pb illustrate the necessity of apportioning safe exposure values to ensure appropriate safety assessments and specifications for food products and ingredients.

PS 3662 **development of improved tlc and hplc-dad methods for identification and quantification of quillaia extract type 2**

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Background and Purpose: Quillaia Extract Type 2 is a natural food additive mainly used as an emulsifier, obtained by aqueous extraction and further purification steps of the bark of *Quillaia saponaria* MOLINA. However, the analytical methods described in various national food additives standards, including the Korean food additives code, CODEX Alimentarius, and Japan's specifications and standards for food additives, lack sufficient specificity for reliable identification and quantification. Therefore, this study aimed to improve the analytical methods for Quillaia Extract Type 2 by optimizing thin-layer chromatography (TLC) and high-performance liquid chromatography with diode array detection (HPLC-DAD). **Methods:** For the identification test, TLC was applied to separate the characteristic saponins of Quillaia Extract Type 2. Various ratios of chloroform, methanol, water, and acetic acid were tested to determine optimal solvent conditions. For the quantification test, the extract was acid-hydrolyzed and analyzed by HPLC-DAD using quillaic acid as a standard compound. **Results:** The optimal TLC system was established as chloroform:methanol:water:acetic acid (20:7.5:2:1, v/v/v/v), which produced four distinct saponin spots within an *R_f* range of 0.1-0.5. The developed HPLC-DAD conditions provided accurate quantification of quillaic acid derived from the hydrolyzed extract. Method validation was performed to verify the analytical procedures, and the results satisfied the Codex Alimentarius criteria, satisfying the coefficient of determination ($R^2 \geq 0.99$), intra- and inter-day accuracy (recovery; 80-120%), and precision (RSD; $\leq 22\%$). **Conclusions:** The optimized TLC and HPLC-DAD methods improved the identification and quantification of Quillaia Extract Type 2. These methods enhance analytical reliability by improving the resolution of overlapping spots or peaks and can serve as a foundation for revising international food additive standards related to Quillaia Extract Type 2.

PS 3663 **Human Exposure to FD&C Red No. 3: Maximum Dietary Intake Assessment Using Latest NHANES Data and Toxicological Context**

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Background and Purpose: FD&C Red No. 3 is a synthetic color additive permitted for use in foods in the European Union and Canada. The acceptable daily intake (ADI) is 0.1 mg/kg/day; derived by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Food Safety Authority (EFSA) from a no-observed-adverse-effect level (NOAEL) of 1 mg/kg/day from a 14-day human study. FD&C Red No. 3 is used to produce a cherry-red color in various foods and beverages. A color additive petition submitted to the U.S. Food and Drug Administration (FDA) in 2022, included data that showed the development of non-malignant thyroid follicular adenomas in male rats fed high doses of FD&C Red No. 3 for 30 months. Although the FDA has long been aware of this finding, since the finding lacks biological relevance to humans, the use of FD&C Red No. 3 as a color additive was approved by the FDA. Despite the FDA reporting that "the available data does not raise safety concerns for humans", the Delaney Clause (FD&C Act; Chapter VII, section 721) prohibits the addition of compounds found to be carcinogenic in humans or animals to foods or pharmaceuticals. There is no acknowledgement in the Delaney clause that some tumor types and mechanisms of carcinogenesis are not relevant to humans, as it was enacted prior to modern understanding of carcinogenesis (*i.e.*, in 1958). Thus, on 15 January 2025, the FDA was bound by the Delaney clause to revoke the authorization of FD&C Red No. 3 as a color additive in foods and pharmaceuticals. Food producers were provided with a deadline of 15 January 2027 to reformulate their products. The objective of this work is to determine the maximum dietary intakes based on the recent U.S. National Center for Health Statistics' National Health and Nutrition Examination Survey (NHANES) data using a conservative approach. **Methods:** Concentration data and types of foods containing FD&C Red No. 3 as a color additive were obtained from the U.S. FDA and other public sources. Concentrations of FD&C Red No. 3 levels range from <1 to 2.4% in various foods. These data were used in conjunction with food consumption data included in the NHANES 2011-2023 to estimate dietary exposures. Intakes for the consumer-only mean and 90th percentile intakes of FD&C Red No. 3 were determined on an absolute (per person) and per kilogram body weight basis for the total population (ages 2 years and older). The mean and 90th percentile intakes were compared to the ADI for FD&C Red No. 3, as determined by JECFA and EFSA (*i.e.*, 0.1 mg/kg/day). **Results:** Total population (ages 2 years and older) mean and 90th percentile intakes of FD&C Red No. 3 were deter-

mined to be 9.0 and 16.6 mg/person/day, respectively (equivalent to 0.2 and 0.3 mg/kg/day, respectively). These intake estimates are 0.2 mg/kg/day higher than the ADI of 0.1 mg/kg/day. Additionally, when compared to the 2016 exposure assessment conducted by the FDA, the mean and 90th percentile intakes were reported as 3.7 and 4.2 mg/person/day (body weight results not reported), respectively, which is notably lower than the current dietary assessment. **Conclusions:** Conservative assumptions were used which may explain the higher intakes: food products were assumed to contain FD&C Red No. 3 at the maximum specified use levels however, the use levels will vary depending on the nature of the food product; food codes that have or likely have FD&C Red No. 3 were selected using the latest NHANES dataset whereas the 2016 FDA publication used an older dataset; and as the NHANES dataset does not indicate if certain food dyes are present in a food code, when selecting food codes for inclusion a conservative approach was taken in which generic or broad food products were all included (unclear if a berry/red flavored product actually contains FD&C Red No. 3 or an alternative red color). The results of the current exposure assessment, which can be considered as worst-case based on the conservative assumptions used, exceeded the ADI by 3-fold but are below the human NOAEL of 1 mg/kg/day. As the current intake estimates were based on many conservative assumptions, it is anticipated that typical intakes would be below the established ADI. Adverse toxicological effects on thyroid hormone levels in humans, which differ from male rats and was observed at 200 mg/kg/day, are not expected to occur at 0.3 mg/kg/day, which is a worst-case estimate.

PS 3664 Development of Supplemental Intake Limits for Nutrients

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Background and Purpose: With increasing use of dietary supplements, including those containing nutrients, it is important to determine levels that may be safely consumed. The Council for Responsible Nutrition (CRN) previously derived upper levels (ULs) for supplemental intake for 28 nutrients. The objectives of the current work were: 1) to update the safety evaluation methodology to provide an enhanced systematic approach to identifying and evaluating available data and 2) to update or derive new ULs in healthy adults for 21 nutrient forms commonly consumed in dietary supplements based on this methodology (calcium, iron, magnesium, potassium, selenium, zinc, vitamin B6, vitamin E, menaquinone-7 (vitamin K2), vitamin A (pre-formed), lutein, zeaxanthin, meso-zeaxanthin, choline, citicoline, folic acid, methylfolate, nicotinic acid, nicotinamide, nicotinamide riboside, and inositol hexanicotinate). **Methods:** The CRN approach based on its principal points of departure for risk analysis was employed, which included a focus on human data from clinical trials (when available); direct evaluation of supplemental intakes (rather than total intakes, when possible); derivation of ULs based on true hazard (vs. nuisance effects); and conservative selection of human NOAEL values that justify selection of an uncertainty factor (UF) of 1.0. For each nutrient, systematic searching and screening of human clinical trials were performed. Data from epidemiological studies were assessed when necessary to evaluate a potential hazard or endpoint of concern. Studies meeting the inclusion criteria were reviewed and data extracted. The critical effect and associated toxicity value (e.g., NOAEL), if available, were identified. The supplemental UL was derived by application of UF(s) to the toxicity value. History of use data were used, if necessary, to identify a highest observed intake (HOI) when adverse effects in humans were not identified. **Results:** A standard operating procedure was developed to ensure systematic and transparent searching and screening of human clinical trials. Upper intake limits for nutrients derived by global regulatory and authoritative bodies were reviewed and summarized within each nutrient update for context. New or revised supplemental ULs/HOIs in adults were derived for all 21 nutrient forms. For example, the UL for vitamin B6 was maintained at 100 mg/day based on human clinical studies in which no adverse effects occurred; excess vitamin B6 (pyridoxine) is associated with adverse neurological effects (e.g., neuropathy) at higher intake levels in human studies. Osmotic diarrhea related to unabsorbed magnesium was identified as the critical endpoint for magnesium. The UL for this nutrient was increased from 400 mg/day to 500 mg/day based on a lack of adverse effects in clinical trials at this dose; however, the magnesium UL does not apply to those with known risk factors related to kidney impairment. HOIs for three xanthophyll carotenoids were also derived based on the lack of any adverse effects across all clinical trials identified: lutein (22 mg/day), zeaxanthin (26 mg/day), and meso-zeaxanthin (17 mg/day). **Conclusions:** This work is part of an overall project to update supplemental ULs for 14 vitamins, 4 minerals, and 10 trace elements previously published by CRN, as well as newly derived supplemental ULs for additional vitamin forms and bioactives. These methods and the resulting ULs/HOIs are valuable tools for manufacturers to guide formulations and can be used internationally by policy makers and authoritative bodies in scientific discussions about setting upper intake levels for nutrients.

PS 3665 Health Promoting Antioxidant activity of Rosemary Extract (*Salvia Rosmarinus*) in Food Preservatives

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Background and Purpose: Rosemary (*Rosmarinus officinalis*) extracts are increasingly utilized as natural antioxidants and antimicrobials in food systems, driven by clean-label trends and the need to replace synthetic preservatives. The efficacy of Rosemary extracts depends on factors such as plant maturity, extraction method, concentration of phenolic compounds, and interactions with other components. These extracts have demonstrated potential applications in meat products, oils, dressings, and functional foods, as well as pharmaceutical and plant-based products. While uses of rosemary extracts have been determined to be safe and cost-effective, challenges remain regarding solubility, sensory impact, variability in active constituents, and regulatory compliance. Formulation strategies, including encapsulation and synergistic blending, are often required to optimize performance. This review presents the antioxidant and antimicrobial properties of rosemary extracts, their safety data, and generally recognized as safe (GRAS) regulatory framework governing the use of natural preservatives in foods and highlighting their role as viable ingredient for food preservation. **Methods:** A comprehensive search strategy was implemented by using PubMed. For this review, a series of searches were conducted to thoroughly assess and compare literature, each utilizing the search key term "Rosemary" combined with the search terms "preservative," using the Boolean operator "AND." We also included "Salvia Rosmarinus" AND "Food." These terms were chosen due to the FDA recognizing rosemary as generally safe as a natural preservative in foods. A majority of peer reviewed studies were, published between 2006 and 2025. Exclusion criteria included studies published in languages other than English, and those outside the specified time frame. Of the 30 studies identified, 24 studies met the criteria for inclusion. The focus was the use of Rosemary extracts in food. We acknowledge potential limitations such as selection bias, lack of generalizability in case studies, and lack of full-text manuscripts that could hinder results. However, despite these limitations, this approach ensures a comprehensive review of current literature on Rosemary extracts in food. **Results:** In summary, Rosemary extracts demonstrate notable antioxidant and antimicrobial properties, making it a valuable natural option for food preservation. Regulatory approval in the EU followed comprehensive safety evaluations, confirming that its NOAEL range poses no significant health risks. Research indicates that the majority of Rosemary extracts antioxidant activity is attributed to its carnosic acid (CA) and carnosol (CL) content, which serves as a benchmark for ensuring consistent efficacy and safety in food applications. Furthermore, the antimicrobial effects of Rosemary extracts and Rosemary essential oils enhance preservation by inhibiting bacterial and fungal growth, reinforcing their role as multifunctional preservatives. **Conclusions:** Rosemary extracts offer a promising approach to natural food preservation because of their bioactive compounds that help prevent spoilage and inhibit microbial growth. In addition to their preservative function, research indicates they may support gastrointestinal health. These findings suggest that the gut health benefits of rosemary extract provide an added advantage when using it as a natural preservative in food products.

PS 3666 Antioxidant, anti-obesity, and anti-aging effects of *Melicoccus bijugatus* seed extract in *Caenorhabditis elegans*

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Background and Purpose: The increasing prevalence of age-associated diseases such as obesity and other chronic disorders linked to oxidative stress poses a major global health concern. Oxidative stress, resulting from an imbalance between the generation of reactive oxygen species (ROS) and the body's antioxidant defenses, plays a central role in cellular damage, accelerates aging, and contributes to the onset of metabolic diseases. Consequently, the identification and development of new therapeutic and preventive agents capable of alleviating oxidative stress, combating obesity, and slowing the aging process have become pressing scientific priorities. These biologically complex substances obtained from plants, fungi, and marine life form a vast reservoir of chemically diverse and bioactive compounds. Because age-related diseases are driven by multiple interconnected biological pathways, natural extracts and their purified constituents offer significant potential for multi-targeted treatments that can simultaneously modulate inflammation, metabolic imbalance, and cellular senescence. Harnessing this immense natural diversity is essential for the discovery of innovative drugs with improved efficacy and potentially fewer adverse effects compared to synthetic alternatives, ultimately advancing both preventive and therapeutic medical approaches. In this study, the aqueous and ethanolic extracts of *Melicoccus bijugatus* seeds were evaluated using *Caenorhabditis elegans* as an *in vivo* biological model. **Methods:** *M. bijugatus* seeds were collected in Bolívar, Colombia (María la Baja), under the relevant environmental permit. Seeds were washed, dehusked, frozen and freeze-dried for 72 h. The pulverized seed material was subjected to ultrasound-assisted maceration using water and chromatographic-grade ethanol as solvents for 24 h. The solvent was removed using a rotary evaporator. Thin-Layer Chromatography was used with specific reagents to detect alkaloids, tannins, flavonoids, triterpenes, saponins, quinones, and cardiotonic glycosides. Gas Chromatography coupled with Mass Spectrometry was used for a detailed chemical profiling. *C. elegans* were maintained on NGM agar plates

with *E. coli* OP50 and synchronized using an alkaline hypochlorite/sodium hydroxide solution to obtain an adult-only population. For lethality assay, L4-stage worms were exposed to 0.1, 1, 10, and 100 ppm of extract for 24 h. Survival was monitored by physical stimulation, comparing results against an ultrafiltered water control. For antioxidant activity assay, worms were pre-treated with the ethanolic extract for 48 h, then exposed to hydrogen peroxide 5 mM. Survival was monitored for 5 h. Lipid accumulation was quantified using Oil Red O (q-ORO) staining. Worms were subjected to three different extract concentrations, involving pre-exposure to the extract, co-exposure with sucrose, or exposure only to the combination, followed by sucrose exposure. Orlistat was used as a positive control. The staining intensity was analyzed via optical microscopy. For lifespan assay, nematodes were treated with the extracts and 5-fluoro-2'-deoxyuridine was added to inhibit reproduction. Worm survival was monitored daily until the death of the last organism, and the data was used to generate a survival curve. **Results:** The extracts showed no basal toxicity, confirming their safety within the experimental system. When exposed to pro-oxidant agents, the ethanolic extract provided significant and dose-dependent protection, with the 10 ppm concentration being the most effective. Furthermore, lipid quantification using q-ORO staining evidenced a reduction in sucrose-induced lipid accumulation, indicating a preliminary anti-obesity effect. Longevity analyses revealed a favorable trend toward increased survival in the treated nematodes, suggesting a potential anti-aging effect. **Conclusions:** Collectively, these results position *M. bicuspidatus* as a promising source of metabolites with antioxidant, anti-obesity, and anti-aging properties, with potential relevance in the prevention of metabolic diseases and the physiological deterioration associated with oxidative stress.

PS 3667 Mutagenic Potential of Higenamine in Cultured V79 Cells

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Background and Purpose: Higenamine (HIG), also known as norcoclaurine, is found in widely available sports and weight-loss supplements. However, there is limited information on HIG's safety. Recently, when exposed to mouse and human liver microsomes and recombinant human CYP enzymes, HIG was observed to oxidize into ortho-quinone and quinone methide. These biotransformation products of HIG might cause DNA damage and lead to mutations. Consequently, this study investigated the mutagenic potential of HIG in cultured V79 cells using the hypoxanthine-guanine phosphoribosyltransferase (HPRT) assay (detecting primarily gene mutations) and the micronucleus (MN) assay (detecting chromosome and genome mutations). **Methods:** V79 cells were exposed to HIG for 4 h both in the absence and presence of S9 fraction derived from livers of β -naphthoflavone/phenobarbital-treated male rats (S9). In the HPRT assay, V79 cells were treated with HIG concentrations ranging from 150 to 423 μ M, 1% DMSO, and the positive controls 4-nitroquinoline-N-oxide (NQO, -S9) and benzo[a]pyrene (BaP; +S9). To assess cytotoxicity, the plating efficiency was determined immediately after treatment. In the MN assay, V79 cells were seeded onto slides and exposed to three HIG concentrations (249-423 μ M), solvent (1% DMSO), and positive controls patulin (PAT; -S9) and BaP (+S9) for 4 h, followed by a compound-free post-incubation period of 16 and 24 h. After staining of DNA and immunostaining for centromere-associated proteins (to distinguish MN containing whole chromosomes and chromosome fragments) and α -tubulin (to evaluate midbodies and mitotic spindles), cells were analyzed by fluorescence microscopy for micronuclei formation, mitotic index, spindle structure, cell death, and recent cell divisions. **Results:** As expected, the positive controls significantly induced 6-thioguanine-resistant mutants in the *Hprt* locus, while HIG did not significantly affect mutant frequency either without or with S9, across a concentration range that caused no reduction up to 68% (-S9) and 61% (+S9) reduction in plating efficiency. In the MN assay, treatment with the highest concentration of HIG resulted in a significant decrease in the frequency of recent cell divisions and the mitotic index by up to 93% immediately after exposure. However, cell proliferation resumed during the post-incubation period. As expected, MN containing whole chromosomes as well as chromosome fragments were observed after treatment with PAT, while BaP mainly induced MN containing chromosome fragments. After treatment with HIG (-/+S9) followed by a 16-h HIG-free post-incubation period, a concentration-dependent increase in MN frequencies from 4.6 to 39.8 (without S9) and from 6.1 to 64.3 (with S9) per 1000 cells. The effect's magnitude decreased at 24 h post-incubation but remained significant. **Conclusions:** HIG did not induce gene mutations at the *Hprt* locus but did induce chromosome mutations *in vitro*. Thus, the mutagenic potential of HIG requires further investigation and should be considered in the safety evaluation of dietary supplements containing HIG.

PS 3668 A combined chemoproteomics and 3D molecular modeling approach identifies small GTPases as potential target proteins of the mycotoxin Ochratoxin A

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Background and Purpose: Ochratoxin A (OTA) is an abundant mycotoxin and food contaminant that presents a health concern due to its nephrotoxicity and renal carcinogenicity in rodents. Although recent findings suggest a causal link between mitotic aberrations, genetic instability and replication stress induced by OTA, the molecular initiating event responsible for these effects remains unclear. To fill this knowledge gap critical for OTA risk assessment, we applied a chemoproteomic strategy to identify direct molecular targets of OTA and characterize early key events in the mechanism of OTA carcinogenicity. **Methods:** OTA and OTB were covalently bound to amino-functionalized agarose beads via their carboxyl group to create an affinity matrix. Lysates obtained from HK-2 renal epithelial cells were incubated with OTA- and OTB-functionalized beads to enrich for OTA- and OTB-binding proteins. Captured proteins representing putative OTA/OTB target proteins were subsequently identified by tandem mass spectrometry. Molecular docking and molecular dynamics simulations were performed to study mycotoxin-protein complex stability over time. **Results:** Our chemoproteomics approach identified several members of the small GTPase superfamily as most significantly enriched OTA and OTB interacting proteins, including representatives of all subfamilies such as RhoA, Ran, Rab5, Sar1a and K-Ras. These small GTPases act as central regulators of essential cellular functions, including vesicular transport and budding (Rab and Arf), nuclear import (Ran), cytoskeletal organization (Rho), as well as cell cycle regulation (Ras) and cell division, thus offering plausible links to key events thought to be critical in OTA genotoxicity. Molecular docking and molecular dynamics simulations predicted OTA binding within the conserved GDP/GTP binding sites of small GTPases, with complex stability over toxicodynamically relevant timeframes comparable to complexes with the natural ligands GTP/GDP. **Conclusions:** Taken together, these findings highlight small GTPase as primary molecular targets of OTA and offer new mechanistic hypotheses that link small GTPases to cellular effects observed in response to OTA.

PS 3669 Complex *Alternaria* toxin mixtures: an underestimated risk for consumer safety?

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Background and Purpose: Since the discovery of aflatoxins in the 1960s, more than 300 fungal metabolites have been characterized as so-called „mycotoxins“. In 1985, the FAO estimated that about 25% of the global food crop are contaminated with mycotoxins. Since then, global climate change has impacted the occurrence spectrum of respective fungi and, at the same time, improvement in analytics has enabled milestones in sensitivity. Nowadays, estimations speculate up to a contamination rate of 90%, at least in trace amounts. In addition to the few regulated mycotoxins such as aflatoxins or ochratoxin A, now numerous additional secondary metabolites can be detected. For most of these potentially „emerging“ mycotoxins data on occurrence and/or toxicity are not sufficient yet for comprehensive risk assessment. Moreover, recent studies demonstrate that mycotoxins are rarely found as single toxins but often occur in mixtures, thus further challenging risk characterization. A special challenge is represented by *Alternaria* mycotoxins with more than 70 compounds out of different chemical classes reported so far. Only a limited number of secondary metabolites formed by *Alternaria* fungi are commercially available and thereby accessible as reference material and test compounds for hazard characterization. **Methods:** Toxicity-guided fractionation combining the alkaline comet assay in HT29 colon carcinoma cells with LC-MS/MS analysis was used to identify genotoxic impact constituents. Key compounds were identified, isolated as single constituents and individually tested. Subsequently, a respective mixture of characterized single toxins was used to determine whether the genotoxic impact of the naturally occurring mixture can be achieved. Finally, a gastromimetic approach was used to determine the proportion of genotoxic *Alternaria* toxins expected to reach the intestinal tissue for absorption. **Results:** Toxicity-guided fractionation demonstrated that the genotoxic potential of naturally occurring *Alternaria* toxin mixtures is strongly imprinted by the presence and structural composition of perylene quinones formed by the fungus. Altertoxin-II (ATX-II) and stemphytoxin III (STX-II) were identified as potent genotoxic mutagens. Both compounds possess a reactive epoxide moiety and can form DNA adducts. But due to the reactivity of the epoxide moiety, reactions with food matrix might already occur during food processing or digestion. Indeed, the addition of ATX-II to tomato-based matrix showed a substantial loss of free and detectable amount of ATX-II during gastromimetic analysis. In contrast, non-epoxide analogues of the perylene quinone class formed in quite considerable amounts by several *Alternaria* strains, altertoxin-I (ATX-I) and alterperyleneol (ALP) were only marginally affected by digestive processes and are therefore expected to reach the small intestine to be absorbed. Both compound were found to possess moderate genotoxic properties but induce comparably potent immunosuppressive effects. **Conclusions:** *Alternaria alternata* is able to grow on a broad spectrum of substrates and growth conditions. But the resulting toxin pattern strongly depends

on the strains, substrates and environmental factors. Further studies are urgently needed to clarify the occurrence of perylene quinones formed by *Alternaria* species in food and feed.

PS 3670 Early Glycation Products Modulate Glucose Homeostasis and Gut Microbiome and Produce Sex-Specific Behavioral Changes in Murine Models of Type 2 Diabetes and Neurodevelopmental Disorders

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Background and Purpose: Glycation products form when proteins or fats react with sugars, and they are widespread in modern diets. Advanced glycation end products produced from prolonged or high-temperature cooking have been linked to negative health effects and increased risks of diabetes. The physiological effects of early glycation products (EGPs) generated through controlled reactions, however, remain much less understood. It is hypothesized that EGP consumption can beneficially influence metabolic health, gut microbiota, and behaviors related to brain function. To test this hypothesis, three complementary mouse studies were conducted.

Methods: In the first study, male and female C57BL/6 mice were fed a Western diet and daily given EGPs (600 mg/kg) or non-reacted (NR; glucose and whey protein only) controls by gavage over six months. Body weight, blood glucose, and food intake were recorded weekly, while glucose tolerance (GTT) and insulin tolerance (ITT) tests were performed monthly. The gut microbiome was analyzed using 16S rRNA gene sequencing. The second study employed both Western diet-fed male and female BTBR-mtB6 mice, a model for autism spectrum disorder, dosed daily with EGPs, NR, or water for six months. In addition to the above endpoints, behavioral tests were conducted for grooming and three-chamber sociability. In the third study, male and female C57BL/6 mice were placed on a Western diet and treated with vancomycin (0.5 g/L in drinking water) to deplete gut microbiota, then administered water, NR, or EGPs (600 mg/kg/day) by gavage for six months. In addition to the above endpoints, behavioral tests included the Y-Maze (spatial working memory), Open Field (locomotor and anxiety-like behaviors), Novel Object Recognition (recognition memory), and Tail Suspension (depression-like behavior). **Results:** EGP-treated C57BL/6 mice demonstrated significant improvements in glucose homeostasis compared to controls, evidenced by lowered blood glucose levels, reduced insulin resistance and beneficial shifts in the gut microbial community. Female C57BL/6 mice also showed reductions in body weight. In BTBR-mtB6 mice, EGP supplementation improved social behaviors in both sexes: females showed increased social interactions, while males exhibited higher social behavior index scores than controls. Additionally, males had significantly improved glucose tolerance and insulin sensitivity with EGP treatment. In the antibiotic-treated C57BL/6 mice, EGPs decreased body weight gain and led to substantial improvements in glucose tolerance and insulin sensitivity, especially among females. EGP treatment also enhanced recognition and spatial memory, and decreased anxiety- and depression-like behaviors, with more pronounced behavioral improvements observed in female mice. **Conclusions:** Across the three murine models, EGPs consistently produced sex-specific improvements in glucose metabolism, neurobehavioral outcomes, and gut microbiota profiles. These findings suggest that early glycation products have potential as dietary interventions for the management of metabolic and neurodevelopmental disorders. The beneficial effects observed may be mediated by both gut microbiome-dependent and independent mechanisms. Continued investigation into EGPs as functional dietary components is warranted. This project was supported by NIH R41DK121553 and partly supported by the Interdisciplinary Toxicology Program at UGA and the USDA National Institute of Food and Agriculture [grant no. 2016-67021-24994/project accession no. 1009090].

PS 3671 Distinct production of advanced glycation endproducts by the microbiome of Crohn's disease patients and healthy controls

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Background and Purpose: Crohn's disease (CD), a subtype of Inflammatory Bowel Disease (IBD), is a chronic relapsing-remitting inflammatory condition and is associated with an altered intestinal microbiota composition. Diet is known to play a role in the onset and development of CD, e.g. by exposure to inflammatory compounds and/or via the intestinal microbiota. One of the dietary factors of interest are advanced glycation endproducts (AGEs), which form during the non-enzymatic glycation of amino acid residues. AGEs can form during cooking, but can also be formed endogenously and be produced by the microbiome. AGEs, and an increased expression of their receptor RAGE, have been associated with IBD. Which specific AGEs are involved and what the contribution of the intestinal microbiome is, is so far not clear. The current study therefore investigates AGE production by the fecal microbiome of CD patients and healthy controls, to identify specific AGEs associated with CD. **Methods:** Fecal samples (1% w/v in simulated ileal efflux medium, containing casein and cysteine but no carbohydrates) from four CD patients and four healthy controls, matched for age, BMI and smoking status, were incubated at 37°C in a well-con-

trolled anaerobic environment. These samples were incubated with one of four added carbohydrate sources (1% w/v): glucose, fructose, galacto-oligosaccharides (GOS), or fructo-oligosaccharides (FOS). Moreover, a non-carbohydrate medium control was included. Samples were collected before (T0), after 4h (T4) and 24h incubation (T24). These samples were immediately diluted 1:10 in ice-cold PBS, sonicated in an ice-bath for 30min and spun down at 18000g for one hour. Supernatant was collected and used to determine the concentrations of 23 AGEs using UPLC-MS/MS on a C18 column and water as eluent. Incubations with the four carbohydrates were also performed without faeces, to correct for non-microbial AGEs formed in the medium. **Results:** Eight AGEs could be identified in the collected samples. All AGE concentrations (CMC, CML, CEL, CMA, MG-H, pyrraline and furosine) increased over time, ranging between 2- and 4-fold for most, up to a 44-fold increase for CMC. The exception was 2-SC, which showed a 2- to 6-fold increase at T4 and then a decrease to T24, for both CD and controls. Six of the eight individual AGEs (CMC, CML, CEL, CMA, pyrraline and 2-SC) were more abundant in the samples of CD patients as compared to the controls. Only MG-H was higher in control as compared to CD samples, whereas furosine was produced by the microbiome of one control sample only. Although differences in individual AGEs between groups were prominent, they were not statistically significant, possibly due to high intra-group variability. Exceptions were CEL after 24h incubation with glucose (mean difference in CD vs. controls: 103 µg/L, p<0.01) and MG-H after 24h incubation with fructose (mean difference in controls vs CD: 36.6 mg/L, p<0.05). Principal component analysis (PCA) revealed a significant clustering of AGEs based on CD status (p<0.001). PCA on AGE production stratified by carbohydrate source showed the most apparent separation between CD patients and healthy controls after FOS exposure, while AGE production profiles from medium only incubations were most similar between CD patients and controls. **Conclusions:** These results indicate that AGE production by the fecal microbiome is significantly different between CD patients and healthy controls, with pronounced interindividual variability within both groups. Interestingly, a subset of AGEs appeared to be produced at higher rates by CD samples. The effect of these on immunity and intestinal barrier function warrants further study. Future work will involve microbiome sequencing of the incubated fecal samples to provide insight into the bacterial taxa responsible for AGE production. Overall, these results show a distinct subset of AGEs produced by CD patients, indicating functional differences in the microbiome between CD patients and healthy controls.

PS 3672 Species specific responses of human, rat and rabbit hepatocytes to the next-generation refrigerant R-1132(E)

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Background and Purpose: In recent years, climate change associated with global warming has become a major worldwide concern. R-1132(E), trans-1,2-Difluoroethylene, is a promising next-generation refrigerant with an extremely low environmental impact, having a global warming potential (GWP) of <1. For the full-scale practical use of R-1132(E), a safety evaluation is indispensable. Previous animal studies showed the different responses among different species, rat, dog, and rabbit. Mortality was only observed in the rabbit after repeated exposure to R-1132(E). Since the liver is a critical organ for metabolizing chemicals, the hepatocyte responses might be different among different species. In this study, we conducted *in vitro* exposure tests of R-1132(E) gas using primary hepatocytes from three species, human, rat, and rabbit, to infer its effects on the liver, the organ responsible for detoxification. **Methods:** Cryopreserved primary hepatocytes from human, rat, and rabbit were thawed, cultured, and exposed to gas phase R-1132(E). A gas-tight Tedlar bag was used for gas exposure. One side of the bag was cut open, culture plates were placed inside, the opening was sealed with a heat sealer, and the bag was filled with gas at the concentrations of 0, 1k, 10k, 15k ppm for 5 days. Gas exposure began one day after cell seeding; gas and culture medium were replaced daily. The culture medium used had been pre-equilibrated with the gas since the previous day. Cells of 0 and 15k ppm were collected on Day 2 and Day 5 after exposure, and protein and RNA were extracted for proteomic and transcriptomic analyses. On Day 5, cellular viability/metabolic activity was assessed using the WST-8-based Cell Counting Kit-8 (CCK-8). **Results:** Microscopic evaluation of cell morphology showed no differences across gas concentrations in human hepatocytes. In rabbits, the cuboidal morphology characteristic of hepatocytes was preserved in the gas-exposed groups. In rats, gas exposure modestly promoted cell detachment and aggregation. However, the morphology changes in rats were not aligned with the cell viability. No statistically significant differences of cell viability (CCK8-assay) were observed in human or rat cells at all exposure levels during exposure. In contrast, rabbit cells in the non-exposed group (0 ppm) showed lower activity than the exposed groups (1k, 10k, 15k ppm), with significant differences particularly at 1k and 15k ppm on Day 5 after exposure. Proteomic and Transcriptomic analysis showed the different cellular responses after exposure among the three species. Rabbit hepatocytes exhibit the different responses compared to human and rat, especially in metabolism, inflammation and elimination. Proteomic analysis identified more than 7,000 protein groups in each species, providing sufficient depth for statistical analysis. Gene Ontology (GO) and pathway analyses comparing the R-1132(E)-exposed (15k ppm) versus non-exposed (0 ppm) groups revealed activation of immune responses, inflammatory responses, transcription factor-related pathways, autophagy, and cytoskeleton/migration across species. Analyses focusing on liver function-related proteins indicated minimal commonality

among species and pronounced interspecies differences. Notably, rabbits exhibited a marked and distinctive response compared with the other species, with strong upregulation of CYP2C isoforms, especially CYP2C3. Transcriptomic results were broadly consistent with the proteomic findings. **Conclusions:** At all tested exposure concentrations, no substantial toxicity indicative of acute hepatocellular death over a period of several days was observed in any species, suggesting that the acute hepatotoxicity of R-1132(E) is low. The omics analyses indicate a high likelihood of R-1132(E) exposure related effects on hepatocellular metabolic functions, immune responses, and inflammatory pathways in all three species. However, the responses in rabbit were different compared to human and rat. Given the substantial interspecies differences observed, caution is warranted when extrapolating animal data to humans, as variations in drug-metabolizing enzymes may yield different metabolites.

PS 3673 Establishing a quantitative and advanced *in vitro* test system to evaluate chemical-induced liver fibrosis

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Background and Purpose: Integrated Approaches to Testing and Assessment (IATAs) are developed to provide safety information about chemicals and eliminate animal testing. Chemical-induced liver fibrosis is characterized by activated hepatic stellate cells that accumulate extracellular matrix proteins which is a key event within the adverse outcome pathway (AOP) for fibrosis. Currently, no IATA is available for liver fibrosis, and therefore, this study aims to systematically develop a quantitative and advanced *in vitro* test system to evaluate chemical-induced liver fibrosis.

Methods: Four monoculture liver test systems (primary human hepatocytes (PHH), immortalized cell lines HepG2 and HepaRG, and human induced pluripotent stem cell (hiPSC)-derived hepatocytes-like cells (HLC)) in 2D and 3D, and two coculture primary cell models in 3D (3D InSight™ Liver Microtissues, InSpherO) have been evaluated on predictivity of acute liver toxicity and fibrosis. All test systems were treated with chemicals known to induce fibrosis: allyl alcohol, thioacetamide, and monocrotaline. After 24h single or repeated exposure across a concentration range, cytotoxicity was determined, and whole transcriptome analysis was performed using TempO-Seq technology. **Results:** The similarity in gene expression of the systems compared to the human liver was established using the liver-specific gene expression panel (LiGEP) algorithm. This showed that primary cell systems were most comparable to the human liver, followed by HepaRG, HLCs, and HepG2, respectively. Moreover, 3D format resulted in higher LiGEP similarity compared to 2D. primary cells showed the highest expression of CYP enzymes, while HepaRG had increased expression of ADH genes. Transcriptomics analysis provided mechanistic insight into the test systems' capability to identify fibrosis development after exposure. Known stressor pathways, such as NRF2 and HSF-1, were activated in PHH and HepG2 upon exposure. Cocultures of primary cells showed additional immune response module activation, serving as the second key event. Under repeated exposure conditions, ECM-related modules were perturbed, showcasing the late key event. **Conclusions:** This shows the importance of the inclusion of non-parenchymal cells for the detection of liver fibrosis-related key events and gene networks. Therefore, our study will further focus on implementing the non-parenchymal cells (e.g., hepatic stellate cells and Kupffer cells) using hiPSC-derived organoids or human-primary-cell-derived spheroids. The development and characterization of advanced liver models, including essential mature cell types, could aid in the recognition of early fibrosis and will therefore drastically improve the prediction of liver toxicity. This work has received funding from Partnership for the Assessment of Risks from Chemicals (PARC) (grant agreement No 101057014).

PS 3674 Co-regulated gene network analysis to uncover mechanisms of liver toxicity liabilities *in vitro*

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Background and Purpose: Next-generation chemical risk assessments require mechanism-based approaches that integrate human-relevant new approach methodologies (NAMs) in order to protect human health and ensure readiness for future regulatory needs. High-throughput transcriptomics has emerged as an ideal platform to effectively capture early biological responses resulting from chemical exposures in human *in vitro* test systems. However, in-depth understanding on the preservation of adverse responses across different test systems, as well as the interpretation of *in vitro* transcriptomic data remain major hurdles for regulatory implementation. Considering the liver as a predominant target for chemical-induced toxicities, our aim is to improve liver toxicity prediction by employing co-expression network analysis across multiple test systems. These networks facilitate biological inter-

pretation of transcriptomic changes and enable evaluation of response concordance, thereby supporting the definition of applicability domains for each system.

Methods: In order to build co-expression networks, toxicogenomics datasets were generated for differentiated HepG2 cultured in amino acid-rich medium (AAGLY-HepG2), HepaRG, and primary human hepatocytes (PHH). Based on the previously established human hepatocyte TXG-MAPr (Callegaro et al., 2021), the minimum required set of compounds for a preserved gene network was determined and used for the generation of new toxicogenomic datasets. The resulting exposure library consisted of 39 hepatotoxic compounds, 8 reference compounds for specific stress response pathways, and 4 growth factors and 4 cytokines to trigger other endogenous signaling pathways of interest. All compounds were tested at three concentrations, and samples were collected for TempO-Seq at 8 and 24 hours after exposure. Differential gene expression analysis was employed using the DESeq2 package. Based on the differentially expressed genes, Weighted Gene Correlation Network Analysis (WGCNA) was used to construct co-expression networks. On these WGCNA networks, preservation analysis using Z-summary statistics was performed to identify robust modules. Also previously established HepG2, PHH, and human induced pluripotent stem cell-derived hepatocyte-like cells (hiPSC-HLCs) TXG-MAPr tools available from <https://txg-mapr.eu/> were taken along for the cross-test system preservation analysis. **Results:** WGCNA networks from PHH TG-GATes data and newly obtained PHH TempO-Seq data displayed ~75% module preservation. Among these preserved modules were important stress-response related modules enriched for unfolded protein-response and inflammatory pathways. Between normal HepG2 and AAGLY-HepG2, we found that ~80% of modules were preserved. HepaRG showed the highest preservation to 'golden standard' PHH, followed by AAGLY-HepG2, HepG2, and lastly hiPSC-HLC. Among the preserved modules, UPR is generally well captured across the different test systems, as well as heat shock and oxidative stress responses. **Conclusions:** A WGCNA-based data-reduction approach effectively captures the preservation of transcriptomic perturbations induced by chemical exposure across different test systems. This framework enables identification of conserved stress-response signatures and provides an overview of the applicability domains of different liver *in vitro* test systems.

PS 3675 Comparative Analysis of Species-Specific Hepatocyte Function and Drug Effects in Static Monolayers, Spheroids, and a Perfused Microphysiological System

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Background and Purpose: Drug-induced liver injury (DILI) remains a major challenge in drug development, highlighting the need for reliable *in vitro* tools to assess hepatotoxicity and improve translation from animal to human studies. Models using hepatocytes from preclinical species are also needed to evaluate species-specific toxicity.

Methods: In this study, we evaluated the basal performance of primary hepatocytes from human, monkey, rat, and dog in several *in vitro* liver platforms, including static monolayers, static microspheroids (Elplasia ULAP), and the microfluidic TissUse HUMIMIC Chip2 microphysiological system (MPS). Cells were maintained for 14 days, and cultures were characterized using functional (albumin, urea) and DILI (AST, LDH) biomarkers. In addition, we tested the effects of several species-specific DILI compounds: chlorpromazine (CPZ; toxic across species), bosentan (BOS; human-specific), and flialuridine (FIAU; human-specific); these were dosed every second day starting on day 6 until the end of the experiment. **Results:** Human hepatocytes in the HUMIMIC Chip2 showed gradually increasing albumin and declining urea over time. These patterns were similar in the static monolayer and spheroid cultures. In hepatocytes from other species, we observed overall declines in these biomarkers. Rat hepatocytes performed comparably across systems, while monkey and dog cells showed slightly higher biomarker levels in the HUMIMIC Chip2. In monolayer hepatocyte cultures, CPZ and BOS induced significant cytotoxicity in human and monkey hepatocytes, as indicated by increased LDH and AST and reduced albumin production. No effects of CPZ or BOS were observed in rat and dog hepatocytes. FIAU treatment resulted in a significant dose-dependent decrease in albumin production in human hepatocytes. In monkey hepatocytes, a significant increase in albumin was observed. Liver injury markers, AST and LDH, were increased in human cells, but in monkey cells, only LDH was increased. No effects were found in either rat or dog hepatocytes on any of the biomarkers. In static microspheroids, CPZ and BOS resulted in dose-dependent decreases in albumin in human and monkey hepatocytes. LDH and AST increases were also observed in human and monkey hepatocytes. Effects on dog and rat hepatocytes were inconsistent. FIAU responses mirrored monolayer results, limited to human and monkey cells. LDH and AST were unchanged, and no effects were observed on rat and dog hepatocytes. In HUMIMIC Chip2 perfused microspheroids, only FIAU was tested due to the limited throughput of this MPS. In human hepatocytes, FIAU reduced albumin and other biomarkers, but variability and low sample size prevented statistical significance. Responses in other species were variable. **Conclusions:** Across platforms, human and monkey hepatocytes demonstrated the highest functional stability and sensitivity to known DILI compounds, while rat and dog hepatocytes showed limited or inconsistent responses. The TissUse HUMIMIC Chip2 supported comparable or slightly improved hepatocyte function relative to static models, though variability and low throughput limited statistical power. FIAU, BOS, and CPZ produced species- and compound-spe-

cific hepatotoxic responses consistent with known *in vivo* outcomes, supporting the translational relevance of these *in vitro* systems. Overall, integrating multi-species hepatocyte models across static and microphysiological platforms provides a promising approach for comparative DILI assessment and preclinical-to-clinical translation.

PS 3676 Preliminary evaluation of donor variability in albumin secretion for fit-for-purpose applications of primary human hepatocytes

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Background and Purpose: Primary human hepatocytes (PHHs) are widely used in toxicology, drug discovery, and advanced microphysiological system (MPS) applications. Albumin secretion is a key marker of hepatocyte function; however, donor-to-donor variability may affect experimental outcomes and influence the suitability of PHHs for different applications. This proof-of-concept study provides preliminary data on albumin secretion across PHH donors as a potential fit-for-purpose guide for use in MPS and ADME-TOX applications. **Methods:** Cryopreserved PHHs from multiple donors were thawed and cultured either as 2D monolayers on collagen-coated plates or self-assembled 3D spheroids. Albumin secretion was quantified over time using BioAnalyzer (2D hepatocytes) or ELISA (3D spheroids). Donors were categorized into higher- and lower-secreting groups based on internal reference ranges, and QC metrics (viability, attachment, CYP activity) were compared. Four representative donors (two per group) were further evaluated for acetaminophen (APAP) cytotoxicity in both formats, with ATP-based assays used to generate dose-response curves and EC₅₀ values. **Results:** PHHs maintained stable morphology for up to 5-7 days (2D) and 3 weeks (3D). Albumin secretion levels (N=26 lots) enabled donor stratification into high and low secretors; no consistent correlation was observed with viability or CYP activity. Both groups exhibited time- and concentration-dependent ATP loss upon APAP exposure in 2D and 3D cultures. EC₅₀ values after 72 h repeated exposure were comparable between groups and formats. Importantly, at least one high-secreting donor has been externally validated in an MPS application, further supporting the link between albumin secretion capacity and suitability for long-term advanced models. **Conclusions:** Both high- and low-secreting donors performed reliably in routine cytotoxicity assays. High-secreting donors above a reference cutoff may be preferentially suited for long-term applications such as NAMs, MPS platforms, and chronic ADME-TOX studies, while lower-secreting donors remain fit-for-purpose for short-term testing, acute toxicity screening, mechanistic pathway studies, and exploratory ADME applications.

PS 3677 Targeted Lipidomics as a High-Throughput New Approach Method for Mechanism-Based Assessment of Drug-Induced Liver Injury In Vitro

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Background and Purpose: Traditional toxicity testing is limited in throughput and mechanistic characterization, underscoring the need for cell-based new approach methodologies. Incorporating mechanistic readouts, such as lipidomics, can enhance the translational relevance of hepatocyte-based assays; however, the addition of this analysis to *in vitro* studies has its limitations. For example, traditional untargeted lipidomic analyses are time-consuming and may also require greater sample volumes. Therefore, this study focused on developing a targeted lipidomics method that can be used to characterize drug-induced liver injury-related lipid responses in small volumes from *in vitro* studies. **Methods:** Lipid species characteristic of normal hepatic function and those associated with adverse drug responses were identified and used to establish the analytical target list. A focused lipid profiling assay targeting 181 lipid species was conducted using LC-triple quadrupole MS in positive electrospray ionization mode with multiple reaction monitoring. Next, primary hepatocytes from human, rat, monkey, and dog were cultured in collagen I-coated 96-well plates and exposed to known human hepatotoxins that act via different mechanisms of action: chlorpromazine (1, 10, and 30 μ M), bosentan (10, 30, 100, and 200 μ M), and flialuridine (1, 10, 30 and 100 μ M). Cells were exposed to test compounds every other day from culture day 4 to day 8. **Results:** We found that test compounds not only resulted in changes in biochemical markers of liver injury (increases in alanine and aspartate aminotransferases and a decrease in albumin production) but also elicited compound- and species-specific lipid perturbations. Chlorpromazine exposure had significant effects on lipid profiles in all species; specifically, increases in diacylglycerols were observed in human, rat, and monkey, but not in dog hepatocytes. Triglyceride levels exhibited species-dependent patterns, increasing in human and monkey hepatocytes, decreasing in dog hepatocytes, and showing chain length-dependent alterations in rat cells. Bosentan exposure induced ceramide accumulation in human hepatocytes, providing insight into a mechanism underlying its human-specific hepatotoxicity. Flialuridine resulted in few effects on lipid profiles, potentially reflecting the low sensitivity of this method for the detection of mitochondrial toxicity. **Conclusions:** The targeted lipidomics platform proposed herein enables high-throughput, mechanism-based characterization of compound-induced lipid responses in cultured hepatocytes. This approach provides a strategy for

mechanistic toxicological evaluation, comparative studies across different species, and demonstrates the potential of lipidomics as a mechanistic complement to traditional *in vitro* hepatotoxicity phenotypes.

PS 3678 Evaluation of Drug-Induced Liver Injury in Microphysiological Human Liver-on-Chip Models

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Background and Purpose: Drug-induced liver injury (DILI) remains a major challenge in drug development, often resulting in late-stage drug withdrawals, substantial financial losses, and serious risks to patient safety. Conventional preclinical models, such as two-dimensional (2D) cell cultures and animal models, frequently fail to predict human-relevant hepatotoxicity due to their limited cellular complexity, lack of physiological perfusion, species-specific differences, and inability to capture immune-mediated mechanisms. To overcome these limitations, we leveraged three-dimensional (3D) human cell-based microphysiological liver models with varying levels of cellular complexity and compared their performance to that of conventional 2D monocultures and co-cultures. In these models, acetaminophen (APAP), trovafloxacin (TVX), and levofloxacin (LVX) were tested to evaluate their ability to reproduce clinically relevant hepatotoxic responses. **Methods:** Liver models for APAP exposure were assembled using upcyte human hepatocytes and liver sinusoidal endothelial cells (LSECs), forming a co-culture liver-on-chip system. APAP was tested exclusively in this model at clinically relevant concentrations (0-10 mM) for up to 7 days. For TVX and LVX, a more complex, triple-cell model was used, incorporating HepaRG hepatocytes, LSECs, and monocyte-derived macrophages (MDMs) as Kupffer cell surrogates to recreate multicellular liver microenvironments with integrated immune function. These drugs were perfused at clinically relevant concentrations (0-20 μ M) for up to 7 days. Endpoint readouts included immunofluorescence and live-cell imaging to assess morphology and toxicity markers, alongside cell viability comparisons with conventional 2D cultures. Effluents from hepatic and vascular channels were analyzed for lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and cytokine release. Mechanistic analyses examined glutathione depletion and mitochondrial reactive oxygen species (ROS) formation to investigate the underlying mechanisms of DILI. **Results:** APAP induced concentration-dependent hepatocellular and vascular toxicity exhibited by reduced cell viability, elevated LDH release, and disrupted morphology at supraphysiological concentrations. Perfused liver-on-chip models showed enhanced sensitivity compared with 2D mono- or co-cultures. TVX exposure caused pronounced hepatic and vascular tissue damage at clinically relevant concentrations, accompanied by glutathione depletion, mitochondrial ROS formation, and pro-inflammatory cytokine release. Similar to *in vivo*, cytokine secretion was dependent on a multicellular immune response, highlighting the potential of the liver model for detecting idiosyncratic immune-mediated toxicity. Similar to APAP treatment, TVX-induced liver injury was more pronounced in the liver-on-chip compared to 2D cultures. In contrast, LVX exposure caused no toxicity. These results underscore the importance of perfusion, cellular complexity, and immune components in these 3D liver-on-chip models for accurately assessing hepatotoxicity. **Conclusions:** Collectively, we have demonstrated that 3D liver-on-chip models exhibit higher sensitivity than 2D cultures in detecting DILI for the tested drug compounds. Importantly, we were able to reproduce TVX-induced liver injury and elucidate associated toxicity mechanisms, which were not observed in mice studies without inflammatory co-stimulation. These findings underscore the potential of human liver microphysiological models as predictive *in vitro* platforms for evaluating drug-induced liver injury. By addressing the limitations of conventional animal models, this approach may improve the detection of undesirable drug-induced adverse events and reduce unnecessary animal use during preclinical testing.

PS 3679 A Translational 3D Spheroid Assay for Preclinical Assessment of Drug-Induced Liver Injury

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Background and Purpose: Drug-induced liver injury (DILI) remains a major challenge in drug development, often leading to compound attrition, post-market withdrawal, and usage restrictions. While traditional 2D hepatocyte cultures are limited in their physiological relevance and longevity, 3D liver spheroid models provide a more *in vivo*-like microenvironment, enabling sustained hepatocyte function and chronic toxicity assessment. In this study, we established a 3D spheroid model using primary human hepatocytes to better predict human DILI risks. **Methods:** Primary human hepatocytes were cultured in an ultra-low attachment platform to form 3D spheroids. Functional validation included monitoring CYP enzyme activity, albumin and urea secretion, and LDH release over four weeks. Transporter functional investigation was performed using benzbramarone (BSEP inhibitor) and PSC833 (P-gp inhibitor), with substrate accumulation measured via Choly-Lys-Fluorescein and Calcein-AM,

respectively. For hepatotoxicity screening, 110 drugs with known clinical DILI classifications were tested. The margin of safety (MOS) for each drug was calculated based on the ratio of human maximum plasma concentration (C_{max}) to the EC₅₀ derived from viability assays. **Results:** The 3D hepatocyte spheroids maintained functional stability throughout the 4-week culture period, as demonstrated by sustained CYP enzyme activity, stable urea production, consistent albumin secretion, and LDH release. Functional transporter studies showed that benzobromarone (a BSEP inhibitor) significantly increased bile acid accumulation without evidence of mitochondrial toxicity. Similarly, PSC833 (a P-gp inhibitor) enhanced intracellular retention of Calcein-AM, confirming P-gp transporter activity. Multiparameter analysis using mBCL (GSH content), Sytox Green (DNA damage), and Mitotracker Deep Red (mitochondrial mass) revealed compound-specific injury patterns following drug treatment. Using a margin of safety (MOS) cut off of 50 for DILI prediction yielded 85.29% sensitivity for positive compounds and 76.92% specificity for negative compounds. **Conclusions:** The 3D primary human hepatocyte spheroid model demonstrates robust functional maintenance and enables comprehensive assessment of drug-induced liver injury. Through chronic exposure studies, this system successfully identified known hepatotoxic drugs. The implementation of an MOS threshold of 50 provided high predictive accuracy for clinical DILI. These findings establish the 3D spheroid model as a valuable tool for evaluating chronic drug toxicity and improving drug safety assessment.

PS 3680 Untargeted Lipidomics Reveals Mechanistic Signatures of Drug Induced Hepatotoxicity in Rat Hepatocytes

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Background and Purpose: Drug-Induced Liver Injury (DILI) remains a leading cause of drug withdrawal from the market. A thorough understanding of DILI mechanisms is critical for ensuring the safety of drug candidates. Predicting mechanism-based toxicity can aid in identifying potential adverse effects and optimizing drug design. However, evaluating hepatotoxicity *in vitro* and translating these findings to clinical outcomes remain challenging. Untargeted lipidomics offers a sensitive approach for comprehensively profiling biological changes, particularly when mechanistic knowledge is limited, making it a valuable tool for elucidating the toxic mechanisms of novel drugs. Primary hepatocytes are regarded as the gold standard for *in vitro* drug toxicity assessment, and rodent plated hepatocytes provide a cost-effective model that can be compared with *in vivo* data prior to clinical trials. **Methods:** Mechanism specific lipidomic changes were investigated in rat plated hepatocytes to explore pathways underlying drug induced hepatotoxicity. Primary hepatocytes were treated for 24 hours with twelve toxic and three non toxic reference compounds representing different mechanisms, including oxidative stress, apoptosis, mitochondrial injury, and cholestasis. Untargeted lipidomic profiling of nonpolar metabolites was conducted using high resolution liquid chromatography coupled with mass spectrometry. Processed data were analyzed using Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) to identify metabolite patterns associated with specific toxicity mechanisms. Key discriminatory metabolites were further evaluated through statistical testing and machine learning to identify potential biomarkers for predicting mechanism specific hepatotoxicity. **Results:** OPLS-DA revealed clear separation among hepatocyte nonpolar metabolites treated with apoptotic, mitochondria-disrupting, and cholestasis-inducing drugs compared with their respective non-toxic controls. Several metabolites showed significant changes even at low concentrations. Potential biomarkers for predicting drug toxicity were identified using machine learning. **Conclusions:** These findings demonstrate the potential of combining *in vitro* rat hepatocyte models with lipidomic profiling to predict mechanism-specific hepatotoxicity and provide early warnings of toxicity risks during drug development.

PS 3681 Phenotypic Profiling of Drug-Induced Liver Injury Using Cell Painting in 2D and 3D Human Hepatocyte Models

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Background and Purpose: Drug-induced liver injury (DILI) remains a critical challenge in pharmaceutical development, emphasizing the need for more comprehensive and physiologically relevant evaluation methods. While conventional 2D primary human hepatocyte (PHH) cultures are widely used for high-throughput toxicity screening, they suffer from rapid dedifferentiation and limited physiological relevance. In contrast, 3D PHH spheroid models exhibit a higher degree of physiological relevance to the native liver, maintaining long-term phenotypic stability and enhanced metabolic function. Conventional approaches in both systems often focus on viability endpoints, offering limited insight into cellular morphological perturbations. This study explores how the Cell Painting assay can complement established viability assessments in both 2D and 3D PHH models by capturing rich, high-dimensional morphological information. **Methods:** PHHs were cultured both as conventional 2D monolayers and as self-assembled 3D spheroids. Both models were exposed to a panel of FDA-categorized DILI compounds representing different concern levels, across eight concentrations spanning sub-cytotoxic to cytotoxic ranges. Cytotoxicity was assessed using the CellTiter-Glo (CTG) assay to determine IC₅₀

values. In parallel, the Cell Painting assay was performed using a multiplexed staining panel. High-content images were acquired on an Operetta CLS system, and quantitative morphological features were extracted to construct phenotypic profiles for both culture formats. **Results:** Both the CTG viability assay and the Cell Painting morphological assay revealed clear, concentration-dependent responses in the 2D PHH and 3D spheroid models. In general, the 3D spheroids exhibited different sensitivity profiles to the DILI compounds compared to 2D cultures. Cell Painting successfully captured phenotypic alterations that correlated with viability readouts in both models. Furthermore, it identified distinct, often more complex, morphological changes at sub-cytotoxic concentrations, with the 3D spheroids frequently showing a richer diversity of phenotypic features. Feature-based analysis in both models indicated compound- and concentration-dependent phenotypic patterns, suggesting potential differentiation among compounds with different DILI concern levels and provided complementary insights into hepatocellular responses to toxic exposure. **Conclusions:** The integration of Cell Painting with viability assessment offers a more informative and multi-dimensional framework for evaluating DILI. This combined methodology adds morphological context to conventional viability data, yielding a more comprehensive understanding of compound-induced toxicity in hepatocytes. By comparing 2D and 3D models, this study highlights the value of more physiologically relevant culture systems for capturing complex toxicological responses. It holds promise for improving early safety assessment by revealing subtle phenotypic perturbations that complement standard viability metrics.

PS 3682 Assessment of Primary Human Liver Microtissue Performance Consistency Across Donor Lots and Production Runs

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Background and Purpose: Hepatotoxicity remains a major cause of drug development failures and post-marketing withdrawals. To enhance the liver safety of drug candidates, researchers are increasingly integrating human-centric *in vitro* test systems into the drug development process. However, the use of the most physiologically relevant models is often constrained by issues of reproducibility, scalability, and high cost. In particular, the reliance on primary cells in these models introduces variability due to donor-to-donor differences. Manufacturing liver models using batches of primary cells pooled from multiple donors can help average out donor-specific differences, thereby reducing variability between primary cell batches. Culturing cells as spherical aggregates is well suited for standardized large-scale production and is particularly relevant for modelling globular organs, or organs composed of contiguous globular subunits, such as the liver. Liver microtissues are spherical aggregates composed of primary hepatocytes and a crude non-parenchymal cell fraction in physiological proportions. They are suitable for uniform, standardized production at scale in 96- or 384-well microplates and can be used both for high-throughput screening in early drug development and for more detailed mechanistic investigations conducted before or during clinical trials. **Methods:** The consistency of liver microtissue performance was assessed by comparing their volume, ATP content, drug metabolism, and inflammatory response across three different 10-donor liver cell lots, each produced in two independent manufacturing runs. The cytotoxic responses to 20 orally administered FDA-approved drugs were also evaluated twice for each cell lot. **Results:** Cytotoxicity dose-response curves for all twenty drugs were consistent across cell lots and production runs, yielding distinct ATP IC₅₀ values for each drug but similar values for the same drug across experiments. Liver microtissue volume, ATP content, and drug metabolism were also consistent across all three cell lots. **Conclusions:** Liver microtissues manufactured from 10-donor cell pools produced consistent results across different cell lots and production runs. Combined with their physiological relevance, miniaturization, and compatibility with scalable production and use, liver microtissues represent a promising analytical platform suited to the rigorous demands of industrial and regulatory applications.

PS 3683 Development of a human-relevant *in vitro* model of early-stage cholangiocarcinogenesis using HepaRG spheroids

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Background and Purpose: Despite its increasing incidence and poor prognosis, cholangiocarcinoma remains poorly understood. One known risk factor for intrahepatic cholangiocarcinoma (ICC) is chronic liver injury, which is often observed in chemical toxicity studies. Therefore, the development of new approach methodologies (NAMs) with high human relevance is essential for more accurate risk assessment. Recent evidence suggests that ICC may originate not only from cholangiocytes but also from progenitor cells or hepatocytes undergoing aberrant differentiation, a process thought to play a key role in early carcinogenic transformation. HepaRG cells, capable of differentiating into both hepatocytes and cholangiocytes, exhibit enhanced hepatic functionality in 3D spheroid culture compared with conventional 2D culture, making them a promising *in vitro* model for studying early events in human cholangiocarcinogenesis. This study aimed to determine whether aberrant differentiation observed in early rat ICC models could be replicated *in vitro* using primary rat hepatocyte (PRH) spheroids, and whether similar cellular responses could be modeled in

HepaRG spheroids to establish a human-relevant NAMs for cholangiocarcinogens. **Methods:** PRHs were isolated and cultured as spheroids using ultra-low attachment plates, followed by exposure to the cholangiocarcinogen thioacetamide (TAA) at concentrations of 0, 5, and 20 mM for 14 days. HepaRG spheroids were treated similarly. Samples from both models were collected on days 3 and 14, and cellular responses were analyzed by mRNA expression profiling and immunofluorescence staining, focusing on markers related to cell differentiation and proliferation. **Results:** In PRH spheroids, TAA exposure induced severe hepatocellular necrosis and down-regulation of hepatocyte markers, Albumin and Hnf4a. In contrast, cholangiocyte markers, Ck19 and Sox9, which were scarcely expressed in controls, were upregulated following TAA treatment, accompanied by the appearance of Sox9-positive hepatocytes. In HepaRG spheroids, TAA similarly suppressed hepatocyte markers and induced necrosis. Ck19-positive cholangiocytes expanded at the spheroid periphery, and cells co-expressing Sox9 and Hnf4a increased, suggesting a transitional phenotype. Gene expression analysis revealed that in PRH spheroids, differentiation-related genes *Yap1*, *Hes1*, and *Bmp4* were downregulated, while *Tgfb1* was upregulated. In contrast, all four genes were upregulated in HepaRG spheroids. Cell proliferation-related genes *Cyclin D1* and *Myc* were consistently upregulated in both models, while growth factor receptor expression showed model-specific patterns: *ErbB2* increased in PRH spheroids, whereas *Met* and *Egfr* were elevated in HepaRG spheroids. **Conclusions:** TAA exposure triggered hepatocyte-to-cholangiocyte phenotypic conversion in both PRH and HepaRG spheroids, highlighting aberrant differentiation as an early hallmark in cholangiocarcinogenesis. Distinct regulation of differentiation-related genes suggests species- and cell-type-specific mechanisms. The shared upregulation of proliferative markers along with increased growth factor receptor expression indicates common initiating events. These findings support the potential of HepaRG spheroids as a human-relevant NAM platform for predicting early cholangiocarcinogenesis.

PS 3684 Role of Hydrolysis in the Cytotoxicity of 3-(3,5-Dichlorophenyl)-2,4-thiazolidinedione (DCPT) and Its Metabolites in HepG2 Cells

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Background and Purpose: Troglitazone (TRO), a thiazolidinedione (TZD) antidiabetic drug, was withdrawn from the market due to severe hepatotoxicity. The TZD ring, a distinctive five-membered heterocycle shared by TRO and related compounds, was subsequently identified as a potential contributing factor to the observed toxicity. To investigate the mechanism behind TZD ring toxicity, we evaluated a structurally analogous compound, 3-(3,5-dichlorophenyl)-2,4-thiazolidinedione (DCPT), using human hepatoma HepG2 cells. In addition, DCPT hydrolysis was analyzed in the presence and absence of HepG2 cells over time using reverse-phase HPLC. **Methods:** The experimental design involved seeding HepG2 cells in 96-well plates, incubating them for 24 hours, and then treating them with DCPT and its potential hydrolysis products, S-(3,5-dichlorophenyl) aminocarbonyl thioglycolic acid (DCTA), 3,5-dichlorophenyl isocyanate (DPI), and mercaptoacetic acid (MAA), at with concentrations ranging from 0 μ M to 200 μ M in complete DMEM/F12 media. Hydroxyacetic acid (HAA), an analogue of MAA, was also evaluated. Cell viability was assessed by the Promega MTS cytotoxicity assay after 1, 4, and 24 hours. For monitoring DCPT hydrolysis, HepG2 cells were seeded in 96-well plates as above for 24 hours, and then treated with DCPT (200 μ M). Parallel DCPT treatment solutions were added to wells containing no cells to evaluate the possibility of spontaneous hydrolysis. Samples from the wells were collected at various time points ranging from 0 to 24 hours, and the presence of DCPT and its hydrolysis products was assessed by HPLC. Results were generated using GraphPad Prism (Version 10.2.3; GraphPad Software Inc. San Diego, CA) and are expressed as means $\pm$ standard deviations ($n=4-5$ for cytotoxicity experiments and $n=4$ hydrolysis experiments). The data were analyzed by a one-way ANOVA followed by a Dunnett's post hoc test and/or Student's t-test. Differences in the means were considered significant when $p < 0.05$. **Results:** MTS assay at 24 hours revealed concentration-dependent toxicity of DCPT (14.33% $\pm$ 2.92% viability at 200 μ M), DCTA (32.73% $\pm$ 17.73% at 200 μ M) and DPI (21.42% $\pm$ 8.85% at 200 μ M), while MAA and HAA exhibited no cytotoxicity. Loss of viability was not as pronounced at earlier time points. DCPT exhibited limited cytotoxicity at 1 hour and became more toxic at 4 hours (83.34% $\pm$ 7.42%). DPI produced severe cytotoxicity at both 1 hour (29.21% $\pm$ 11.06%) and 4 hours (23.37% $\pm$ 3.91%). Co-incubation of DCPT with either MAA or HAA produced similar losses of cell viability to DCPT alone. The HPLC data revealed that DCPT hydrolysis was evident as early as 30 minutes. By 4 hours, the majority of DCPT (83.34% $\pm$ 7.4%) had been hydrolyzed to DCTA. DCTA levels peaked at 2 hours and then slowly decreased over 24 hours. DPI was sporadically detected at 4 hours and became more prominent at 24 hours. Degradation of DCPT to DCTA and DPI did not appear to be different in the presence or absence of cells. **Conclusions:** These results suggest that individual treatment with DCPT, DCTA and DPI resulted in severe cytotoxicity in HepG2 cells at 24 hours. Since DCTA and DPI, two hydrolysis products, are formed rapidly and onset time of cytotoxicity is early, they may contribute to DCPT cytotoxicity in the HepG2 cells. The similar degradation pattern observed in media with and without cells indicate that DCPT hydrolysis proceeds largely spontaneously. Altogether, these findings demonstrate that DCPT toxicity in HepG2 cells may result, at least in part, from its spontaneous degradation to cytotoxic intermediates, providing insight into the role of TZD ring metabolism in hepatocellular injury.

PS 3685 3D Spheroid Formation of Rat and Human Cryopreserved Hepatocytes to Develop Model Systems for Liver Disease and Drug Development

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Background and Purpose: 3D liver spheroid models are fast becoming useful models for drug development and testing, due to their extended culture lifespan, metabolic stability, and compatibility with high-throughput assays. The goal of these studies was to assess spheroid formation and functional performance over 21 days in culture of cryopreserved primary hepatocytes from: 1) two strains of rat; 2) non-diseased human liver (Non-Alcoholic Steatohepatitis Activity Score (NAS) ≤ 3 ; and 3) diseased human liver (NAS ≥ 4). **Methods:** Cryopreserved rat hepatocytes (Wistar (RW; $n = 1$ lot, 8 donors) (ThermoFisher) and Sprague Dawley (RSD; $n = 1$ lot, 5 donors) (ThermoFisher)) or Primary Human Hepatocytes (PHHs) ($n \geq 2$ non-diseased donors and $n \geq 2$ diseased donors) were thawed, centrifuged, and counted. Cells were then resuspended in TruVivo® Flex Plating Medium (rat) or Human Hepatocyte Plating Medium (human) and seeded at 3,000 hepatocytes/well in a 96-well Nunclon Sphera™ U-shaped bottom plate. These plates were centrifuged at 100 g and placed in an incubator at 37°C/5% CO₂ for 5 days. Beginning on day 5, half volume media exchanges were performed every other day for the remaining culture period, using TruVivo Flex Culture Medium (rat) or Human Hepatocyte Culture medium (human). Albumin and urea levels were determined by ELISA (Abcam) and colorimetric biochemical assay (Stan Bio), respectively. Human Cytochrome P450 3A4 (CYP3A4) activity was measured using P-450 Glo kit (Promega). CellTiter Glo assay (Promega) was used to measure ATP levels. These assays were performed on days 7, 14, and 21. CDFDA (ThermoFisher) was used to stain bile canaliculi. Nile red staining (Abcam) for lipid was performed according to the manufacturer's instructions. **Results:** Spheroids formed with rat hepatocytes by culture day 7 and were maintained over a 21-day period. Urea levels in RW and RSD ranged from 875 $\pm$ 37.0 and 812 $\pm$ 52.6 μ g/10⁶ cells/day on day 7 to 236 $\pm$ 20.2 and 33.9 $\pm$ 5.84 μ g/10⁶ cells/day on day 21, respectively. ATP was measured in both strains on days 7 (RW: 23 $\pm$ 3.2 $\times 10^6$ RLU/10⁶ cells/mL; RSD: 2.0 $\pm$ 1.1 $\times 10^6$ RLU/10⁶ cells/mL) and 21 (RW: 7.5 $\pm$ 1.8 $\times 10^6$ RLU/10⁶ cells/mL; RSD: 1.8 $\pm$ 0.43 $\times 10^6$ RLU/10⁶ cells/mL). Both non-diseased and diseased PHHs formed spheroids by culture day 7 and had expected morphology for at least 21 days of culture. Albumin synthesis was similar between non-diseased and diseased spheroids on days 7 (76.4 $\pm$ 12.1 and 78.3 $\pm$ 8.00 μ g/10⁶ cells/day) and 21 (50.0 $\pm$ 28.2 and 71.7 $\pm$ 5.03 μ g/10⁶ cells/day). Urea levels for non-diseased and diseased PHHs ranged from 658 $\pm$ 41.1 and 439 $\pm$ 5.02 μ g/10⁶ cells/day on day 7 to 211 $\pm$ 34.4 μ g/10⁶ cells/day and not detected on day 21, respectively. No major differences were seen in CYP3A4 activity between non-diseased (day 7: 5.5 $\pm$ 1.0 nm/10⁶ cells/min; day 21: 27 $\pm$ 3.7 nm/10⁶ cells/min) and diseased spheroids (day 7: 6.8 $\pm$ 0.40 nm/10⁶ cells/min; day 21: 32 $\pm$ 7.5 nm/10⁶ cells/min) over time. ATP levels differed between non-diseased spheroids (day 7: 14 $\pm$ 1.9 $\times 10^6$ RLU/10⁶ cells/mL; day 21: 18 $\pm$ 1.9 $\times 10^6$ RLU/10⁶ cells/mL) compared to diseased spheroids (day 7: 10 $\pm$ 0.88 $\times 10^6$ RLU/10⁶ cells/mL; day 21: 7.6 $\pm$ 0.5 $\times 10^6$ RLU/10⁶ cells/mL). Human bile canaliculi networks formed within both types of human spheroids by day 7 and became more extensive over the culture period. Nile red staining showed steatosis in both normal and diseased donors. **Conclusions:** In conclusion, rat hepatocytes formed spheroids by day 7 and demonstrated functionality via urea production throughout the 21-day culture period. Although differences were seen between strains, additional lots need to be tested. Spheroids were also formed using non-diseased and diseased PHHs by day 7 that maintained albumin and urea function and CYP3A4 activity throughout the 3-week culture period. Although differences in urea and ATP levels were measured, it was unexpected to determine similar albumin levels along with CYP3A4 activity in spheroids derived from diseased versus non-diseased PHHs. In addition, bile canaliculi were detected, and lipids were detected from both non-diseased and diseased spheroids. Overall, these spheroids demonstrate characteristics that can be used to develop a model system for studies of liver disease and toxicity of drugs and chemicals.

PS 3686 Precision-Cut Liver Slices provide a human, immune-competent tissue model for mechanistic assessment of immune-related Drug-Induced Liver Injury

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Background and Purpose: Immune-related mechanisms in drug induced liver injury (irDILI) are pre-clinically understudied. Sitaxsentan, an endothelin-A and B receptor antagonist (ERA) was withdrawn from the market due to hepatotoxicity. The benefit of anti-inflammatory corticosteroid treatment during sitaxsentan-induced liver injury, particularly in severe cases, suggests immune-mediated mechanisms which are, however, still elusive. Here we aim to unravel early immune reactions to ERA and the well-known DILI APAP using human Precision-Cut Liver Slices (PCLIS) *ex vivo*. **Methods:** Immune competence of human PCLIS was characterized by flow cytometry and cytokine responses to anti-CD3/28, LPS, poly I:C and concavalin A. Sitaxsentan, ambrisentan, and APAP were tested for immune modulation using multiplex cytokine analysis and qPCR of inflammatory markers after 48h cultivation. Cytotoxicity was

assessed by LDH release, KRT18 and CDKN1a expression, while metabolic activity using WST-1 assay and CYP450 function (CYP3A4, CYP2C9) were measured as liver functionality parameters. **Results:** Flow cytometry revealed substantial immune cell presence (20-40%), dominated by T cells (66%, CD8/CD4 ratio 2.5) and 17% NKT cells. Human PCLiS secreted distinct cytokines upon cultivation with immune stimuli, confirming immune reactivity. Sitaxsentan (255 μ M) preferentially induced IL-12p70 (1.4-fold), IL-13 (1.13-fold), and IL-2 (1.07-fold), indicating Kupffer cell activation and potential NKT involvement. Ambrisentan (23.5 μ M) lacked this profile, consistent with its lower clinical toxicity. APAP induced IFN- γ response and at high doses reduced metabolic activity, with decreased *KRT18* and *CDKN1a* gene expression. ERAs enhanced CYP3A4 and CYP2C9 gene expression without cytotoxicity at tested concentrations. **Conclusions:** Our model reflects APAP-induced hepatitis across key events of AOP 362, while sitaxsentan-induced inflammation may involve alternative mechanisms. Early IL-12p70 secretion point to Kupffer cell activation, warranting further studies deciphering immune cell activation of liver tissue-resident cells. PCLiS provide essential mechanistic insights into immune-mediated hepatotoxicity, supporting translational preclinical safety assessment, enhancing human relevance, reducing animal use, and enabling mechanism-based prediction of DILI.

PS 3687 Microphysiological liver-on-chip integrating HepaRG™ cells and liver sinusoidal endothelial cells (LSECs) for drug-induced liver injury assessment

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Background and Purpose: Drug-induced liver injury (DILI) remains a leading cause of drug attrition and withdrawal, highlighting the need for more predictive *in vitro* liver models. Conventional 2D hepatocyte cultures often fail to reproduce the complex microenvironment and intercellular interactions that impact hepatic responses to xenobiotics. In particular, the crosstalk between hepatocytes and liver sinusoidal endothelial cells (LSEC) plays a crucial role in maintaining liver homeostasis, influencing metabolism, transport, and inflammatory responses. To better mimic these physiological features, we developed a microphysiological system (MPS) combining human HepaRG hepatocytes and primary LSEC under dynamic flow conditions. The aim of this study was to assess whether this co-culture model improves the prediction of drug-induced cytotoxicity compared to conventional static monocultures. **Methods:** The MPS chip consists of two channels: one containing HepaRG™ cells and the other containing LSECs, separated by a communicating hydrogel (20 mg/mL alginate solution). Hepatocyte differentiation was evaluated by immunofluorescent labeling of HNF4a and by assessing bile canaliculi formation using the fluorescent probe Fluobite™ 1. Albumin secretion was quantified by ELISA and labeled by immunofluorescence. To assess hepatotoxicity, cells were exposed to 2 mM APAP for 48 or 72 hours to mimic toxic stress conditions. Cellular viability was measured via LDH release, while oxidative stress and mitochondrial integrity were monitored using MitoTracker live staining. Activities of seven major CYP450 enzymes were determined by incubating cells with Xenomix™ CYP substrates and measuring substrate clearance by LC-MS/MS. In addition, CYP3A4 activity was assessed using a live staining approach. **Results:** HNF4a and albumin immunolabeling, together with quantitative measurement of albumin, demonstrated a high level of differentiation and functionality of HepaRG™ cells within the liver-on-chip model. This was further confirmed by the presence of bile canaliculi structures formed by HepaRG™ hepatocytes. Albumin secretion was significantly reduced following APAP exposure compared with control conditions, as shown by both ELISA quantification and immunofluorescence analysis. This decline reflects an impaired hepatic synthetic function, a hallmark of hepatocellular injury. A strong increase in LDH release was observed after 48 hours of exposure to 2 mM APAP, confirming cytotoxicity induced by APAP overload. MitoTracker staining showed a pronounced reduction in mitochondrial signal in treated cells, consistent with elevated oxidative stress and mitochondrial dysfunction, in line with the well-established mechanism of APAP-induced hepatotoxicity. CYP3A4 staining revealed a significant decrease in enzyme expression following APAP treatment, accompanied by increased signal heterogeneity and tissue disorganization. Similarly, enzymatic activity assays indicated a general reduction across multiple CYP450 isoforms in APAP-treated hepatocytes, further supporting the loss of metabolic competence under toxic stress conditions. **Conclusions:** We successfully established a co-culture liver-on-chip model integrating HepaRG™ cells and primary LSECs. The system effectively reproduced key features of APAP-induced liver injury, demonstrating its relevance for hepatotoxicity assessment. Future developments should focus on evaluating additional DILI-associated compounds and incorporating other hepatic cell types, such as Kupffer and stellate cells, to more closely recapitulate the hepatic microenvironment and enhance the physiological relevance of the system.

PS 3688 Systematic characterization of transcriptional reprogramming by non-genotoxic carcinogens after single or repeated exposure of hiPSC-derived hepatocyte-like cells

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Background and Purpose: Exposure to environmental chemicals is an important risk factor for cancer. The standard 2-year rodent bioassay limits the rapid and cost-effective evaluation of possible carcinogenic risk and may not represent human relevant mechanisms of chemical carcinogenesis. Both genotoxic as well as non-genotoxic carcinogenic can drive tumorigenesis. While genotoxic carcinogens are relatively easy to detect using non-animal approaches, the complexity of mechanisms and repeated dosing that drive non-genotoxic carcinogen (NGTxC) cancer formation creates challenges to predict carcinogenic risk using non-animal approaches. The liver is the most important target of chemical carcinogenesis. High-throughput transcriptomics allows the identification of the mode of action (MoA) of NGTxC for the liver. We utilized human-induced pluripotent stem cell (hiPSC)-based hepatocyte-like cell (HLC) cultures and systematically defined the potential differences in transcriptional reprogramming of HLCs after single 24 hr treatment or 8-day repeated exposure to six different chemicals. **Methods:** Six compounds with different modes of action were selected: cyclosporin A, nitrofurantoin, potassium bromate, ochratoxin A, phorbol 12-myristate 13-acetate (PMA), and etoposide. hiPSC-derived HLCs were treated at different concentrations for either 24 h (single exposure) or every 48 h for eight days (repeated exposure). Cytotoxicity was measured using propidium iodide staining at two time points through live-cell confocal imaging. Concentrations for transcriptomics analysis were based on the highest non-cytotoxic concentrations after 8-day repeated exposure. Samples were collected at 24 h and 8 days, and followed TempO-Seq targeted RNA sequencing. The DESeq2 package was applied for differential gene expression analysis. The data were further analysed using hiPSC-derived HLC TXG-MAPr application tool to identify up- and down-regulated gene modules to get deeper insights into the different responses from the two time points. **Results:** The transcriptomics data showed robust differentiations of hiPSCs into HLCs, maintaining transcriptomic stability over eight days of repeated exposure. Transcriptomic analysis revealed time- and compound-dependent responses: cyclosporin A showed a similar number of differentially expressed genes (DEGs) at both time points; nitrofurantoin, potassium bromate, and ochratoxin A exhibited a significantly increased number of DEGs and stronger concentration responses following repeated exposure, while etoposide and PMA showed decreased DEG numbers. Gene network activity analysis using the HLC TXG-MAPr provided more mechanistic insight into each compound at two time points. The module correlation analysis indicated that compound responses from the same time point overall clustered together. The regulations of gene network modules markedly differed among compounds. E.g., modules associated with endoplasmic reticulum stress were upregulated under cyclosporin A treatment, whereas potassium bromate induced oxidative stress-related modules. Comparing the module patterns of the same compound across two exposure time points, some modules exhibited consistent regulation at both time points, while some modules displayed unique regulatory patterns or even reversed after repeated exposure. The data indicate that the assessment of MoA for NGTxC characterization is highly dependent on the treatment scenario. **Conclusions:** hiPSC-derived HLCs represent a robust human *in vitro* test method to assess NGTxC MoA. 8-day repeated exposure uncovered altered and more pronounced transcriptomic responses with the selected compounds that were not apparent after single exposure. This study highlights that repeated exposure could provide more insightful toxicological profiles of compounds for identifying and characterizing NGTxC.

PS 3689 Trends in Intrinsic Clearance of Drugs in *In Vitro* Liver Test Systems: Insights from a CRO's Microsome and Hepatocyte Data

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Background and Purpose: Drug developers have long aimed to design molecules with low metabolic turnover. *In vitro* assays, such as metabolic stability studies, are commonly used as an initial approach to assess how rapidly compounds are metabolized and to estimate turnover rates to guide early drug development. We therefore used *in vitro* metabolism study data to examine whether there has been a shift over time toward drug candidates with lower turnover. **Methods:** To evaluate trends in metabolic turnover, we analyzed more than 400 sponsored metabolism studies conducted by XenoTech (now part of BioIVT) between 2000 and 2023, focusing on human liver microsomes and hepatocytes as test systems. Intrinsic clearance (CL_{int}), a key determinant of hepatic metabolism and systemic exposure, was used when reported or calculated from half-life data to normalize results. The drug candidates were then categorized into groups representing high, medium, and low turnover compounds based on the CL_{int} values adjusted to the typical incubation times of the two test systems. **Results:** In microsomal assays, approximately 40-48% of compounds exhibited $CL_{int} > 23 \mu\text{L}/\text{min}/\text{mg}$ protein, while a similar proportion showed low intrinsic clearance of $< 5.78 \mu\text{L}/\text{min}/\text{mg}$ protein. These proportions remained consistent over the time periods analyzed. In contrast, 60-80% of compounds tested in hepatocytes had half-lives exceeding 120 or 240 minutes corresponding to $CL_{int} < 5.78$ and $< 2.89 \mu\text{L}/\text{min}/\text{million cells}$. Notably, there was a slight

increase in the proportion for these low-turnover compounds in the most recent time period (2016-2023) compared to 2008-2015. However, the overall number of studies decreased over these years complicating the interpretation. **Conclusions:** Analysis of *in vitro* test systems revealed no significant trend toward lower turnover in micro-somal assays, while hepatocyte studies suggest a modest increase in low-turnover compounds. Taken together with the overall decrease in study numbers and tested drug candidates, this could also indicate that drug developers are looking for test systems that are better suited to measure low-turnover compounds.

PS 3690 Microphysiological Systems as Predictive Tools for Liver Toxicity and Metabolic Function: A Multi-platform Comparative Study

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Background and Purpose: To advance the utility of *in vitro* liver models, it is essential to characterize their robustness, reproducibility and comparative performance. This study evaluated the baseline function and drug-induced responses of primary human hepatocytes (PHH; BioIVT) across three *in vitro* liver models of varying complexity: the TissUse Humimic Chip2 microphysiological system (MPS), 24-well micro-patterned co-cultures (MPCCs) of PHH and 3T3-J2 fibroblasts, and 96-well static monolayers of PHH. **Methods:** The Humimic Chip2 MPS employed in-chip formation of PHH microspheroids and was assessed exclusively in BioIVT hepatocyte maintenance medium (HMM). Both the MPCC and 96-well models, which offer higher throughput, were tested under two media conditions, HMM and a co-culture medium (CoCM) optimized to support both hepatocytes and stromal fibroblasts, yielding five total culture conditions for comparison. In all models, PHH were maintained for 10 days prior to drug treatment. Hepatotoxicity was evaluated using Troglitazone (243 µM) and Fialuridine (25.6 µM), administered every second day for two weeks. Functional endpoints (albumin secretion, lactate dehydrogenase (LDH) activity, blood urea nitrogen (BUN) production, and alanine (ALT), aspartate (AST) aminotransferase activity, and CYP3A4 activity) were monitored over time. Drug metabolism function was assessed using Acetaminophen and Warfarin (both at 1 µM), with LC-MS/MS quantification of the kinetics of compound disappearance from the media. **Results:** Baseline function analyses indicated that albumin secretion was highest in the TissUse MPS. LDH activity was strongly influenced by media composition, with higher baseline levels observed in MPCCs cultured in CoCM. Following drug exposure, the 96-well and MPCC models demonstrated comparable toxicity profiles over time, whereas the TissUse system exhibited delayed and muted toxicity responses. In the 96-well and MPCC models, hepatotoxic responses were much more pronounced with HMM as compared to CoCM, suggesting that media formulation significantly impacts *in vitro* toxicity outcomes. In addition, baseline CYP3A4 activity was approximately 3-fold higher in MPCCs vs 96-well monocultures when cells were cultured in HMM, indicating that co-culture with fibroblasts enhances hepatocellular metabolic function. The use of CoCM further increased baseline CYP3A4 activity, producing roughly a 4-fold elevation in both MPCC and 96-well models relative to their counterparts in HMM, underscoring the combined impact of media composition and cellular context on metabolic competency. These findings suggest that while increased model complexity (e.g., Humimic Chip2 MPS) may enhance baseline hepatocyte function, media composition may be a dominant determinant of model performance. In addition, the incorporation of fluid recirculation may attenuate toxic responses. **Conclusions:** Overall, this study underscores the importance of harmonizing culture conditions across platforms, particularly through standardized media formulations, as a critical step toward enabling the comparison of advanced *in vitro* liver models predictively without the confounding effects of non-cellular factors.

PS 3691 Novel Role of Macrophage Migration Inhibitory Factor in Hepatocyte Organellar Stress

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Background and Purpose: Alcohol-related Liver Disease (ALD) is a major cause of chronic liver disease and poses a significant socio-economic burden globally. Our lab found that hepatocyte-specific Macrophage Migration Inhibitory Factor (Mif) drives ethanol-induced liver injury in mice. Mif is a pleiotropic cytokine/chemokine that contributes to acute and chronic liver injury. The current study sought to investigate the mechanism by which Mif drives liver injury in response to ethanol feeding and exposure and expand on the current knowledge of the Mif signaling axis in ALD. **Methods:** Livers of hepatocyte-specific Mif knockout mice and Mif replete mice were sequenced and transcriptomes were analyzed by Gene Set Enrichment Analysis. Knockout clones of AML-12 hepatocyte of Mif and homologue d-dopachrome tautomerase (Mif2) were generated to interrogate the mechanisms of Mif-mediated hepatocyte damage. Cells were exposed to ethanol, hydrogen peroxide or ER stress inducing SERCA inhibitor Thapsigargin (TG) for 6, 24 and 48 hours and hepatocellular function and viability were determined. **Results:** Protection from liver injury due to ethanol exposure in hepatocyte Mif deficient mice was observed after 10 days of ethanol feeding. Surprisingly, Mif deficiency was associated with marked improve-

ment in expression of gene sets associated with mitochondrial function, opposed to canonical Mif-driven inflammation. Stable Mif knockout clones had improved mitochondrial function compared to wild type when exposed to ethanol, hydrogen peroxide or TG, as well as decreased mitochondrial DNA damage. A divergent response was noted between Mif and Mif2, wherein the rescue effect of Mif deficiency was not found in Mif2 clones which were more susceptible to mitochondrial dysfunction and cytotoxicity from either stimulus. **Conclusions:** These findings further illustrate a novel protective role of hepatocyte-specific Mif knockout in ALD and a divergent role for Mif2 in similar contexts. Moreover, a distinct mechanistic role might exist for Mif in organellar stress outside of its canonical role of a cytokine/chemokine. Further research will define the specific mechanisms by which Mif leads to organellar damage and the homologue-selective roles Mif or Mif2 might play in cytotoxicity.

PS 3692 Hepatic Stellate Cell Activation Drives Hepatocyte Sensitivity to *in vitro* Drug Toxicity

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Background and Purpose: Hepatic Stellate Cells (HSC) play a critical role in hepatic homeostasis. In healthy liver, hepatic stellate cells exist in a quiescent state, storing Vitamin A and maintaining extracellular matrix. HSC activation occurs in response to liver injury, resulting in matrix deposition and cytokine release. This activation has the potential to sensitize the liver to hepatotoxic drugs such as Trovafloxacin and Chlorpromazine. Human iPSC-derived HSC offer a consistent, renewable source of quiescent HSC to interrogate acute and chronic liver injury. **Methods:** iCell Hepatic Stellate Cells (FUJIFILM CDI) were thawed and maintained as 3D spheroids in ultra-low attachment (ULA) plates to maintain quiescent state. Flow cytometry, RNA expression, and ELISA were used to characterize HSC quiescence and using the markers CD271, LRAT, Type I Collagen, and α-SMA. Vitamin A storage was determined by fluorescent intensity of cell lysates. iCell Hepatic Stellate Cells were cultured with either isogenic iCell Hepatocytes 2.0 or primary human hepatocytes. Co-cultures were assayed for spheroid formation, drug metabolism, hepatocyte maturity, and culture longevity. HSC were also co-cultured with hepatocytes in 3D on PhysioMimix "liver-on-a-chip" platform from CN-bio. **Results:** High expression of CD271 and LRAT combined with low expression of Type I Collagen, α-SMA, GFAP, and Desmin indicated iCell HSC can be maintained in a quiescent state. Upon activation by plating into 2D culture or addition of TGFβ, secretion of Type I Collagen was increased 8-fold by protein ELISA, while Vitamin A storage was significantly decreased. Trovafloxacin and Chlorpromazine treatment of mono-culture HSC led to detectable activation after 2 days at concentrations as low as 100 nM. Primary human hepatocytes cultured with iCell HSC became spheroid competent and maintained ALB secretion for 7 days. iCell Hepatocytes 2.0 cultured with isogenic quiescent HSC became sensitive to *in vitro* treatment Trovafloxacin and Chlorpromazine at 10 µM after 2 days. Improvements in hepatocyte functionality (from both primary and iPSC-derived) were observed with HSC co-culture, but further optimizations are required. **Conclusions:** Identifying and harnessing HSC activation state is a critical missing link in many liver toxicology workflows. The practical application of incorporating hepatic stellate cells can be technically and scientifically challenging, however, understanding HSC/Hepatocyte interactions is critical for these studies. Quiescent iPSC-derived iCell Hepatic Stellate Cells are a critical piece of liver biology providing the alternative to inherently variable primary HSC. Functional *in vitro* liver biology may be possible with complex Organ-on-a-Chip systems, media optimization, and culture conditions.

PS 3693 Development and Characterization of a Patient-Derived Liver Organoid Biobank

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Background and Purpose: The liver, as the largest solid internal organ, is responsible for numerous essential functions. In addition, liver diseases account for about 2 million deaths annually worldwide (1). Traditional models for studying liver biology, such as 2D cultured primary cells, 3D spheroids and cell lines, do not accurately represent the *in vivo* metabolic and structural complexities. Additionally, animal models are often ineffective, costly, time-consuming and associated with ethical concerns. Thus, there is a critical need for advanced, tissue-mimicking liver models. Organoids are self-organizing 3D-cultured structures that replicate the structure and functionality of the original tissue (2). Patient-derived organoids (PDOs) closely resemble the source tissue and may mimic individual responses to therapies. In this study, we report the development and characterization of a biobank comprising 11 individual liver PDO lines, reflecting donor variability and enhancing the relevance of these models for toxicology applications. **Methods:** Liver tissue were obtained with donor consent. PDOs were generated by modifying a previously published protocol (3). In brief, samples were minced, digested, filtered, mixed with Matrigel (Corning) and cultured in liver PDO-specific growth media. When appropriate, undifferentiated (immature) PDOs were differentiated (matured) using media tailored for this purpose. **Results:** PDO lines were successfully expanded and cryopreserved. The

immature PDOs could be thawed and further expanded using our standard operating procedures, confirming the robustness of these lines. All PDO lines expressed liver-specific biomarkers, as confirmed by qPCR and confocal microscopy. When matured, these lines showed increased albumin and urea production, as well as inducible cytochrome P450 (CYP) activity, confirming their functional maturity. Levels of these phenotypes were donor dependent, suggesting that the biobank could represent the patient pool. Each PDO line was also established as microbial and viral contamination free and confirmed as unique through short tandem repeat analysis. **Conclusions:** This liver PDO biobank provides a representative snapshot of patient populations and may serve as a valuable tool for pharmaceutical clients. To date, we have successfully established and characterized 11 liver PDO lines, with further characterization ongoing. Our liver PDOs will significantly enhance the understanding of liver biology and disease mechanisms, offering substantial applications in drug testing, including DMPK and ADME/Tox studies. Future efforts will focus on scaling up, developing liver-specific assays, and generating PDOs from patients with specific liver conditions, such as hepatocellular carcinoma, MASLD, and NASH. References: 1. Asrani et al., 2019. J. Hepatol. 2. Zhao et al., 2022. STAR Protocols 3. Broutier et al., 2016. Nature Protocols

PS 3694 FUNCTIONALLY RELEVANT hPSC-DERIVED HEPATOCYTES AND LIVER ORGANOIDS FOR HEPATOTOXICITY AND LIVER BIOLOGY MODELING

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Background and Purpose: Functionally relevant human hepatocyte models are critical for drug safety and efficacy screening, cell therapy, and disease modeling. However, currently available *in vitro* models, such as primary human hepatocytes and immortalized cell lines, often rapidly de-differentiate or lack metabolic maturity. We developed workflows for the generation and culture of human pluripotent stem cell (hPSC)-derived hepatocytes and liver organoids to address these challenges, as convenient, scalable, and patient-representative alternatives to conventional models that can be banked and used for long-term studies. **Methods:** hPSC-derived hepatocyte-like cells (HLCs) were generated over 21 days using STEMdiff™ Hepatocyte Kit; hPSCs were first patterned to definitive endoderm cells, then differentiated to hepatic progenitors, and finally matured to HLCs. HLCs were also replated in Matrigel® domes to establish passageable liver organoids using STEMdiff™ Hepatic Organoid Growth Medium. These organoids were further matured in 3D culture using STEMdiff™ Hepatic Organoid Differentiation Medium (ODM). Hepatic functions were characterized in HLCs and organoids using an albumin ELISA kit for assessment of albumin secretion and the P450-Glo™ CYP3A4 Assay and Screening System for measurement of baseline and calcitriol or ketoconazole-modulated CYP3A4 activity. HLCs and organoids were also used for proof-of-concept toxicity testing, where dose-response viability data was generated using the CellTiter-Glo® 3D Cell Viability Assay following treatment with known hepatotoxic compounds ketoconazole, rifampicin, and acetaminophen. Organoids were also used for cholestasis modeling via an imaging-based readout, using 5-chloromethylfluorescein diacetate (CMFDA) for assessment of bile transporter functionality in the presence of compounds troglitazone and cyclosporine A, which are known to inhibit bile acid efflux. **Results:** hPSC-derived HLCs expressed hepatic proteins (66 ± 8% ALB+A1AT+; mean ± SD) and exhibited mature functionality, including CYP3A4 enzymatic activity and albumin secretion (n = 2 - 15). Subsequent culture and differentiation as organoids resulted in significant downregulation of fetal hepatocyte gene *AFP* (5.7-fold decrease; p < 0.0001), and increased expression of several mature hepatic genes, including *ALB*, *ASGR1*, *CYP3A4*, *CYP2C9*, *UGT1A1*, and *OTC* (n = 2 - 20). ODM-differentiated cultures also exhibited increased albumin secretion (2.9-fold increase; p = 0.0199) as well as CYP3A4 activity that was modulated using ketoconazole (average 2.0-fold decrease) and calcitriol (average 2.5-fold increase). HLC-derived organoids were sensitive to ketoconazole-, rifampicin-, and acetaminophen-induced hepatotoxicity (average ODM-differentiated organoid IC₅₀ = 58 µM, 527 µM, and 1.8x10⁴ µM, respectively; n = 2), and also exhibited dose-dependent responses to high liver injury-causing compounds, troglitazone and nefazodone. In contrast, organoids exhibited low or no responses to related low/no liver injury-causing compounds pioglitazone and buspirone, respectively. Organoids could also model bile transport and efflux. Fluorescence intensity, corresponding to the product of cytosolic CMFDA cleavage, in cultures treated with 50 µM troglitazone or 12.5 µM cyclosporine A was significantly higher (3.0-fold and 2.7-fold increase respectively; p < 0.0001) than the DMSO vehicle control, while 50 µM pioglitazone-treated cultures exhibited fluorescence intensity comparable to the vehicle control (1.1-fold increase; p = 0.9999) (n = 2). **Conclusions:** hPSC-derived HLCs and hepatic organoids represent a convenient, scalable, and patient-representative model of hepatic function, and could successfully be used in toxicity screening applications to model compound-specific responses. Organoids could be used to distinguish between low and high liver injury-causing compounds as well as between cholestatic and non-cholestatic compounds that modulate bile efflux and transport. These results demonstrate the utility of STEMdiff™ Hepatocyte Kit and STEMdiff™ Hepatic Organoid Media in hPSC-derived liver modeling and drug screening applications.

PS 3695 Chronic Cadmium and Glucose Crosstalk Drives Hepatic Lipid Dysregulation through PPAR Signaling and Membrane Remodeling in Hepatocellular Carcinoma cell lines

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Background and Purpose: The incidence of hepatocellular carcinoma (HCC) is increasing in the Western world over the past decades. Within the United States, rise of HCC cases has been mainly observed in the Southwest part of country including State of Texas, New Mexico, and Arizona. In New Mexico elevated numbers of HCC cases are seen among Hispanic and Native American populations of the states. Historically, these populations are prone to key risk factors of HCC such as diabetic and obesity. However, those risk factors alone are not sufficient to explain the rapid increase of HCC incidence observed in the past decades. We hypothesize additional environmental risk factor (i.e. heavy metal exposure) plays a key role in the Southwest United States including New Mexico. Cadmium (Cd) is a major key metal pollutant of global implication. Environmental cadmium (Cd) exposure and metabolic stressors such as hyperglycaemia are emerging as critical co-factors in the pathogenesis of metabolic-associated fatty liver disease (MASLD). However, their combined mechanistic impact on hepatic lipid homeostasis remains poorly understood. **Methods:** In this study, we chronically exposed human hepatocellular carcinoma cell lines (HepG2 and HUH7) to low-dose Cd (1 µM) for 24 weeks under normoglycemic (5.5 mM) or hyperglycemic (15 mM) conditions to mimic physiologically relevant, prolonged environmental and metabolic challenges. **Results:** Chronic Cd exposure alone modestly elevated lipid synthesis markers (ACCT1, FASN) and increased intracellular lipid content, suggesting enhanced *de novo* lipogenesis. Hyperglycaemia further exacerbate this lipogenic response. Interestingly, while Cd exposure alone increased the fatty acid transporter CD36, its expression markedly declined under combined Cd + hyperglycaemic conditions, indicating a possible adaptive downregulation of lipid uptake in response to lipid bilayer remodeling or oxidative stress. The concomitant upregulation of lipogenic enzymes and suppression of CD36 suggest a metabolic reprogramming that favors lipid synthesis over uptake, potentially contributing to intracellular lipid disequilibrium and mitochondrial burden. RNA-seq data analysis revealed transcriptional perturbations in PPAR-mediated dysregulation of lipid transport, and oxidation. Pathway enrichment highlighted alterations in linoleic acid and glycerophospholipid metabolism, aligning with metabolomic analysis from Cd-exposed systems that demonstrate disrupted TAG, DAG, and phosphatidylcholine (PC) profiles which were exacerbate in the hyperglycemic and cadmium exposed cells. Furthermore, we observed several remarkable differential lipidomics metabolites enriched these pathway. **Conclusions:** These results support a “two-hit” paradigm where environmental Cd exposure and metabolic dysregulation combinedly disturb hepatic lipid homeostasis, potentially linking disease to environmentally driven liver carcinogenesis.

PS 3696 The effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on human liver cells-derived spheroids

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Background and Purpose: Three-dimensional (3D) *in vitro* liver models are essential tools in toxicology, as they support the Replacement, Reduction, and Refinement (3R) principle by reducing animal testing while providing physiologically relevant data. In contrast to traditional two-dimensional culture, 3D liver models, such as spheroids, more accurately replicate the complex structure and function of the liver *in vivo*, maintaining drug metabolism, enzyme activity, and cell-cell interactions over an extended period of time. These models improve the accuracy and relevance of toxicology studies to human risk assessment. This is particularly important, given the well-documented *in vivo* hepatotoxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice, which manifests as hepatic inflammation, steatosis, and fibrosis. This study examined the dose dependent effects of TCDD on human liver-derived spheroid viability, cytotoxicity, and gene expression. **Methods:** Spheroid models included (i) human hepatocytes only, and (ii) quad-cellular spheroids consisting of hepatocytes, liver endothelial cells, stellate cells, and Kupffer cells. Spheroids were treated with DMSO vehicle or TCDD (0.1, 1, 10, and 100 nM) for 24 hours. Effects on cytotoxicity, viability, *CYP1A1* and *CYP1A2* expression, as well as ethoxyresorufin activity were determined. Immunofluorescence staining confirmed the presence of the different cell types within the spheroids. **Results:** TCDD did not affect viability or LDH release in hepatocyte-derived spheroids. Moreover, TCDD dose-dependently induced *CYP1A1* and *CYP1A2* expression and ethoxyresorufin activity, consistent with AhR activation by TCDD. **Conclusions:** Human 3D liver spheroids provide a robust and mechanistically valuable model for elucidating AhR-mediated hepatotoxicity. This approach represents a critical step toward more predictive and human-relevant toxicological testing. Funded by SRP P42ES004911.

PS 3697 Integrated Assessment of Drug-Induced Cholestasis Using Sandwich-Cultured Human Hepatocytes: Combining Kinetic High-Content Imaging, Cytotoxicity Endpoints, and Transporter Inhibition for Early Discovery De-Risking

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Background and Purpose: Drug-induced liver injury (DILI) is a major challenge in drug development, contributing to clinical trial failures and post-marketing withdrawals. The liver's central role in xenobiotic metabolism makes it highly susceptible to adverse drug reactions. DILI can result from mechanisms such as hepatocellular toxicity, mitochondrial dysfunction, oxidative stress, and immune-mediated responses. A complex subtype, cholestatic DILI (cDILI), involves impaired bile flow due to inhibition of hepatic transporters (e.g., BSEP, MRPs, OATPs), leading to toxic intracellular bile acid accumulation. Sandwich-cultured hepatocyte models (SCH-PHH) and vesicular transport assays are established *in vitro* approaches for assessing bile acid transporter inhibition and cholestatic risk. These assays form part of new approach methodologies (NAMs) designed to improve human relevance and reduce reliance on animal testing. To enhance predictive power, NAMs increasingly incorporate advanced tools such as high-content imaging (HCI), which enables multiparametric phenotypic analysis. When combined with cytotoxicity endpoints like ATP depletion, HCI provides a more comprehensive view of hepatotoxic potential, complementing transporter inhibition data. **Methods:** Primary human hepatocytes were seeded in collagen-coated 384-well plates and overlaid with Matrigel to establish SCH-PHH. Cultures matured for four days before exposure to a proficiency set of 59 compounds (cDILI, DILI, and no-DILI) across concentration ranges for 24 h, with or without a bile acid/free fatty acid cocktail. Post-incubation, CMFDA staining enabled kinetic HCI of bile canaliculi morphology and bile acid accumulation. ATP-based viability assays complemented phenotypic data. Transporter inhibition profiles for BSEP, MRP2, MRP3, and MRP4 were generated for all compounds. **Results:** cDILI compounds exhibited strong BSEP inhibition (0.19-1,000 μ M) and variable MRP inhibition (MRP2: 21.9-708 μ M; MRP3: 3.34-2,610 μ M; MRP4: 0.6-226.7 μ M). DILI compounds showed moderate effects (BSEP: 79.8-10,000 μ M; MRP2: >100-399.7 μ M; MRP3: >100-281.3 μ M; MRP4: 1.7-10 μ M), while no-DILI compounds demonstrated minimal inhibition (BSEP $\geq$ 100 μ M; negligible MRP inhibition). Kinetic HCI revealed bile canaliculi disruption and bile acid accumulation in cDILI compounds, correlating with transporter inhibition and cytotoxicity. Integrated phenotypic and mechanistic data improved discrimination of cholestatic risk compared to single-endpoint assays. **Conclusions:** This NAM-based approach combining SCH-PHH, kinetic HCI, cytotoxicity, and transporter profiling enables mechanistic assessment of cholestatic liability. The integrated dataset supports early identification of cDILI risk, enhancing predictive power and reducing reliance on animal testing in drug discovery.

PS 3698 Effects of chronic overnutrition on mitoNEET in mouse livers

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Background and Purpose: Mitochondrial dysfunction after prolonged consumption of high fat diets contributes to the onset of nonalcoholic fatty liver disease (NAFLD), the most common liver disorder globally. While lean livers tolerate moderate stressors well, fatty livers do not. How chronic steatosis impacts mitochondria remain elusive. mitoNEET (mNT) is a homo-dimeric mitochondrial outer membrane protein that regulates mitochondrial redox, bioenergetics, autophagy and iron homeostasis. **Methods:** Hepatocytes were isolated from male C57BL/6J mice either on a chow diet (CD) or on a high fat diet (HFD: 60 Kcal % fat, Research Diet # D12492) for 8 weeks. Cells were treated with dithiobis (succinimidyl propionate), a covalent crosslinker, to separate mNT monomers (mNTm) and oligomers (mNTo) from mNT dimers (mNTd). Changes in mNT expression, autophagy, mitochondrial membrane potential ($\Delta\psi$ m) and steatosis were assessed by immunoblot and imaging analysis. Some experiments were conducted in whole mouse livers. Since isolated hepatocytes do not represent cell-to-cell interactions, three-dimensional architecture, and zonal structure in the liver, we also generated precision-cut liver slices (PCLS) from mouse livers using a Krudieck automatic tissue slicer (Alabama R & D, Munford, AL). Thickness of individual PLCL was 200 microns. To investigate roles of $\Delta\psi$ m in steatosis-mediated disassembly of mNTd, hepatocytes from CD mice were incubated with 500 nM of carbonyl cyanide m-chlorophenylhydrazone (CCCP), a mitochondrial uncoupler, for up to 90 min. **Results:** The expression of mNTm and mNTo, nonfunctional forms of mNT, in HFD hepatocytes and livers was significantly greater than that of CD counterparts. Confocal imaging analysis with tetramethylrhodamine methylester demonstrated that $\Delta\psi$ m of HFD hepatocytes was 28.1% of CD hepatocytes. Confocal microscopy of rhodamine-123 confirmed substantial mitochondrial depolarization in PCLS from the HFD group, not from the CD group. Treatment of lean hepatocytes with CCCP progressively depolarized $\Delta\psi$ m and accumulated mNTm followed by mNTo, suggesting the disassembly of mNTd by mitochondrial depolarization. HFD hepatocytes failed to respond to autophagic stimuli such as starvation and rapamycin, which was, however, reversed by genetic deletion of mNT. **Conclusions:** Chronic overnutrition depolarizes mitochondria, leading to the dissociation of mNTd into mNTm and the formation of mNTo. The accumulation of nonfunctional mNT consequently impairs autophagy, thereby sensitizing steatotic livers to moderate stressors.

PS 3699 Multidrug Resistance-Associated Protein 4 (MRP4) Deficiency Disrupts Hepatic Lipid Homeostasis and Exacerbates Metabolic Dysfunction-Associated Steatotic Liver Disease

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Background and Purpose: Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) is characterized by dysregulated hepatic lipid metabolism, resulting in fat accumulation ($\geq$ 5%) in the liver without significant alcohol consumption. MRP4 (ABCC4) is a sinusoidal efflux transporter that exports a broad range of xenobiotics and endogenous substrates. Recent studies indicate that loss of MRP4 function alters hepatocellular signaling networks that regulate energy balance and lipid handling, contributing to metabolic disturbances, including obesity and diabetes. Consistent with its highly inducible and protective role in the liver, MRP4 expression is markedly elevated in hepatic tissues of obese individuals, implying a compensatory response to metabolic stress. Given these observations, we aimed to define how MRP4 deficiency disrupts hepatic lipid homeostasis using complementary *in vivo* and *in vitro* MASLD models. **Methods:** To assess the systemic impact of MRP4 deficiency, an *in vivo* high-fat, high-sucrose diet (HFHSD) study was performed using wild-type (WT) and *Mrp4*^{-/-} C57BL/6J mice. Animals were maintained on either a standard chow diet or HFHSD for 24 weeks. At study completion, serum ALT and triglycerides were quantified using commercial assay kits to evaluate liver injury and systemic lipid metabolism. Liver tissues were examined histologically to assess steatosis and inflammation. Hepatic expression of genes involved in lipid metabolic pathways was measured by RT-qPCR. To translate the *in vivo* findings into a human-relevant system, an *in vitro* hepatic MASLD model was established using the HC-04 human hepatic cell line. Steatosis was induced with oleic and palmitic acid (OA: PA, 2:1 molar ratio) at concentrations ranging from 100 to 400 μ M. MRP4 activity was pharmacologically inhibited with MK-571 (2.5-20 μ M). Cell viability was determined by MTT assay, and intracellular lipid accumulation was visualized and quantified using Oil Red O staining. **Results:** As previously reported, *Mrp4*^{-/-} mice displayed elevated serum ALT and triglycerides levels following HFHSD feeding compared with WT mice, indicating enhanced liver injury and systemic dysregulation of lipid metabolism. H&E staining showed increased steatosis and inflammation in *Mrp4*^{-/-} livers. RT-qPCR analysis showed altered expression of key lipid-regulatory genes, including *Ppara*, *Fxr*, *Cd36*, *Apob*, *Scd1*, *Cpt1a*, *Dgat2*, and *Glut2* in liver tissue under both chow and HFHSD diet conditions. Guided by these *in vivo* findings, we developed an *in vitro* MASLD model using HC-04 hepatocytes. MK-571 treatment for 24 hours did not cause cytotoxicity. Free fatty acid (FFA) treatment alone (100-400 μ M OA: PA) did not affect cell viability either. Based on these results, MK-571 at 10 μ M, with or without FFAs, was selected for functional studies. After 24 hours, MK-571 alone induced notable lipid droplet accumulation, whereas the MK-571 and FFA combination did not produce a further increment in lipid accumulation. However, extending exposure to 48 hours resulted in greater lipid accumulation by the combined treatment compared to MK-571 alone. These preliminary observations indicate that acute inhibition of MRP4 activity promotes steatosis in human hepatocytes, consistent with phenotypes observed in *Mrp4*^{-/-} mice. **Conclusions:** Together, these *in vivo* and *in vitro* findings demonstrate that loss of MRP4 function disrupts hepatic lipid homeostasis and promotes steatosis. *Mrp4*^{-/-} mice exhibit increased lipid accumulation and altered expression of genes regulating lipid uptake, oxidation, and export, while pharmacological inhibition of MRP4 enhances lipid deposition in human HC-04 hepatocytes. These data support a model in which MRP4 activity is required to maintain metabolic balance through the export of endogenous signaling molecules.

PS 3700 Integration of HLA-Typed PBMC into a Long-term, Functional Hepatocyte Co-culture, HEPATOPAC®, for Detection of Immune-Mediated Drug-Induced Liver Injury

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Background and Purpose: Preclinical prediction of idiosyncratic drug induced liver injury (iDILI) is important in drug development both to safeguard patients from serious adverse outcomes and to reduce late-stage candidate drug failure. Standard preclinical *in vitro* models fail to identify DILI potential for several reasons including lack of relevant immune cell types in the model and/or factors that are specific to particular patient populations. Retrospective studies have identified compound interaction with T-cells in patients with specific human leukocyte antigen (HLA) types as a contributing factor for iDILI in some compounds which showed liver toxicity in the clinic. Based on these observations, we developed a co-culture model which incorporates human PBMC into a long-term micropatterned hepatocyte co-culture model (HEPATOPAC) in order to better model peripheral immune system contribution to DILI development. In these studies, culture conditions were optimized to maintain both hepatocyte and PBMC viability and function for up to 7 days. To investigate the utility of this model for immune DILI studies, HEPATOPAC cultures with and without HLA-typed PBMC were treated with amoxicillin-clavulanate (AC), which is known to cause DILI in patients having specific risk associated HLA-types (DRB1*15:01-DQB1*06:02). The TC50 in cultures with and without PBMC was compared to determine if the presence of PBMC increased toxicity as indicated by a shift of the concentration-response curve. **Methods:** HEPATOPAC plates were manufactured using HLA-typed single donor human hepatocyte lots. After maturation, 75,000 allo-

genic PBMC/well were added. The impact of HLA-mismatch (low resolution allele group match vs. mismatch) was investigated for up to 10-days of co-culture. Urea production was used to assess hepatocyte function, CellTiter-Glo® 2.0 Cell Viability Assay was used to assess general toxicity, and PMA/ionomycin (6.3 ng/mL PMA and 250 ng/mL ionomycin) or CD3/CD28 beads (8 µL/well) induced cytokine production was used to assess PBMC function. For AC toxicity studies, PBMC and hepatocytes having the DRB1*15:01-DQB1*06:02 DILI risk HLA-types were used. Cultures with and without PBMC were treated with AC. The clinical C_{max} for a 875 mg (A)/125 mg(C) dose given every 12 hours (A: 11.6 ± 2.78 mcg/mL and C: 2.2 ± 0.99 mcg/mL): was used to generate a concentration curve using multiples of C_{max} (500x, 250x, 125x, 50x, 10x, 1x, 0.1x and vehicle control). Cultures were treated with AC for 48 hours. Hepatocyte specific toxicity was determined by measuring urea production. For analysis, data was normalized to inter-group vehicle controls. Concentrations were log transformed and a nonlinear fit of the transformed data was performed in GraphPad Prism to determine the TC_{50} . **Results:** PBMC-HEPATOPAC co-cultures that were HLA-matched or mismatched showed no difference in hepatic urea production, ATP content, or cytokine production (IL-2, IL-6, IFN-γ) following stimulation with either PMA/ionomycin or CD3/CD28 dynabeads. Cultures with and without PBMC maintained similar urea production through day 7 of co-culture, however, hepatic function was reduced by day 10. AC-treatment reduced viability of hepatocytes as measured by urea production in a concentration dependent manner. The TC_{50} of AC in cultures without PBMC was 24 mM (A)/ 42.4 mM (C), while the TC_{50} of AC in cultures containing PBMC was significantly lower at 15 mM (A)/26.5 mM (C). **Conclusions:** The long-term micropatterned hepatocyte co-culture, HEPATOPAC, supports co-culture with HLA-typed PBMC while maintaining hepatocyte function and PBMC responsiveness for up to 7 days. Incorporation of PBMC into the model did not alter baseline hepatic function or cytokine production but resulted in a significant leftward shift in the amoxicillin-clavulanate concentration-response curve, consistent with increased susceptibility observed in patients carrying risk-associated HLA alleles. The ability to integrate functional immune cells with metabolically competent hepatocytes enables evaluation of immune contributions to idiosyncratic DILI. This approach expands the utility of HEPATOPAC as a physiologically relevant platform for mechanistic investigation and preclinical detection of immune-mediated hepatotoxicity.

PS 3701 Impairment of Chaperone-mediated autophagy leads to TRIM44-mediated TLR4 deubiquitination and hepatic inflammation

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Background and Purpose: Tripartite motif-containing protein 44 (TRIM44), a TRIM family member, participates in ubiquitination and protein degradation through E3 ligases. Chaperone-mediated autophagy (CMA) selectively targets cytosolic proteins to lysosomes, and CMA impairment has been associated with metabolic dysfunction-associated steatohepatitis (MASH). TLR4 is a key receptor mediating hepatic inflammation, and its dysregulation contributes to MASH progression. In this study, we investigated the role of CMA in regulating TRIM44 degradation in the liver. **Methods:** Using RNA interference, we silenced LAMP2A and HSPA8 to observe effects on TRIM44 protein levels. We examined TRIM44 accumulation in response to altered LAMP2A expression in LAMP2A knockdown mice and mice fed a CDAHFD. **Results:** Sequence analysis of TRIM44 revealed a KFERQ-like motif. We verified the interaction between TRIM44 and heat shock protein family A (Hsp70) member 8 (HSPA8/HSC70) in HEK293 cells, indicating that CMA contributes to TRIM44 degradation. Mutations within the KFERQ-like motif reduced the binding between TRIM44 and HSPA8, underscoring the motif's importance in CMA recognition. Knockdown of lysosome-associated protein 2A (LAMP2A) or HSPA8 by siRNA increased TRIM44 protein levels. TRIM44 accumulated in lysosomes upon leupeptin treatment. Co-treatment with siLAMP2A altered this accumulation, demonstrating that TRIM44 degradation depends on CMA. Additionally, a cycloheximide chase assay in primary mouse hepatocytes transfected with siLAMP2A showed a reduced rate of TRIM44 degradation in CMA-deficient cells. Ubiquitination assays demonstrated that overexpressing TRIM44 decreased TLR4 ubiquitination, potentially enhancing TLR4 signaling and promoting hepatic inflammation. In a MASH mouse model, reduced LAMP2A expression coincided with TRIM44 accumulation. **Conclusions:** Our results demonstrate that TRIM44 is a substrate of CMA, and further suggest that CMA-regulated TRIM44 may influence hepatic inflammation by deubiquitinating TLR4.

PS 3702 Effect of cytokines on human angiotensinogen acute phase response: an *in vitro* study

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Background and Purpose: Human angiotensinogen (hAGT) is an acute phase response type II (APR II) protein modulated by interleukin-6 (IL-6) and glucocorticoids. The advent of presence of the response element of nuclear factor-kappa B (NF-κB) in its promoter opens the possibility of modulation by tumor necrosis factor-alpha and interleukin-1beta (IL-1β), which can activate NF-κB. Angiotensinogen is the source of angiotensin II, playing a significant role in blood pressure regulation, and increased AGT levels are found among hypertensives. Besides, AGT participates in the fibrotic

transformation of the liver after hepatotoxicant-mediated liver injury. Levels of circulating cytokines (i.e., IL-1β, IL-6, and IL-8) are increased among chronic alcoholics. **Methods:** The cells were grown in DMEM media supplemented with 10% FBS and antibiotics in a humidified chamber with 5% CO₂. The cells were treated with 10 ng/ml cytokines (IL-1β, IL-6) and/or 50 ng/ml phorbol 12-myristate acetate (TPA) for 24, 48, 72 and 96 hrs. The control samples were not treated with any reagent. The media were collected, while cells were washed with PBS and lysed in PBS containing triton X-100. The cellular levels of protein were determined by dye binding method. The proteins were size separated on denaturing 10-12% acrylamide gel, transferred to PVDF membrane. The levels of AGT in secreted samples and cellular levels were determined using Western blotting technique using mouse monoclonal antibody against human angiotensinogen (sc-374511). **Results:** It was observed that IL-1β, PMA, and IL-6 increased the level of secreted hAGT and cellular levels from both HepG2 and Huh7 cells from 24 -96 hrs. The effects of IL-6 on AGT secretion from both hepatocytes were more sustained compared to IL-1β or PMA. The cellular AGT levels were increased commensurate with their secretion after IL-1β, PMA, or IL-6 treatment. The secreted AGTs were glycosylated, and the non-glycosylated form was not observed with any treatment. **Conclusions:** Based on the sustained effects of IL-6 on AGT secretion from hepatocytes compared to IL-1β or PMA, it is concluded that human AGT remains a type II acute phase response (APR II) protein.

PS 3703 Pioglitazone-Mediated Upregulation of Hepatic Low-Density Lipoprotein Receptor-Related Protein 1 (LRP1): Exploring a Metabolic Link to Alzheimer's Disease

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Background and Purpose: One risk factor for Alzheimer's Disease (AD) is the accumulation of Amyloid-Beta (Aβ) in the brain. Enhancing Aβ clearance is a potential method of reducing the risk of AD. The Peroxisome Proliferator-Activated Receptor Gamma (PPARγ#120516;;) pathway plays a role in lipid metabolism and may increase Aβ clearance by upregulating low-density lipoprotein receptor-related protein 1 (LRP1). LRP1, a member of the low-density lipoprotein receptor (LDLR) family, facilitates Aβ clearance from the brain by ejecting Aβ into the bloodstream to then get degraded by the liver. Pioglitazone, a type-2 diabetes medication, is a PPARγ#120516;; agonist shown to increase LRP1 expression in the hippocampus, suggesting it may be a potential target for reducing Aβ accumulation. **Methods:** A liver model was adapted in this study to observe whether pioglitazone can increase LRP1 expression, which in turn could promote hepatic endocytosis of Aβ and eventual degradation. HepG2 cells were treated with one of two pioglitazone concentration ranges (0µM - 100µM or 0µM - 25µM). After 24 hours of pioglitazone treatment, cell viability was measured using the MTT Cell Viability Assay, and protein expression was measured through Western Blot analysis. **Results:** Compared to the control, LRP1 expression increased at lower concentrations of pioglitazone. Proprotein convertase subtilisin/kexin type 9 (PCSK9) expression also increased, consistent with previous findings. **Conclusions:** These results suggest that pioglitazone can increase hepatic LRP1 expression, which may facilitate Aβ clearance and highlight its potential as a repurposed therapy for AD. These findings also emphasize the metabolic connection between the liver and brain, providing insight into how drugs targeting hepatic pathways may influence neurodegenerative disease progression.

PS 3704 Oleic Acid as a Potential Modulator of LRP1 and BACE-1 in the Treatment of Alzheimer's Disease

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Background and Purpose: Alzheimer's disease (AD) is a neurodegenerative disease caused by the buildup of amyloid beta (Aβ#120515;;) plaques in the brain. According to the Alzheimer's Association, 7 million people are diagnosed with AD every year. However, no cure has been found and at best, drugs only slow the progression of the disease. The scale of AD diagnosis and lack of treatment makes finding a treatment for AD imperative. Removal of Aβ#120515;; plaques is key to treat AD, so any proteins that can modulate Aβ#120515;; is a potential drug therapeutic target. One such protein is the lipoprotein receptor related protein 1 (LRP1). LRP1 is found in the liver and brain. Liver LRP1 binds to peripheral Aβ#120515;; and the Aβ#120515;; is endocytosed by the liver to be processed (van Uden et al., 1999). Liver LRP1 reduces peripheral Aβ#120515;; that can travel to the brain, which if left unchecked, can initiate pathogenesis of AD in the brain. The relationship between the liver and the brain through the blood brain barrier is key in Alzheimer's disease development, and is commonly known as the liver-brain axis. Another method of AD treatment is to target key proteins involved in Aβ#120515;; formation, such as beta-secretase cleaving enzyme 1 (BACE-1). Aβ#120515;; plaque formation occurs when amyloid precursor protein (APP) is cleaved into Aβ#120515;; by various secretases. The most important secretase in this cleavage process is BACE-1 because BACE-1 catalyzes the rate-limiting step in Aβ#120515;; cleavage (Gorji et al., 2017). This means that once APP is cleaved by BACE-1, the formation of Aβ#120515;; is inevitable. Therefore, treatments that target BACE-1 are imperative because drugs that down-regulate BACE-1 will downregulate APP cleavage into Aβ#120515;; plaques. Many

studies have focused on BACE-1 regulation in the brain, but very few have focused on liver BACE-1 regulation. Minimal research has been conducted on the effects of natural remedies targeting key proteins in the treatment of AD. Oleic acid (OA) is a monounsaturated fatty acid that is known to target BACE-1, while little is known on its effects on LRP1 regulation in the liver. Diabetic models suggest that OA's effects increase insulin sensitivity through LRP1 upregulation (Y. Ding et al., 2016). Taken together, it can be hypothesized that OA may prevent/delay AD development by regulating liver BACE-1 and LRP1. **Methods:** HepG2 liver cells were seeded, grown, and then treated with Oleic Acid suspended in media for 24 and 48 hours. Cells were then used for either MTT Viability Assay or prepped for Western Blot Protein Analysis, Imaging, and Image J software analysis. **Results:** MTT assay showed that the cells did not die from treatment, so Oleic Acid was not toxic towards liver cells. Western Blot analysis of Actin showed equal loading. LRP1 did not show much change, levels stayed relatively constant throughout 24 and 48 hour treatments. PS1 decreased, while PS2 remained consistent. BACE-1 decreased in the 24 hours, as did APP. Both BACE-1 and APP didn't change in the 48 hour treatment. **Conclusions:** OA down-regulated PS1 at higher concentrations, but LRP1 levels slightly decreased. BACE-1 decreased, which means less APP is getting processed, and this was confirmed because APP trends are similar to BACE-1. Overall, OA may have protective effects against AD through the β -secretases but not through LRP1 regulation. Since LRP1 is slightly downregulated, OA may be best used as a preventative measure instead of as a direct treatment to AD. More research is needed, perhaps performing the same experiment on a BBB or mouse model will modulate AD related proteins as expected.

PS 3705 Nitroalkene therapy for allergen-induced hepatic inflammation and dysregulated metabolism

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Background and Purpose: Environmental allergens including house dust mite (HDM) trigger airway inflammation and downstream extrapulmonary organ and metabolic dysfunction. Allergic asthma is a complex disease characterized by chronic airway inflammation. The noncommunicable disease affects >260 million people worldwide and causes ~250,000 deaths per year. HDM is a primary trigger for the allergic asthma response with sensitization present in 50-85% of patients. Injured bronchial epithelium results in airway cells interacting with immune cells to promote inflammation through Type 2 (T2) mediated airway hyperresponsiveness. In compromised asthmatic airway epithelium, cell-cell contacts are disrupted, leading to increased allergen permeability. HDM contains proteases that trigger the immune response, mediate lung epithelial cell activation, and compromise barrier integrity. Preliminary data from our lab utilizing RNA sequencing and proteomic approaches has also demonstrated that mice with HDM-induced allergic airway disease (AAD) have altered hepatic metabolism compared to their control counterparts regardless of dietary influence. These changes are predominantly related to Phase 1 and 2 metabolic enzyme expression and redox signaling. Observed in epidemiological asthma studies, systemic inflammation is correlated with downstream extrapulmonary organ and metabolic dysfunction consistent with cases of obesity and liver disease. We have observed increased hepatic inflammation (e.g., NF- κ B) and dysregulated hepatic metabolism (e.g., glutathione-related pathways) in our mouse model of HDM-induced AAD. It is unclear whether these changes are a direct effect of HDM on hepatic function or a result of increased pulmonary and systemic inflammation. Nitroalkenes (CP-6 and CP-7) are potential pleiotropic therapies for co-occurring asthma and liver disease. CP-6 has been shown to improve glucose metabolism and lower hepatic triglyceride levels in an obese mouse model for liver disease. Additionally, CP-6 treatment reduced small airway resistance in our obese AAD mouse model. The nitroalkene, CP-7 is a new chemical entity and promising AAD therapeutic. Unpublished data from our lab shows that CP-7 reduced airway inflammation, total cell counts, and protein concentrations in bronchoalveolar lavage fluid from AAD mice. **Methods:** To begin to answer this question, human liver cells, HepG2, were treated with HDM to observe its direct inflammatory effect and optimize parameters for treatments in primary human hepatocytes. HepG2 cells were also treated with either CP-6 or CP-7 in combination with HDM to determine if they abrogate HDM-directed gene expression changes in phase 1 and phase 2 metabolic enzymes, proinflammatory markers, and redox-regulated signaling pathways. **Results:** Hepatic mRNA expression of inflammatory markers (*NFKB1/p105*, *RELA/p65*, *MAPK1*, and *Nrf2*) increased with HDM treatment and are restored by nitroalkenes ($p < 0.05$, $p < 0.0001$, $p < 0.01$, and $p < 0.0001$, respectively). Likewise, with HDM treatment hepatic mRNA expression of glutathione-related pathways and receptors (*GCLM*, *NR1I3/CAR*, and *NR1I2/PXR*) increase and are restored by nitroalkenes (trend, $p < 0.0001$, and $p < 0.0001$, respectively). Ongoing studies investigating how HDM treatment impacts glutathione-related enzymes, including glutathione-S-transferases and peroxidases, are being conducted. **Conclusions:** Preliminary findings thus far support evidence from the AAD model for lung-liver crosstalk through increased liver inflammation and the need for therapeutics that target multiple signaling pathways. Treatment with HDM and nitroalkene therapies will be recapitulated in primary mouse hepatocytes, primary human hepatocytes, and 3D microphysiological systems (MPS) for a holistic assessment of hepatic inflammatory, redox, and metabolism markers. Thus far, measured changes are predominantly related to phase 1 and 2 metabolic enzyme expression and redox signaling. Overall, further characterization of the extrapul-

monary toxicology of the environmental allergen house dust mite is warranted for subsequent exploration into the pathways directly involved in lung-liver crosstalk in the context of allergic asthma.

PS 3706 Scaling Without Compromise: Liver MPS for Lead Optimization Screens to Investigative Toxicology

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Background and Purpose: Drug-induced liver injury (DILI) remains the most common cause for acute liver failure in the USA and Europe and is a leading cause of attrition of compounds in drug development. Prediction of clinical safety is still a major hurdle in drug discovery and development, with many late-stage attrition events still occurring due to limited translatability of current *in vitro* or *in vivo* preclinical models. Microphysiological systems (MPS), otherwise known as organ-on-a-chip (OOC), provide a solution to this challenge by enabling the long-term culture and in-depth examination of highly human relevant liver microtissues. However, scaling and throughput of OOC have in the past been perceived as a challenge. Here, we present two solutions based on their different contexts of use. Firstly, a higher throughput approach, the PhysioMimix® Multi-chip Liver-48 plate can be applied within the lead optimization process for screening of compounds, up to 288 samples (or chips) per run. Secondly, due to its enhanced tissue size and sampling volume, the Liver-12 plate can further characterize DILI events at a deeper level and their associated mechanisms of toxicity through a wide range of liver-specific endpoints. **Methods:** Using the PhysioMimix® Core System, primary human hepatocytes and Kupffer cells were cultured in 3D microtissues on engineered scaffolds in either the Liver-48 or Liver-12 plates under perfusion for eight days. The Liver-48 microtissues were subjected to 4-day exposure to two tool compounds recommended by the IQ MPS Consortium for DILI validation (troglitazone and pioglitazone) tested at seven concentrations alongside positive control (chlorpromazine) and vehicle control (0.1% DMSO). Each condition was tested in triplicate. For mechanistic studies, the Liver-12 microtissues were subjected to 10-day exposure to three tool compounds (diclofenac, troglitazone and pioglitazone). An equivalent dose to clinical concentration and 1.5x the Minimal Important Difference (MID) clinical concentration doses were tested alongside vehicle control (0.1% DMSO). Treatments were randomised across the plates and each condition tested in triplicate. **Results:** Liver-48 plate microtissues were able to successfully differentiate the DILI risks of the tool compounds, using liver functionality (albumin, urea, ALT, AST) and viability (ATP) markers. The DILI profile of troglitazone was found to match those predicted by Liver-12 plate microtissues, demonstrating equivalence between the two plate types. The results of pioglitazone testing accurately predicted this compound to be safe. By assessing a range of test compounds recommended by the IQ MPS Consortium for DILI validation, the Liver-12 was able to correctly capture differing mechanisms of toxicity and mirror clinical outcomes. Oxidative stress, mitochondrial dysfunction, steatosis, dysregulation of bile acid synthesis or transport and inflammatory responses were investigated using a wide range of clinically relevant biomarkers. **Conclusions:** OOC/MPS has long been considered a useful tool for better predicting human outcomes, however providing scalable throughput to meet lead optimization requirements and recoverable material at scale to enable biomarker detection sensitivity, plus deep mechanistic insights - has been a challenge. Here we demonstrate that the PhysioMimix Liver MPS can be scaled to enable specific contexts of use. By incorporating human-relevant insights into the earlier lead optimization phase of drug discovery using the Liver-48 plate facilitates the design of more refined pre-clinical studies. Conversely, when the context of use shifts from compounds screening to investigative toxicology, the Liver-12 DILI assay provides the optimal solution. Data-rich analyses from a wide range of clinically relevant biomarkers can be derived to make informed decisions to modify drug design and de-risk clinical progression of drug candidates. Together, this enables more efficient drug development and safer medicines in the future.

PS 3707 Clinically Benchmarked 3D Human Liver Spheroids Advance DILI Prediction and Within-Class Differentiation

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Background and Purpose: Drug-induced liver injury (DILI) remains a leading cause of attrition and market withdrawal. Conventional 2D systems often miss clinically relevant liabilities especially when medicinal chemistry teams need series-level guidance. Traditional animal models may not fully recapitulate human-specific DILI risk, underscoring the need for more predictive, human-relevant platforms. By leveraging multicellular 3D liver spheroids, which enable crosstalk between hepatocytes and non-parenchymal cells, we establish a platform that enhances mechanistic insight and supports series-level differentiation for improved risk assessment and candidate prioritization. **Methods:** A multicellular 3D human liver spheroid platform (NAMkind™ Liver; primary hepatocytes with non-parenchymal support) was run under repeat-dose exposure (7-21 days). Drug toxicity was revealed by reduced hepatocellular function (decline in albumin levels) as well as overt cytotoxicity (LDH

release) and metabolic stress (depletion of ATP levels). We benchmarked against a 50-compound clinical reference set and profiled within-class analogs to emulate SAR decision-making at lead optimization. A proprietary machine-learning calibration integrated multivariate assay features with clinical outcomes to obtain probabilistic risk scores, confidence intervals, and series-level prioritization. **Results:** The platform achieved 91% accuracy for clinical DILI classification. It discriminated analogs within the same chemotype, aligning *in vitro* rank-ordering with known clinical risk and supporting medicinal-chemistry triage. **Conclusions:** This NAMkind™ Liver 3D spheroid assay provides robust, mechanism-aware DILI prediction beyond the limitations of animal models. For small molecules and practical analog rank-ordering to guide SAR. This advances a NAMs-aligned, translational Context-of-Use Case for early candidate selection.

PS 3708 Human Liver Organoid Models: Advancing Drug Toxicity Assessment

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Background and Purpose: Current drug toxicity assays predominantly rely on the HepG2 hepatocarcinoma cell line, which lacks key liver-specific metabolic functions, exhibits limited cytochrome P450 enzyme activity, and poorly reflects the physiology of healthy human hepatocytes. To establish a more physiologically relevant *in vitro* system, we developed a novel differentiation medium that enables adult stem cell-derived liver organoids, which primarily consist of cholangiocytes, to mature into hepatocyte-like cells suitable for drug toxicity testing, thereby bridging the gap between tumour cell models and *in vivo* systems. **Methods:** In this study, we sought to identify a differentiation condition that robustly generates human hepatocyte-like organoids. We established liver organoids from five different donors and subsequently differentiated them towards a hepatocyte-like state. The organoid model was characterised before and after differentiation using RNA sequencing, cytochrome P450 activity assays, and immunofluorescence staining. Finally, we benchmarked our differentiated organoid model against HepG2 and primary human hepatocytes, based on their ability to detect Fialuridine toxicity, a compound historically withdrawn from clinical trials due to unpredicted hepatotoxicity. **Results:** We successfully established a reliable and physiologically relevant human hepatocyte organoid model. By applying our novel differentiation medium, the adult-derived organoids matured into hepatocyte-like cells that exhibited characteristic hepatic morphology and demonstrated functional cytochrome enzyme activity. Compared to the commonly used HepG2 cell line, our organoid-derived hepatocytes showed higher metabolic activity and more accurate dose-dependent responses to known hepatotoxic compounds. **Conclusions:** The results indicate that the developed organoid system more closely resembles *in vivo* liver physiology than the transient primary hepatocyte cell model and hepatocarcinoma-derived HepG2 cells. This new hepatocyte-organoid model provides a stable platform for assessing drug toxicity in preclinical drug screening.

PS 3709 Human 3D Liver Spheroids Establish a NAM for ADC DILI Risk Assessment

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Background and Purpose: Antibody-drug conjugates (ADCs) can present hepatic liabilities arising from conjugate design and clinical exposure paradigms. There is a need for human-relevant, throughput-compatible assays to generate early translational signal even when mechanistic uptake or payload analytics are not yet available. Here, we introduce a multicellular 3D human liver spheroid platform that enables functional profiling and comparative ranking of intact ADCs, facilitating early risk assessment and supporting decision-making in ADC development. **Methods:** A multicellular 3D human liver spheroid platform (NAMkind™ Liver; primary hepatocytes with non-parenchymal support) was run under repeat-dose conditions (7-21 days). Intact ADCs were evaluated across clinically relevant exposure ranges using ATP (CellTiter-Glo), LDH, and albumin as a functional triad. **Results:** Intact ADCs produced concentration- and time-dependent changes across ATP/LDH/albumin, enabling initial hepatic risk profiling and comparative ranking of conjugates. Signal patterns differentiated functional impairment versus overt cytotoxicity, providing actionable readouts for program teams while orthogonal mechanistic assays are being planned. **Conclusions:** The NAMkind™ Liver 3D spheroid platform offers a translational, mechanism-aware approach for intact-ADC DILI risk assessment, providing decision-quality functional data ahead of deeper mechanistic deconvolution, supporting NAMs-aligned development.

PS 3710 Development of iPSC-Derived Human Liver Organoids for Preclinical Drug Testing and Toxicology Studies

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Background and Purpose: Over 90% of drug candidates fail during clinical trials, often due to insufficient efficacy and unmanageable toxicity. Drug-induced liver injury (DILI) is frequently missed in preclinical testing due to a lack of robust and predictive liver models. We report the *de novo* development of iPSC-derived human liver organoids as scalable, reliable, and physiologically relevant preclinical toxicology models. **Methods:** Using an efficient and reproducible iPSC-differentiation protocol, we generated long term expandable, cryopreservable bipotential 3dGRO™ Human iPSC-derived Liver Progenitor Organoids, which differentiate into mature liver organoids (MLOs) containing multiple liver cell types. **Results:** The iPSC-derived mature liver organoids display long-term stability, secrete albumin and urea, and express key biomarkers of mature hepatocytes (albumin, CYP3A4, HNF4a, PCK2) and cholangiocytes (Sox17, Sox9, CK7, MRP2). They also exhibit functional Phase I/II liver enzymes (CYP3A4, CYP2C9, CYP1A2, ALT, AST, GST) and active bile salt/drug transporters. Compared to primary hepatocytes, our 3dGRO™ Human iPSC-derived Mature Liver Organoids demonstrate comparable functionality and outperform HepG2 cells and liver spheroids. In DILI assays, MLOs responded to drug treatment with elevated liver enzymes, confirming their potential for toxicology screening. **Conclusions:** These liver organoids represent a powerful tool for high-throughput drug screening, DMPK and toxicological studies, with future applications including disease modeling of hepatocellular carcinoma, MASLD, and NASH.

PS 3711 DEVELOPMENT OF HUMAN IPSC-DERIVED HEPATOCYTES FOR DRUG DISCOVERY, TRANSLATIONAL RESEARCH AND TOXICITY TESTING

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Background and Purpose: Hepatocytes are responsible for the primary functions of the liver, including detoxification and xenobiotic metabolism, making them essential for *in vitro* toxicology. Despite their critical role, their use is hampered by significant challenges with traditional models, such as the scarcity and variability of primary human hepatocytes (PHHs), the poor physiological relevance and metabolic function of immortalised cell lines, and the poor scalability and high lot-to-lot variability of conventional human induced pluripotent stem cells (hiPSCs) directed differentiation methods. These limitations hinder the ability to effectively predict drug-induced liver injury (DILI), heavily contributing to the 90% attrition rate of drug candidates in clinical trials and underscoring the need for better liver models. Here, we used opti-ox™, a deterministic cell programming technology, to generate consistent, scalable, hiPSCs-derived hepatocytes, termed ioHepatocytes, and demonstrated their suitability for toxicology, research and disease modelling. **Methods:** ioHepatocytes were generated through forward programming of hiPSCs using proprietary opti-ox™ technology and were characterized by immunofluorescence, transcriptomics, and assays for hepatic function (e.g., albumin secretion) and metabolic competency (CYP450 activity). DILI prediction was validated by assessing viability (CellTiter-Glo) after challenge with known hepatotoxins. **Results:** ioHepatocytes display classic cobblestone morphology with distinctive nuclei and well-defined borders. Cells express key pan-hepatocyte markers including ALB, HNF4A, ASGR1 and SERPINA1, and present a transcriptomic signature similar to PHHs. Additionally, ioHepatocytes derived from hiPSCs with opti-ox™ demonstrate high batch-to-batch consistency. Functionally, ioHepatocytes perform critical hepatocyte functions including albumin secretion, glycogen storage, and accumulation of lipids. Most importantly, ioHepatocytes show expression of genes involved in phase I, II and III of drug metabolism and have functional cytochrome P450 enzymes (CYP3A, CYP2B6 and CYP1A2). When challenged with compounds known to cause toxicity, ioHepatocytes show a clear dose-dependent decrease in cell viability that strongly correlates with the known DILI severity of the compounds. Importantly, the toxic response very closely mimicked that of PHHs, validating the predictive capability of ioHepatocytes. **Conclusions:** ioHepatocytes offer a highly consistent, functional and scalable human hepatocyte model, overcoming the limitations of existing technologies. Their demonstrated ability to respond to toxic insults similarly to PHHs suggests their use as a reliable, predictive tool for improving DILI risk assessment in drug development.

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Background and Purpose: Hepatotoxicity can occur as a consequence of exposure to adulterants in foods and dietary supplements. Traditional animal studies are too inefficient to assess all potential hepatotoxicants in the food supply. Therefore, more cost effective and efficient new approach methods (NAMs) have emerged to address this urgent public health need, and facilitate the replacement, reduction, and refinement of *in vivo* studies. Described, herein, is the development of a three-tiered NAM for the assessment of potential hepatotoxicity, with each tier offering increased toxicological and mechanistic data. To demonstrate the applicability of this tiered approach, a known hepatotoxicant, usnic acid (UA), was evaluated as a performance compound. **Methods:** In the first tier, primary human hepatocytes (PHH) seeded in 96-well plates were exposed to a 10-point concentration range of the potential toxicant for 48 hours. Tier 1 examined markers of both liver function and viability. Concentration-response modeling was then applied to determine an effective concentration (ACC) at which liver function was altered without impacting cell viability. In Tier 2, PHH again were seeded in 96-well plates and exposed to a single effective concentration, as identified in Tier 1, of the potential toxicant for 48 hours. Tier 2 consisted of imaging of pertinent functional biomarkers to gather mechanistic data about the potential hepatotoxicant. Tier 3 incorporated four distinct liver cell types (PHH and non-parenchymal cells (NPCs): liver sinusoidal endothelial cells (LSECs), Kupffer cells, and stellate cells) in a microphysiological system (MPS), a liver-on-a-chip. After initial exposure, effluents were collected every 2-3 days, for 9 days. The same endpoints assessed in Tier 1 and Tier 2 were evaluated in Tier 3, with additional endpoints added for the NPC channel. **Results:** For assay development, UA was applied at a 10-point concentration range from 0-50 μM to demonstrate Tier 1 performance. A decline in hepatocyte function and cytotoxicity was observed after 48 hours of exposure. The least sensitive functional endpoint (urea content) had an ACC at 9.6 μM , while the most sensitive cytotoxicity endpoint (alanine transferase (ALT)) had an ACC at 15.9 μM after 48 hours. Therefore, 10 μM UA was selected for subsequent evaluation in Tiers 2 and 3. In Tier 2, UA exposure disrupted transporter activity (80% decrease) and phospholipid content (20% decrease). Tier 3 similarly showed a significant decrease in hepatocyte function resultant to UA exposure, with the most sensitive functional endpoint (albumin) being reduced by 82% after 9 days of exposure compared to pre-exposure values. Cytotoxicity significantly increased compared to day-matched control after 48hrs with a more pronounced increase after 7 days of exposure. Most notably, ALT levels were 3000% higher than control. Cells collected after 9 days of exposure demonstrated a 40% reduction in glutathione, a 60% reduction in adenosine triphosphate (ATP) content, as well as a significant disruption of mitochondrial membrane potential and transporter activity. An inflammatory cytokine panel also exhibited significant decreases (53-58%). **Conclusions:** Application of this tiered approach demonstrated that a known hepatotoxicant can be appropriately evaluated with enhanced toxicological and mechanistic information gained from each tier. Tier 1 allowed for a rapid evaluation of effects on functional endpoints, with the determination of an effective concentration for assessment in subsequent tiers. Tier 2 expanded the mechanistic information collected in Tier 1, allowing for a more thorough investigation of functional endpoints, albeit with fewer concentrations. Tier 3 furthered the cell types included, as well as relevant physiology, demonstrating greater biological complexity which allows for further enhanced toxicological and mechanistic information. Further investigation into the use of this tiered approach using more complex materials is required, however, this method has potential to more efficiently evaluate foods and dietary supplements for possible hepatotoxicity.

PS 3713 Evaluating the hepatotoxic potential of Green Tea and Kava Root Extract in primary human hepatocytes

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Background and Purpose: Evaluation of botanical safety is a rising need due to increased use in dietary supplements and other consumer products. Hepatotoxicity can sometimes occur when consumers are exposed to potentially toxic constituents in botanicals. Traditionally animal studies have been utilized for safety assessment. However, animal studies are often limited by the time and cost necessary to evaluate compounds. New Approach Methods (NAMs) are becoming more widely used to reduce animal testing and can potentially aid in decreasing the time and cost commitment of studies. However, NAM designs also have important considerations such as stability of the treatment compounds and their interaction with experimental components. In this study, our goal was to determine the ability of a higher throughput NAM to identify potential hepatotoxicity of complex materials using two botanical extracts, green tea and kava root, which have been reported to cause hepatotoxicity in humans. **Methods:** To evaluate the hepatotoxicity of green tea extract (GT) (Indena) and kava root extract (Kava) (Mountain Rose Herbs) UHPLC/MS was performed to confirm the concentrations of constituents and the solubility in dimethyl sulfoxide (DMSO). Subsequently, a 10-point concentration-response was created based on the solubility of the extracts in DMSO and the necessity to maintain a minimal level of DMSO (0.25%) in cell culture medium. For GT, concentrations ranged from 1.22-313 $\mu\text{g/mL}$ and for Kava, concentrations ranged from 0.059-15

$\mu\text{g/mL}$. Solubility, stability, and interaction with the cell culture media and plate were evaluated at treatment concentrations, in the absence of cells, to ensure the cells receive the intended concentrations of the extracts. Primary human hepatocytes (PHH) from three separate donors were seeded into 96-well plates and treated with the extracts for 48 hrs, with media exchanged every 24 hrs. A positive performance compound of 20 mM acetaminophen (APAP) was also included on each plate. After 48 hrs, key hepatotoxicity endpoints (i.e., changes in secretions of albumin, urea, lactate dehydrogenase (LDH), alanine transaminase (ALT), adenosine triphosphate (ATP), and glutathione (GSH)) were measured. **Results:** GT showed hepatotoxicity within 48 hrs in this study. ATP and GSH were the most sensitive endpoints with decreased levels starting at 19.5 $\mu\text{g/mL}$; whereas albumin was reduced and ALT was increased starting at 39.1 $\mu\text{g/mL}$. However, the stability of GT in hepatocyte media was low. After 24 hrs in glass vials, recovery of (-)-Epigallocatechin gallate (EGCG), the most abundant constituent in GT, was reduced, albeit variably. Additionally, after incubation in the 96 well plates, without cells, recovery was reduced even further, at least 80% less than expected. Unlike GT, Kava showed minimal hepatotoxicity. There was a slight reduction in albumin starting at 7.5 $\mu\text{g/mL}$ and a very slight increase in ALT at the same concentration. ATP was reduced slightly starting at 0.234 $\mu\text{g/mL}$ and GSH was reduced starting at 1.88 $\mu\text{g/mL}$. Albumin, ATP, and GSH were only reduced about 30% compared to control at the highest concentration. In all experiments APAP strongly affected all endpoints evaluated. Conversely to GT, Kava was stable in hepatocyte media in glass vials. However, similar to GT there was some interaction with the cell culture plate. Three constituents; methysticin, flavokawain A, and flavokawain B, lost 40-90% when incubated without cells within 24 hrs. **Conclusions:** New approach methods can help increase the throughput necessary to evaluate complex materials such as botanical extracts. However, evaluation of the stability and interaction of the compounds of interest in the experimental design should be examined. Here, two opposite situations occurred. GT appeared to affect PHH in a dose dependent manner, however, closer evaluation of the stability of GT in the media and potential interaction with the culture plate questions the accuracy of the GT concentration the cells received. Alternatively, Kava did not appear to have a great effect on PHHs. However, three constituents, including flavokawain B, which is believed to be the hepatotoxic component of kava root, appeared to interact with the culture plate, which may explain the limited toxicity seen in this model.

PS 3714 ML/AI assisted digital measure of pica behavior in emetic-induced rat emesis model

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Background and Purpose: Drug-induced emesis is a major symptom of gastrointestinal toxicity in both humans and laboratory animals. In drug development, preclinical assessment of emesis risk remains challenging due to the lack of suitable animal models and sensitive behavioral or functional biomarkers. While humans, dogs, and several other mammals exhibit emetic reflexes, commonly used toxicological species such as rodents do not. Instead, rodents rely on alternative defense mechanisms, such as pica, to cope with toxicosis. In rats, daily kaolin consumption is typically measured as an indicator of pica behavior. However, environmental stressors can also trigger pica, potentially confounding research outcomes. Although useful, the kaolin pica method faces challenges, including measurement difficulties, individual variability, and limited diagnostic specificity. **Methods:** In this study, we employed computer vision and machine learning (ML)/artificial intelligence (AI) algorithms to analyze continuous video recordings of Wistar Han rats in their home cages, enabling automated identification of pica behavior—specifically, kaolin consumption—over a 24-hour period following administration of copper sulfate, apomorphine, and semaglutide ($n = 6$ per treatment group). Rats were pair-housed, with one animal identified by an ear tag. In each cage, one animal was dosed with the emetic and the other with vehicle. A convolutional neural network model was trained on approximately 500 images and validated using manual annotations of feeding behavior sampled every other minute from the video data. All pica events were verified by human observers. **Results:** Copper sulfate (40 mg/kg, p.o.), apomorphine (10 mg/kg, i.p.), and semaglutide (0.12 mg/kg, s.c.) induced kaolin consumption behavior, particularly during nighttime (combined, $p < .001$ compared with 18 vehicle controls). Pica behavior occurred between 2 to 9 hours post-dosing and lasted from several seconds to approximately one minute per bout, with most animals exhibiting only a single bout. Pica appeared earlier in copper sulfate-treated animals and later in semaglutide-dosed animals. Additional toxicological effects—such as apomorphine-induced stereotypic peripheral cage rotation—and pharmacological responses—such as semaglutide-induced reductions in feeding behavior frequency and duration—were also observed within 24 hours post-dose. The AI-generated results were reasonably accurate and comparable to manually analyzed data, though some false positives indicate the need for further model training. The models achieved F1 scores ranging from 0.27 to 0.55 for feeding and drinking behavior. **Conclusions:** This study demonstrates the utility of ML/AI-assisted digital measurement—or digital biomarkers—for monitoring pica behavior in rat toxicity and pharmacology studies using an emetic-induced emesis model.

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Background and Purpose: The ICH M7 guideline recommends an initial *in silico* assessment of pharmaceutical impurities using two complementary (Q)SAR methodologies to predict bacterial mutagenicity. It also suggests expert review if warranted (i.e., cases where model predictions are discordant, inconclusive, or outside the applicability domain). This study investigates whether a large language model (LLM) can assist in generating expert-level justifications for mutagenicity predictions based on *in silico* outputs. **Methods:** A dataset of 684 chemicals with known experimental results was analyzed using CASE Ultra statistical and expert rule-based models. Outputs were further analyzed with the Konsolidator tool, which uses structural alerts in the query compound to identify structural analogs and generate a final call: POSITIVE, NEGATIVE, or NO CALL. From this dataset, 75 compounds (25 per category) were randomly selected. Each Konsolidator report was submitted to ChatGPT v4o for interpretation under a standardized prompt, which asked for an executive summary aligning with ICH M7, including a mutagenicity prediction, ICH M7 classification, and supporting rationale. ChatGPT was configured without memory and allowed to browse the web. The same 75 compounds were also independently evaluated by a human expert under conditions of a blind experiment. The results produced by ChatGPT and the human expert were compared with experimental outcomes for each compound to evaluate performance. **Results:** The human expert achieved the highest prediction accuracy for bacterial mutagenicity (80%) and 100% coverage. ChatGPT's performance was notable, with 61% accuracy and 91% coverage. ChatGPT also demonstrated a marked ability to correctly identify negative compounds with a specificity of 79%. In contrast, its ability to correctly identify positive compounds was low (sensitivity of 38%). The LLM's handling of "NO CALL" cases were particularly interesting. It provided a conclusive interpretation in 21 out of 25 cases (84%), with an accuracy rate of 64%. All outputs referenced structural alerts and analogs, but the quality and depth of analysis varied across compounds. A recurring issue involved the ICH M7 classification: ChatGPT misclassified 9 compounds, labeling them as ICH M7 Class 3 while predicting them as negative. Of these, 7 were originally predicted as NO CALL and 2 were POSITIVE; only the POSITIVE mentioned "Class 3." Still, ChatGPT correctly predicted 8 of these 9 outcomes. It also labeled 3 compounds as ICH M7 Class 4, despite not being provided an API or other compounds for comparison. **Conclusions:** This study highlights the strengths and limitations of generative AI in regulatory toxicology, as well as the role of human experts in this field. While the human expert outperformed the LLM in both accuracy and coverage, the LLM's ability to process and summarize complex *in silico* data, particularly in NO CALL cases, was promising. The study was limited to the use of a single LLM, a fixed prompt, and a manual input process, which may not reflect variability or scalability in real-world applications. Future work should explore automated workflows, alternative LLMs, and the interpretation of raw (Q)SAR outputs and outputs from other software. Overall, LLMs may serve as valuable tools for generating structured summaries that assist expert workflows, but they should not yet be used as a standalone tool for interpreting *in silico* results.

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Background and Purpose: The increasing complexity of regulatory toxicology and the volume of scientific literature present significant challenges for timely and auditable human safety assessments. This work explores the feasibility of an agentic artificial intelligence (AI) framework to simulate a virtual Human Safety (HuSa) team, enabling automated, multi-agent collaboration for pesticide risk assessment and adverse effect screening. **Methods:** A proof-of-concept system was developed in Python using large language models (LLMs) configured as domain-specific agents representing toxicology, dietary, occupational, and director roles. The system orchestrated structured, multi-round 'virtual meetings' where agents analyzed regulatory assessments and peer-reviewed literature. Key design elements included document chunking, numeric and citation enforcement, and iterative decision-making under predefined rules. **Results:** The virtual HuSa team successfully synthesized evidence from regulatory documents and literature, producing auditable outputs with traceable evidence chains. The system demonstrated the ability to classify study relevance, summarize findings, and evaluate potential regulatory implications. A second proof-of-concept extended the approach to adverse effect screening, identifying findings warranting further consideration under regulatory criteria. **Conclusions:** This work illustrates the potential of agentic AI to augment regulatory toxicology workflows by improving efficiency, transparency, and reproducibility. Future development will focus on creating a more user-friendly interface and enhancing model specialization, establishing a strong foundation for integrating AI-driven decision support into human safety risk assessment.

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Background and Purpose: In the European Union, the Safe and Sustainable by Design (SSbD) concept is a cornerstone of the EU Chemicals Strategy for Sustainability, aiming to promote innovation of safer and more sustainable chemicals while ensuring a high level of protection for human health and the environment. Achieving this goal requires systematic access to transparent, reproducible, and mechanistically anchored data. New Approach Methodologies (NAMs) are transforming chemical safety assessment by reducing reliance on animal testing while providing mechanistic insight into toxicity pathways. Despite their scientific maturity, the widespread regulatory uptake of NAMs remains limited due to challenges in accessibility, interoperability, and contextual fit-for-purpose application. The NAMs.Network (<https://nams.network/>) initiative addresses these challenges by creating a digital ecosystem that supports the searching, integration, and application of NAMs within regulatory frameworks. It connects regulatory needs with curated assays, Adverse Outcome Pathways, and computational models, ensuring transparent evidence generation across diverse endpoints. In this study, we present a practical, regulation-oriented workflow that leverages NAMs.Network to identify and apply ready-to-use NAMs for specific regulatory contexts: assessing the safety of flame-retardant (FR) alternatives. Our objective was to conduct a critical review of existing NAMs and to demonstrate how they can support SSbD implementation. **Methods:** A regulation-oriented workflow was developed using the NAMs.Network platform to identify and apply NAMs supporting SSbD assessment of FR alternatives. Regulatory needs were translated into a structured evidence plan and data-gap matrix to pinpoint missing or insufficient information. The NAMs.Network was queried for suitable *in vitro* assays, computational models, and other mechanistically anchored tools addressing these gaps. Collected NAMs were critically evaluated for scientific robustness, mechanistic relevance, validation status, regulatory applicability, data quality and transparency, and availability of SOPs. **Results:** To identify and apply ready-to-use NAMs within the SSbD framework, the first step involved defining the regulatory decision context, specifying the intended uses of FRs, and their exposure scenarios. Based on applicable EU regulations and supporting guidance documents, a requirements map was constructed to outline the relevant endpoints necessary for regulatory compliance. This map served as a structured evidence plan, defining what needs to be demonstrated for different regulatory purposes, including screening or comprehensive hazard assessment. In the second step, the map was translated into a data-gap matrix, which systematically captured available data and pinpointed information gaps across hazard domains. Particular attention was paid to regulatory-driven endpoints with limited existing data, such as neurotoxicity, developmental and reproductive toxicity, and endocrine disruption. Using the NAMs.Network platform, these gaps were queried against a curated inventory of available NAMs. More than 20 candidate methods were identified and subsequently subjected to a critical evaluation according to predefined criteria. Based on this evaluation, methods were categorized as "ready-to-use", indicating direct applicability in a regulatory context, or "conditionally applicable", requiring further data generation, optimization, or validation to reach regulatory readiness. This structured and transparent approach enabled the translation of regulatory needs into actionable safety assessment strategies within the SSbD paradigm. **Conclusions:** The application of the regulation-oriented workflow demonstrated that NAMs.Network can effectively support the systematic identification and integration of ready-to-use NAMs for assessing the safety of alternative FRs. By linking regulatory requirements to mechanistically anchored evidence, the approach enables transparent, reproducible, and efficient safety evaluation.

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Background and Purpose: Drug-induced liver injury (DILI) remains a leading cause of drug attrition, highlighting the limitations of traditional preclinical models and static computational tools in predicting human hepatotoxicity. **Methods:** We developed a dose-enabled AI/ML framework that integrates physicochemical descriptors, predicted off-target interactions, and exposure as a continuous variable to generate dynamic, mechanistically interpretable risk profiles. A curated dataset of compounds annotated for most (M-DILI), no (N-DILI), and less (L-DILI) hepatotoxicity was used to train ensemble classifiers. **Results:** Physicochemical features such as aqueous solubility (logS) and fraction of sp³ carbons (Fsp³) differentiated risk classes, consistent with mechanisms of hepatic accumulation and promiscuous binding. Off-target networks enriched for cytochrome P450s, nuclear receptors, and adrenergic proteins further stratified high-risk compounds, while predicted metabolites captured bioactivation liabilities. Incorporating dose enabled simulation of dose:response trajectories, estimation of hepatotoxic thresholds (Probability score EC₅₀), and identification of latent risks. Acetaminophen and fluoxetine transitioned into high-risk categories at low exposures, whereas streptomycin remained below toxic thresholds even at elevated doses. Although trained on binary endpoints (M-DILI vs. N-DILI), the model generalized to L-DILI compounds, yielding a bimodal risk distri-

bution that reflected ordinal toxicity relationships. The ensemble classifier outperformed individual models (AUC = 0.88; balanced accuracy = 0.80), demonstrating that predictive strength emerges from the convergence of chemistry, biology, and dose. **Conclusions:** By embedding exposure into model architecture, this framework reframes predictive toxicology from static classification toward exposure-informed, mechanistically grounded prediction, offering a scalable, non-animal tool aligned with FDA's New Approach Methodologies.

PS 3719 Novel Machine Learning Algorithms Allow Fast and Accurate Automated Counting of Primary Hepatocyte Samples

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Background and Purpose: Primary hepatocytes are a critical cell type in toxicity and drug metabolism workflows. Given the sensitive nature of these experiments, accurate and precise hepatocyte counts are essential for achieving reproducible results for downstream applications. We have developed a machine learning-based model for counting hepatocytes and determining viability on an automated cell counter to 1) help standardize results across laboratories and 2) reduce user to user variability. This study aims to test if an automated machine learning model can provide the same level of counting accuracy for hepatocyte quantification against the established gold standard method of manual hemocytometer counts by trained scientists. Data sets were generated independently by internal DeNovix scientists and collaborating scientists at a leading supplier of commercial hepatocytes (BiolVT). **Methods:** This study evaluated the viability and concentration of cryo-preserved and freshly isolated hepatocyte cells. The cells, sourced from common toxicology species (human, rodent, and canine), were isolated or thawed according to recommended protocols. Subsequently, they were stained with either 0.4% trypan blue or a combination of 12 uM Acridine Orange and 140 uM Propidium Iodide (AO/PI). Cell concentration and viability were then determined using either a hemocytometer manual counting technique using trypan blue and a Neubauer grid by trained technicians on a 4x brightfield microscope or with an automated cell counter with a machine learning based algorithm using AO/PI staining to distinguish between live and dead cells. **Results:** Results of hepatocyte counts and sample viability determinations between the counting methods were compared using the unpaired two tailed Welch's t-test in GraphPad Prism version 10.6.1, for Mac OS GraphPad Software, Boston, Massachusetts USA. Calculated p-values across data sets of all three species showed p-values greater than an alpha value of 0.05. Additionally, these p-values were supported by the calculated 95% confidence intervals for each of the Welch's t tests performed. Overall, the hepatocyte count data collected in this study between the two methods showed no significant statistical differences in the mean values of sample concentration or percent viability. **Conclusions:** Automation provides significant advantages over manual counting in both time and consistency, especially when accounting for multiple technicians and variations in technique and training within or between various labs. The CellDrop's Hepatocytes App also quickly and accurately provides additional information about the sample not captured in manual counting such as the level of debris, free floating nuclei, and the number of non-parenchymal cells vs hepatocytes present.

PS 3720 Beyond spatiotemporal modeling: applications of machine learning for traffic-related airpollution toward non-exhaust emissions

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Background and Purpose: Traffic-related air pollution (TRAP), including tailpipe emissions and non-exhaust emissions, is a major health concern in urban areas and is associated with various adverse health outcomes. As tailpipe emissions decrease due to regulatory efforts, non-exhaust emissions have become a more significant source of pollution. Machine learning (ML) has been widely used to better characterize and quantify traffic-related air pollutants, enhancing understanding of their compositions and spatiotemporal distributions related to health implications. In this study, we hence reviewed publications on applying ML for traffic-related air pollution to summarize recent applications of ML in traffic-related air pollution studies with a special focus on non-exhaust emissions and cover wider applications beyond spatiotemporal modeling. **Methods:** Over 50 peer-reviewed papers related to ML and TRAP were collected from Google Scholar and PubMed using key words, including "machine learning", "traffic-related air pollution", "deep learning", and "air pollution". Widely used features/variables that are involved in TRAP modeling were collected and summarized, with special emphasis on traffic-related features. We further summarized major applications of ML in TRAP research, and discussed existing challenges and opportunities. **Results:** We identified four major applications of ML in TRAP research: (1) TRAP spatial modeling, (2) identification of contributing factors to TRAP, (3) characterization of non-exhaust emissions, and (4) source tracking and apportionment. We found that random forest models were predominantly used in TRAP spatial modeling while deep learning models were often used to process image-based air pollution data and/or capture temporal information. ML models

have also been used to identify and classify types of PM components, especially from brake and tire wear, using image and spectrum data from instrumental analysis. The interpretation of ML models can improve our understanding of how traffic related features such as traffic patterns and driving characteristics contribute to TRAP. **Conclusions:** Machine learning has shown great potential in TRAP research, which helps generate high-resolution spatial exposure maps and improve our understanding of TRAP exposures. With the advancement of ML methods and their further integration with environmental health, ML will continue to contribute to better assessing the effects of TRAP exposure on human health. Furthermore, the use of ML models coupled with the diverse traffic-related air pollution measurements will provide essential information for risk assessment and inform public health policies.

PS 3721 Harnessing Artificial Intelligence To Advance Clinical Pharmacology And DMPK In Drug Development

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Background and Purpose: Drug development relies heavily on the accurate interpretation of pharmacokinetic, pharmacodynamic, and safety data to guide clinical dose selection and regulatory decision-making. However, these processes are often hindered by the complexity and volume of unstructured information within regulatory submissions, reviews, and product labeling. Extracting relevant data to inform modeling, FIH dose estimation, or risk assessment typically requires extensive manual effort, limiting efficiency and reproducibility. Recent advances in artificial intelligence (AI), particularly natural language processing (NLP) and machine learning, provide new opportunities to systematically identify, extract, and contextualize pharmacological and regulatory data from historical documentation. Integrating these approaches within regulatory intelligence platforms has the potential to streamline analysis, improve data transparency, and support evidence-based decision-making across Clinical Pharmacology and DMPK disciplines. **Methods:** PharmaPendium AI was built upon a structured database of over 5,000 approved drugs encompassing FDA and EMA reviews, labeling, and approval packages. Advanced natural language processing (NLP) and machine learning algorithms were applied to extract, normalize, and classify pharmacokinetic and pharmacodynamic data, dose rationale information, and safety findings from unstructured text. The system was designed to support natural language queries, enabling scientists and regulatory professionals to obtain context-specific information efficiently. Targeted modules were developed to identify transporter- and enzyme-mediated drug-drug interactions (DDIs), evaluate hepatotoxicity risks by mining Risk Evaluation and Mitigation Strategy (REMS) and clinical hold documents, and retrieve precedent-based language and rationales used in regulatory decision-making. **Results:** The platform's capabilities were assessed through a case study involving a novel tyrosine kinase inhibitor targeting colony-stimulating factor 1 receptor (CSF1R), developed to improve upon pexidartinib for treatment of tenosynovial giant cell tumor (TGCT). AI-driven text mining aggregated preclinical selectivity data, pivotal trial outcomes (e.g., ENLIVEN study), and hepatotoxicity observations to inform dose optimization strategies. Analysis of REMS and regulatory correspondence identified mechanistic insights into liver toxicity, supporting refinement of liver monitoring protocols. Extraction of transporter and enzyme data informed DDI risk assessments, while analysis of biomarker-related content supported patient stratification approaches. These outputs provided multidisciplinary teams with data-driven evidence to guide dosing, safety monitoring, and submission preparation. **Conclusions:** Integration of AI-based text mining and data normalization into regulatory intelligence platforms can substantially improve the accessibility and utility of pharmacological and regulatory data. The PharmaPendium AI framework demonstrates how machine learning and NLP can enhance decision-making across Clinical Pharmacology, DMPK, and Regulatory Affairs by reducing manual data retrieval, improving safety signal detection, and supporting evidence-based regulatory interactions. Broader implementation of such tools may contribute to more efficient, transparent, and scientifically grounded drug development processes.

PS 3722 Interpretable Deep Learning Framework for Predicting Protein Corona Adsorption and Abundance on Nanoparticles

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Background and Purpose: Nanoparticles (NPs) hold great promise for diverse biotechnological applications, including molecular sensing and therapeutic delivery. However, their interactions within biological systems remain unpredictable, as spontaneous protein corona (PC) formation alters surface properties and governs biological identity, transport, and cellular uptake. Understanding and predicting PC composition is essential, but it remains challenging due to inconsistent data and complex interactions between NP properties and experimental conditions. This study aims to develop an interpretable machine learning framework to predict protein adsorption and abundance on NPs. By integrating comprehensive physicochemical and experimental descriptors, the framework elucidates key determinants of PC formation and provides a systematic basis for rational nanomedicine design. **Methods:** Data were obtained from a curated PC database (PC-DB) that consists of 817 NP-PC

samples, and 2,497 proteins identified across 4 different species (human, mouse, rat, and bovine), as described in detail in our previous publication. For this analysis, only human-derived proteins were included, and those detected in more than 5% of samples were retained to reduce sparsity. Eleven physicochemical and experimental features were selected as model inputs after cleaning and encoding categorical variables. Two deep neural network (DNN) models were developed: a binary classifier to predict protein adsorption and a regression model to estimate relative abundance. Model training incorporated Synthetic Minority Oversampling Technique (SMOTE) for data balancing, logarithmic transformation and scaling of abundance values, and Bayesian optimization with early stopping to improve robustness. The applicability domain (AD) was defined using the Euclidean distance in the standardized feature space to identify reliable prediction regions and detect out-of-domain samples. Model interpretation was performed using SHapley Additive exPlanations (SHAP) to identify NP and experimental features driving protein adsorption and abundance predictions. **Results:** The adsorption (classification) model achieved a mean accuracy of 0.90 and an AUC of 0.96 on independent test sets, showing strong reliability across diverse NP-PC pairs. The abundance (regression) model reached an average R^2 of 0.52 despite the highly variable and skewed protein distribution, indicating realistic capture of biological trends. AD analysis indicated that most of the test samples fell within the defined domain, and predictions within this region showed markedly higher accuracy and lower error compared to out-of-domain samples. SHAP analysis revealed distinct feature importance patterns between tasks: protein adsorption was primarily driven by nanoparticle physicochemical properties such as core material and surface modification, while protein abundance was more strongly influenced by experimental parameters, especially incubation and centrifugation time. **Conclusions:** This study presents an interpretable deep learning framework for predicting both the presence and abundance of proteins on NP surfaces. By integrating curated experimental data with standardized preprocessing and SHAP-based interpretation, the models captured key physicochemical and procedural factors that determine PC formation. These findings provide a data-driven foundation for understanding nano-bio interactions and offer practical guidance for designing safer and more reproducible, and functionally optimized nanoparticles in biomedical applications.

3723 Uncertainty Quantification in Toxicity Machine Learning Models for Use in MegaTox

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Background and Purpose: Uncertainty quantification is crucially important for predictive machine learning (ML) models, and particularly relevant to toxicity predictions, where prediction confidence can impact life-or-death decisions. Regardless of how a model performs in cross validation or on a test set, it is useful to know the uncertainty associated with a particular prediction. There are two flavors of uncertainty to consider: aleatoric and epistemic. Aleatoric uncertainty is due to noise in the training data (e.g. measurement error). Epistemic uncertainty is due to lack of knowledge (i.e. the sample is out of domain). We now describe a novel approach to quantifying uncertainty in ML models and apply it to a wide array of toxicity endpoints. **Methods:** We have developed a new approach to uncertainty quantification called Domain Classification (DC) modeling. We begin with a dataset with "inactive" and "active" compounds (e.g. IC_{50} or LD_{50} above or below a threshold). We also draw a random sample of molecules from the ChEMBL database. We then train three binary classification models to predict the probability that a given molecule came from one set versus another: ChEMBL vs. inactive, ChEMBL vs. active, and inactive vs. active. Predictions from these models are combined into four probabilities: P(inactive), P(active), P(ambiguous) and P(out-of-domain), where the latter two represent aleatoric and epistemic uncertainty. Since the prevalence of the out-of-domain distribution (random ChEMBL molecules) is unknown, we can define an uncertainty window based on P(out-of-domain), which sets bounds on the probability prediction for any given molecule. **Results:** We demonstrate the efficacy of this approach by building DC models for acetylcholinesterase (AChE) inhibition, for which we have a large and well-understood training set. We show that, for test sets specifically chosen to be distinct from the training set, the true probability of activity (i.e. the fraction of active compounds in the test set) stays within the bounds defined by the DC uncertainty window; moreover, we show that the uncertainty window widens as a function of the average domain coordinate (e.g. Tanimoto distance) of the test set. We also observe a significant improvement in cross validation model performance metrics if we filter out samples where the uncertainty window overlaps with both classes (equivalently, if we only count samples where either P(inactive) or P(active) is greater than 50% regardless of uncertainty probability). For the AChE model, this leads to a balanced accuracy improvement of 5.4% (specificity and recall improvement of 2.2% and 8.7%, respectively) over the full training set, at the cost of discarding about 25% of cross validation predictions due to uncertainty. We then extend the DC modeling approach to a wide range of toxicity models, including over 40 "safety screen" targets, skin irritation and sensitization, eye irritation, acute inhalation, cardiotoxicity (hERG, hypertension, arrhythmia, etc.), DART, carcinogenicity, genotoxicity, endocrine disruption, LD_{50} , DILI, nephrotoxicity, and CYP inhibition. These ML models represent a small selection of those in our MegaTox software, a commercially available web app which predicts properties of molecules using ML models integrated into the software. Having demonstrated our DC method successfully for AChE inhibition, we are now in the process of validating it against this wider array of toxicological

endpoints. **Conclusions:** MegaTox provides a platform to deploy a customized set of models enabling prediction of toxicological properties from molecular structure. Our continual development of additional algorithms and molecular descriptors points to the benefits of an extensible platform for toxicology predictions that is modular and can be rapidly updated as technology advances. We now describe the development of novel uncertainty-aware models for a wide array of toxicity endpoints. We are also applying this uncertainty technique to help guide the generative design process in drug discovery projects, where we are using this approach with drug targets.

3725 A second-generation artificial intelligence-assisted physiologically based pharmacokinetic model to predict nanoparticle tumor delivery in mice

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Background and Purpose: Nanoparticle (NP)-based drug formulations are characterized by high drug loading, extended circulation time, and targeted delivery, holding great promise for cancer treatment. However, the low delivery efficiency of nanoparticles (NPs) to tumors remains a challenge and hinders clinical translation. This study aimed to develop the second-generation artificial intelligence (AI)-assisted physiologically based pharmacokinetic (PBPK) model for predicting NPs' tumor delivery efficiency (DE) based on NPs' physicochemical properties and experimental strategies. **Methods:** A generic PBPK model was applied to describe NPs' tumor kinetics (N=152), and six different tumor-related PBPK parameters were determined. Local sensitivity analysis was conducted to determine the influence of parameters on PBPK predictions. By combining published (N=378) with current datasets (N=152), a total of 530 datasets were used to construct machine learning (ML) and AI models for predicting the tumor-related parameters. Incorporating the ML model-predicted parameters into the generic PBPK framework, an AI-assisted PBPK model was developed to predict NPs' tumor kinetics. The AI-assisted PBPK model performance was internally evaluated by comparing observed DEs (maximum DEs, DEs across different time points, and DE at 24h) with the model predictions. In-house experimental data of tumor DEs for gold nanoparticles in MCF-7 tumor-bearing mice were generated and used to externally validate AI-assisted PBPK model. **Results:** The generic PBPK model can capture the tumor kinetics for various NPs, which achieved the overall determination coefficient (R^2) of 0.92. Distribution (PT) and permeability (PATC) coefficients at the tumor site were identified as the important parameters in predicting tumor DEs. A total of 30 ML/AI models are developed. The deep neural network (DNN) model was the best-performing model and selected for estimating tumor-related parameters. The AI-assisted PBPK model achieved R^2 of 0.57, 0.42, and 0.57, for maximum DEs, DEs across different time points, and DE at 24h, respectively. For the external validation, most predicted concentrations were within the two-fold error of the observed data, indicating acceptable predictive accuracy. **Conclusions:** This study improved performance in predicting tumor-related parameters and enhanced practical applicability over the first-generation model, which can serve as a screening tool for tumor DE predictions and NP formulation optimizations, informing the clinical translation of NPs.

3726 Prediction of Ocular Toxicity using Machine Learning Models

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Background and Purpose: The ocular surface is routinely exposed to various chemicals, some of which can lead to negative outcomes ranging from mild irritation to permanent blindness. To reduce the risk of damage from exposure, chemicals (e.g., cyclohexanol, and sodium oxalate) must be tested for ocular toxicity. However, traditional animal-based toxicology testing is expensive, time-consuming, and associated with ethical concerns, prompting researchers to turn to new methods for testing toxicity, such as computational models. Specifically, the goal of the study is to develop machine learning models to predict whether a compound with unknown mechanism of toxicity is toxic to the human eye by applying chemical structure data, bioactivity assay (e.g., assays that screen for small molecule agonists of the hypoxia (HIF-1) signaling or thyrotropin-releasing hormone receptor (TRHR) signaling pathways) data, or a combination of both to the models. **Methods:** In this study, three classification algorithms were used to build the models: random forest, support vector machine (SVM), and XGBoost. Feature selection was applied to some of the models to improve model performance. **Results:** Model performance varied according to the data type applied to the model, the model used, and the train/test split generated. Models only using assay data did not perform as well as models using only structural data or both data types. Interestingly, the latter two model types had comparable performances with the structural data models sometimes outperforming the models using the combined data set. The top-performing model, with an area under the receiver operating characteristic curve (AUC-ROC) value of approximately

0.75, was derived by applying feature selection to the random forest algorithm trained on a combination of structural and assay data. **Conclusions:** This random forest model is a strong candidate for future investigation to improve performance and for large-scale ocular toxicity screening. The optimized model will be applied to virtually screen the Tox21 10K compound library to identify potentially ocular toxic compounds. The predicted toxic compounds will be prioritized for experimental validation.

PS 3727 Integrating Machine Learning Approaches for Neurotoxicity Prediction: Combining Tox21 Assay Datasets and Chemical Structures

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Background and Purpose: Environmental and pharmaceutical chemicals pose significant neurotoxicity risks, potentially causing cognitive, behavioral, and neurological disorders. Traditional *in vivo* testing is costly, time-consuming, and often poorly predictive of human outcomes. To address this, the U.S. Tox21 consortium, a collaborative initiative among several federal agencies, has developed a suite of *in vitro* assays for quantitative high-throughput screening (qHTS) of the Tox21 10K compound library. This study evaluates the predictive performance of Tox21 assay data and chemical structure for neurotoxicity assessment. **Methods:** We constructed predictive models using five machine learning algorithms: Random Forest, Naïve Bayes, eXtreme Gradient Boosting, and Support Vector Machine and Neural Network. Model performance was evaluated using the area under the receiver operating characteristic curve (AUC-ROC). **Results:** These models were then applied to the full Tox21 10K compound library, and the compounds with the highest predicted probability of neurotoxicity were selected for experimental validation. Key contributor to neurotoxicity prediction included the activation of glucocorticoid receptor. In parallel, structural features such as pyridine rings and tertiary amine-aliphatic nitrogen bonds were strongly associated with neurotoxic potential. **Conclusions:** Overall, this study demonstrates the effectiveness of integrating assay data with chemical structure information to enable reliable *in silico* prediction of neurotoxicity, offering a scalable and animal-free approach for chemical safety assessment.

PS 3728 Combined evaluation using three *in vitro* testing methods for skin sensitization of medical device materials

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Background and Purpose: The evaluation of skin sensitization hazards is required for all body-contacting medical devices. Currently, the Guinea Pig Maximization Test (GPMT) is widely used for evaluating skin sensitization hazards. From the perspective of animal welfare, the development of alternative methods to replace GPMT is strongly desired. However, there are not internationally standardized alternative methods for the evaluation of medical devices. A unique feature of skin sensitization evaluation for medical devices is the use of organic solvent extracts and extract solutions obtained with polar and non-polar solvents. Furthermore, because skin sensitization is elicited through multiple biological responses *in vivo*, no single alternative method can fully substitute for GPMT. Therefore, it is important to scientifically examine how to combine the results of various alternative methods to accurately predict GPMT results. Unfortunately, there has been little research addressing this issue. In this study, we evaluated organic solvent extracts from medical device materials using three alternative methods listed in the OECD test guidelines—Amino acid Derivative Reactivity Assay (ADRA), Epidermal Sensitization Assay (EpiSensA), and human Cell Line Activation Test (h-CLAT)—and investigated how best to combine these results to predict GPMT outcomes with high accuracy. **Methods:** The nine medical device materials (Rubber A and B, Catheter, Catheter hub, Medical adhesive bandage, Polymer pellet A, B, A+B, and C) were selected as test samples. Each material was extracted with acetone, methanol, or *n*-hexane according to ISO 10993-10, and the extracts were obtained by evaporating the organic solvents. These extracts were re-dissolved in solvents appropriate for each assay and subjected to testing. The GPMT was conducted at an AAALAC-accredited facility, and guinea pig skin reactions were scored according to Japanese guidelines. ADRA was performed according to OECD TG 442C, and the depletion rates of NAC and NAL were measured by HPLC. EpiSensA was conducted following the developer's protocol, using a three-dimensional skin model (LabCyte EPI-MODEL24), and the expression of four genes (*ATF3*, *GCLM*, *DNAJB4*, *IL-8*) was analyzed by Real-time PCR. h-CLAT was performed according to OECD TG 442E, and the expression of CD86 and CD54 in THP-1 cells was measured by flow cytometry. To predict the results of GPMT, two combined approaches were adopted: the "2 out of 3" approach and the Sequential Testing Strategy (STS) approach. The "2 out of 3" approach, based on OECD Test Guideline No. 497, determined the outcome when at least two of the three alternative methods yielded concordant results. The STS approach, based on papers ^{1,2}, determined the outcome based on the results of h-CLAT and ADRA. 1) Y. Nukada *et al.* Data integration of non-animal tests for the development of a test battery to predict the skin sensitizing potential and potency of chemicals. *Toxicol. in Vitro*, 27 (2013), pp. 609-618. 2) O. Takenouchi *et al.* Test battery with the human cell line activation test, direct peptide reactivity assay and DEREK based on a 139 chemical data set for

predicting skin sensitizing potential and potency of chemicals. *J. Appl. Toxicol.*, 35 (2015), pp. 1318-1332. **Results:** In the GPMT, 7 out of 9 materials showed positive, while 2 materials showed negative. The concordance rates between each alternative method and the GPMT were as follows: ADRA showed concordant results for 7 materials, with discrepancies observed for polymer pellet A (GPMT positive) and rubber B (GPMT negative). EpiSensA showed concordance for 8 materials, with a discrepancy for catheter hub (GPMT positive). h-CLAT showed concordance for 7 materials, with discrepancies for catheter and medical adhesive bandage (both GPMT positive). These discrepancies may have been influenced by factors such as the solubility of organic solvent extractables in the test solvents. When combining the results of the alternative methods, the "2 out of 3" approach achieved complete concordance with GPMT for all 9 materials. In contrast, the STS approach showed concordance for 8 materials, with a discrepancy for rubber B. **Conclusions:** The combination of three alternative methods (ADRA, EpiSensA, and h-CLAT) demonstrated the potential to accurately predict GPMT outcomes for medical device materials. In particular, the "2 out of 3" approach may serve as a highly accurate alternative to GPMT.

PS 3729 Development of an Artificial Intelligence tool to support processing data from Extractables & Leachable studies for toxicological risk assessments.

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Background and Purpose: Currently, extractable and leachable testing per ISO 10993-1 and ISO 10993-18 requirements generate reports containing numerous chemicals. In many instances, processing the data involves selection of a replicate that reflects largest amount, grouping similarly structured chemicals to evaluate potential additive effects and lastly, identification of the chemicals for a toxicological evaluation. Depending on the number of chemicals extracted, this refinement process can take a significant amount of time. The reports are accessible PDFs with chemical grouping data in tables, requiring manual extraction and interpretation. This process has the potential to be streamlined by the application of "Artificial Intelligence" (AI) tools. The purpose of the AI tool is to develop a comprehensive solution for extracting, analyzing, and processing data from leachables and extractables studies, especially from scientific PDF reports. **Methods:** The software developed is a proprietary artificial intelligence tool that automates the processing of extractables and leachable data, based on a scientific rationale for grouping similar chemicals in terms of structure or toxicological activity. The artificial intelligence tool uses chemical attributes such as structure and functional groups, mirroring a human expert's decisions for toxicological risk assessments, saving time and improving accuracy and efficiency in the process. It leverages AI, document parsing, and data science tools to automate and streamline the extraction and analysis of chemical data from complex documents. The tool was developed using Google Cloud & ADK including, Google Cloud Build Console and Vertex AI Workbench and Cardinal Health in-house extractable studies data. Over 6,000 chemical constituents spanning 30+ different devices have been used to train and refine the artificial intelligence tool. **Results:** The artificial intelligence tool developed was able to process data from extractable studies. It also generated scientific rationales for grouping certain chemicals together. In order to mitigate the effects of possible artificial intelligence errors, a separate artificial intelligence tool critiqued the grouping from the first data output. **Conclusions:** This artificial intelligence tool provides opportunities for decreasing effort spent on the processing stage of extractables and leachable studies. Continuous effort to refine the tool is ongoing.

PS 3730 Chemical Characterization of Medical Devices: How Creativity and Care Play a Role in Effective Study Design

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Background and Purpose: Biocompatibility testing of medical devices per ISO-10993-18 is a resource intensive process. Careful front-end study design leverages efficiencies to reduce timelines and minimize the total number of devices required for testing. Creative strategies for optimizing device extraction, determining appropriate testing conditions, building protectivity into the AET, and planning for foreseeable misuse are crucial for executing the most effective studies. Furthermore, new draft guidance and recent changes to 10993-1 should be addressed during the study design phase to ensure that up-to-date testing requirements are satisfied prior to submission to a regulatory body. A well-designed and well-executed chemical characterization study plan will both facilitate the generation of an accurate TRA and avoid the need for re-testing. Crucially, testing laboratories must follow best practices to save time and cost while ensuring studies are protective of patient safety. **Methods:** In this study we seek to demonstrate how chemical characterization testing can be optimized on a case-by-case basis and how creative study design is essential in creating efficiencies. **Results:** A variety of creative approaches to chemical characterization will be presented as case studies, as well as primers on foreseeable misuse and recent changes to ISO 10993-1 which may affect future biocompatibility study design. **Conclusions:** Testing laboratories must actively partner with manufacturers and toxicologists in the design and execution of chemical

characterization studies to maximize the value of these results to the TRA. Careful and creative study design will help guide the medical device product from the testing phase to regulatory submission as efficiently as possible.

PS 3731 Extractable and Leachable Tin from Medical Devices: Influence of Speciation on Toxicological Limits and Considerations for Chemical Characterization

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Background and Purpose: Assessment of inorganic elements by inductively coupled plasma mass spectrometry (ICP-MS) is a standard component of the characterization of extractable and leachable (E&L) medical device constituents performed in accordance with ISO 10993-18:2020. However, ICP-MS does not provide speciation of the element detected and chromatographic screening methods for organic compounds included in E&L studies may not detect or identify organometallics. Tin (Sn) is an element included in ICP-MS analyses based on ICH Q3D Guidance for Elemental Impurities for Pharmaceuticals. While the ICH Q3D focuses on the toxicity of inorganic tin, E&L tin from medical devices could be from inorganic or organic compounds, each differing in biological activity and toxicity. In this study, we evaluated potential sources of tin from medical devices and derived tolerable intake levels for commonly used species. To highlight the potential for mischaracterization of toxicological risk when there is inadequate information on device materials of construction and manufacturing, a case study is presented. **Methods:** Potential sources and species of tin as a medical device constituent were examined through a review of commonly reported device materials, additives, and manufacturing processes. Standard analytical techniques for E&L studies were evaluated for their ability to characterize different tin species. Toxicological reference values (NOAELs, LOAELs) were compiled from sources including ATSDR, NTP, OECD, and ECHA dossiers for different tin species including stannous chloride, octyltins, and butyltins. Tolerable intakes (TIs) were derived for tin species when sufficient data was available using the ISO 10993-17:2023 framework, incorporating standard uncertainty and route-to-route extrapolation factors for chronic parenteral exposure. To characterize the toxicological risk associated with different tin species, a simulated-use chemical characterization study where tin was detected by ICP-MS was considered. Materials of construction that may be contributing tin to the chemical release profile, and what species of tin could be a potential E&L constituent were examined. A maximum estimated exposure dose (EED_{max}) for tin was calculated and a comparative analysis of margin of safety (MOS) values was performed by comparing the EED_{max} to the derived TIs for the different tin species. **Results:** Both inorganic and organic tin compounds are potential E&L from medical devices and analytical techniques vary in their sensitivity and specificity. Inorganic tin compounds are of relatively low toxicological concern while organotins exhibit toxicity at much lower exposure levels. Derived TIs spanned four orders of magnitude, ranging from 16 µg Sn/kg/day for inorganic tin to 0.006 µg Sn/kg/day for dibutyltin. For a representative extract containing 11.92 µg Sn/device by ICP-MS analysis, toxicological risk characterization varied from acceptable levels of risk for inorganic tin to potentially unacceptable levels of risk for certain organotins. These findings demonstrate that tin speciation is a critical driver of toxicological risk assessment outcomes. **Conclusions:** Tin speciation substantially influences allowable exposure limits and overall assessment of toxicological risk. Standard ICP-MS analysis does not allow for definitive characterization of tin as an E&L medical device constituent and chromatographic screening methods require additional considerations to detect and identify organotins. Incorporating knowledge of manufacturing processes and material chemistry provides a scientifically defensible basis for characterizing the toxicological risk of exposure to tin as an E&L medical device constituent.

PS 3732 Hazard Evaluation and Structural Analysis of Organophosphorus in Medical Device Extractables and Leachables Datasets

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Background and Purpose: Medical devices (MDs) are composed of a wide range of materials that have the potential to release chemical constituents, including organophosphorus compounds (OPs), during patient use. OPs are listed as one of the cohort of concern (COC) chemical classes by the International Organization for Standardization/Technical Specification (ISO/TS) 21726 (ISO, 2019). OPs are neurotoxins often used as pesticides and nerve agents with a well-studied mechanism of action involving inhibition of the acetylcholinesterase (AChE) enzyme. In MDs, OP-related compounds are commonly used as plasticizers and have been identified from extraction studies (Builee *et al.*, 2025; Borton and Coleman, 2025). As COCs are expected to be potentially toxic and their risk cannot be assessed using a threshold of toxicological concern (TTC), assessments of OPs identified in MD extractables can involve an additional effort to identify suitable surrogates. The ISO/TS 21726 (ISO, 2019) does not provide details on how to identify OPs that are potentially neurotoxic. A TTC of 0.3 µg/kg-bw/day has been derived for OPs based on 31 OP insecticides (Munro *et al.*, 1999; Kroes *et al.*, 2004), and has been reaffirmed by the European Food Safety Authority (EFSA) using neurotoxicity data of 59 OPs and 14 carbamates (EFSA, 2012). Our aim is to evaluate toxicity and potency of

OP species identified in MDs, and to identify chemical structural features that can help to better identify OPs with neurotoxic concern. **Methods:** To better understand potential hazards associated with OPs identified in MDs, we set out to investigate the following with published MD-constituent lists (Borton and Coleman, 2025; Builee *et al.*, 2025): 1. Identify overlap between OPs reported in MD extractables with OPs used to derive (Munro *et al.*, 1999) and verify (EFSA, 2012) the OP TTC; 2. Identify risk points of departure (PODs) based on available data and compare with the OP TTC; and 3. Identify structural features associated with neurotoxic outcomes. **Results:** Approximately 600 unique chemicals were identified in the published MD-constituent lists. Four OPs were uniquely identified in the MD-constituent list to be in the Munro and/or the EFSA dataset; 2 of 31 OPs from the Munro dataset and 4 of 51 OPs from the EFSA dataset were on the list of MD-constituent OPs. MD-constituent OPs that were in the Munro and/or EFSA dataset were thiophosphate-related compounds. Our preliminary results suggest that all toxicity PODs for MD-constituent OPs are at least three orders of magnitude higher than the approximate 5th percentile no observed adverse effect level (NOAEL) of 0.03 mg /kg-bw/day from which the OP TTC was derived (Munro *et al.*, 1999; Kroes *et al.*, 2004). **Conclusions:** Our preliminary findings show that the OP TTC that was derived based on insecticides (Munro *et al.*, 1999) is sufficiently protective for neurotoxicity; however, these findings suggest that there may be room to further refine the OP TTC with chemicals detected from MDs.

PS 3733 Integrating Total Organic Carbon (TOC) Analysis into Chemical Characterization for Toxicological Risk Assessment: A Case Study Evaluating the Relationship Between TOC and Gravimetric Residues

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Background and Purpose: ISO 10993-17 recommends the use of conservative, quantitative exposure data for the purposes of toxicological risk assessment. ISO 10993-18, Section 6.3 identifies total organic carbon (TOC) analysis and non-volatile residue (NVR) determination (via gravimetric analysis) as acceptable methods for quantifying organic extractables from medical devices. While both techniques are non-specific and primarily used to demonstrate exhaustive extraction, few studies have systematically compared their relative performance or explored their utility in informing exposure assessment in toxicological risk assessments. **Methods:** In this case study, test articles (titanium alloy orthopedic plates and screws) were exhaustively extracted under five conditions. For this orthopedic system, the materials of construction are well characterized. These devices provide a unique opportunity to assess whether TOC-derived total organic carbon values can be used to estimate total extractables and inform toxicological risk assessments by applying TOC results to known manufacturing process chemicals. Three organic solvent extractions (non-polar, mid-polar, and polar solvents) were performed for 72 hours with agitation at 50°C, representing exhaustive extraction conditions for a long-term implant device per ISO 10993-18. Two aqueous extractions were performed to evaluate TOC compatibility: (1) 24 hours with agitation at 50°C and (2) 30 minutes with sonication at room temperature. Gravimetric (NVR) analysis was performed on all extractions, and TOC analysis was performed on all aqueous extracts. A separate spiking study was conducted using compounds of varying volatility and solubility to evaluate the classes of analytes detectable by TOC. **Results:** Across all extractions, the highest total residue by NVR was obtained from the 72-hour aqueous extraction (340 µg). The highest TOC residue was measured in the 30-minute sonicated water extraction (141 µg). At first, this study suggests NVR is the more efficient means of quantifying gross residue. However, TOC measurements only quantify carbon-containing chemicals. When adjusted to account for non-carbon elements based on the device's extractables profile, the estimated total extractables increased to approximately 324 µg, which more closely matches the NVR results. These findings indicate that TOC can serve as a viable alternative to NVR. TOC can quantify the gross residues in aqueous extractions of metallic devices as well as NVR for the purposes of an exposure assessment. When the contact chemicals of a medical device are known, TOC values can serve as the quantitative exposure value for each chemical constituent for the purposes of toxicological risk assessment. The use of TOC as the exposure value for each contact chemical results in a highly conservative exposure estimation. **Conclusions:** In conclusion, TOC analysis can be effectively leveraged as a conservative exposure value for toxicological risk assessment of metallic devices. When adjusted for non-carbon composition, TOC provides a conservative estimate of total extractables and offers an efficient means to evaluate manufacturing residuals in support of biological safety assessments.

PS 3734 Considering Physiological Environment in Study Selection for Toxicological Evaluation of Implantable Devices: Case Study of Polyurethane Foam-Coated Implants

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Background and Purpose: ISO 10993-17:2023 states the point of departure (POD) selected for deriving a constituent-specific tolerable intake (TI) should represent the most clinically relevant study and account for the route, duration, and conditions of exposure. Although not explicitly stated, the physiological environment of the peri-im-

plant space can be an important consideration for the toxicological evaluation of some medical device constituents. For example, regulatory agencies have expressed concern over the potential release of toluene diisocyanate (TDI), and hydrolysis to the rodent carcinogen toluene diamine (TDA), from polyurethane (PU) foam-coated implants. In lifetime cancer bioassays, carcinogenic outcomes have been reported in rodents administered TDI via oral gavage, but not other routes of exposure. Because pH, among other factors, influence PU hydrolysis pathways, selecting a POD for TI derivation from an oral gavage study can misrepresent degradation kinetics and overestimate patient risks to implanted device constituents. The purpose of this study is to demonstrate the importance of considering the physiological environment of the peri-implant space and portal of entry in key studies when evaluating the toxicity of medical device constituents using PU degradation products as an exemplar. **Methods:** Published literature demonstrating PU degradation profiles in similar medical devices, and toxicological profiles were reviewed. *In vitro* degradation data and theoretical estimates of TDA formation were compared to physiological conditions. Carcinogenicity data from lifetime cancer bioassays with rodents exposed to TDI via oral gavage and inhalation were integrated into a risk model considering device aging-related pathways. **Results:** In oral gavage studies, TDI hydrolyzes to TDA as a result of the highly acidic gastric juices, which are not representative of the physiological environment of skeletal tissue or the peri-implant space, which are near-neutral with low water turnover. In the peri-implant space, physiological conditions favor the formation of urea and polyurea adducts rather than hydrolysis to TDA. The physiological environment of the respiratory tract, however, better approximates that of the peri-implant space. In an inhalation lifetime cancer bioassay with rodents, TDI was clearly not carcinogenic, which is consistent with literature indicating low *in vivo* hydrolysis of TDI to TDA. **Conclusions:** POD selection for TI derivation should consider the physiological environment of the implanted device and route of exposure in the key study to ensure toxicity data are clinically relevant. For some medical device constituents, such as PU foam degradation products, failing to consider the physiological environment could result in the overestimation of patient risks related to the intended use of the device.

PS 3736 Evaluation of the carcinogenic potential of nickel-containing implants and associated alloys

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Background and Purpose: Alloys are produced to have unique physicochemical properties, including hardness, toughness, and corrosion resistance, relative to their individual constituents. Nickel (Ni) is a metal used in various medical device alloys to improve corrosion resistance and enhance mechanical properties. The toxicology and carcinogenic potential of metallic nickel and nickel alloys have been reviewed previously; however, an important consideration in this toxicological and carcinogenic assessment is that Ni-containing alloys warrant a separate evaluation compared to the individual metallic ingredients. The purpose of this analysis, therefore, is to evaluate the potential local and systemic carcinogenic response to Ni-containing implants in preclinical studies, including the testing of alloys used for potential human implants. **Methods:** A standardized protocol was employed to systematically retrieve preclinical carcinogenicity data on Ni-containing materials relevant to implant materials, including stainless steel, nickel-titanium (NiTi), and cobalt (Co)-containing alloys. Eligibility criteria included the use of relevant material type, form, and route of exposure for Ni-containing implant materials. Methodology and results from relevant preclinical studies were documented to consider the test article (material type), Ni content, material form/shape, animal model/test species, route of exposure, study duration, local and systemic tumor results, and other considerations for study results, including statistical analysis (or lack thereof) and details of the control group. The assessment also integrated a discussion of relevant clinical data and conclusions from previous regulatory reviews. **Results:** Our systematic evaluation identified eight relevant preclinical studies assessing the carcinogenicity of Ni-containing implants. Four preclinical studies assessed the carcinogenicity of stainless-steel materials, five evaluated Co-containing materials with varying Ni levels, and one provided limited data on nitinol (a NiTi alloy), with some studies addressing more than one material. While two studies demonstrated foreign-body effects that were not specific to Ni, all studies demonstrated no significant increase in local or systemic tumors when compared to controls. Overall, the preclinical data on Ni-containing alloys relevant for medical implants demonstrate a lack of carcinogenic potential. The epidemiological evidence identified by previous regulatory reviews demonstrates no consistent increased overall cancer risk and no consistent indication of site-specific cancers that would be causative of any implant factor related to potential Ni exposure. **Conclusions:** Overall, the preclinical data on Ni-containing alloys relevant for medical implants demonstrate a lack of carcinogenic potential, as no significant increase in local or systemic tumors specific to nickel were observed in various experimental designs. The clinical observations are consistent with preclinical studies, which demonstrate no consistent increased overall cancer risk and no consistent indication of site-specific cancers that would be causative of any implant factor related to potential Ni exposure.

PS 3737 Material Equivalence in Medical Devices: A Risk-Stratified, Weight of Evidence Approach

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Background and Purpose: The ability to establish material equivalence is increasingly vital for medical device manufacturers navigating regulatory change management, supply chain disruptions, and product innovation. While ISO 10993 supports material equivalence as a basis for biological evaluation, it does not provide guidance for analytical test selection or acceptance criteria. Such ambiguity triggers inconsistent risk assessment practices, regulatory uncertainty, and inefficient use of resources. We present a risk-based, orthogonal framework leveraging material characterization tests (e.g. FTIR, DSC, TGA, and GPC) with analytical acceptance criteria to define equivalence. Our approach integrates first-principles material science, material-derived sources of biological harm, measurement uncertainty, and statistical equivalence approaches. **Methods:** We conducted a systematic literature review of standards/guidance (ISO 10993; FDA; ASTM; ISO), previous round-robin studies, academic/technical publications, and prior Weight-of-Evidence frameworks (e.g. Street et al 2024). Analytical techniques, from bulk characterization to trace extractables analysis were systematically ranked for suitability in equivalence determination. Risk stratification is enabled by nature/duration of patient contact as well as based on historical evidence ("a priori" knowledge). Materials under investigation are assessed for depth of prior biocompatibility data, clinical history of safe use, and the extent of formulation and process characterization. This informs a risk-based, tiered approach to analytical testing, where materials in low-risk applications with robust prior evidence and well-characterized processes are eligible for streamlined testing and risk-adjusted acceptance criteria. Conversely, novel/implantable materials or those with substantial changes undergo comprehensive orthogonal analysis or additional biological/chemical testing. **Results:** The proposed material equivalence framework is an adaptable decision flowchart for experimental design and analytical test method selection, tailored to the specific material, device, and process context. Acceptance criteria are quantitatively defined for FTIR, DSC, TGA, and GPC, with each metric grounded in measurement uncertainty and statistical rigor. Criteria are mapped from analytical capability to biological relevance: FTIR detects new patient-contacting chemistries; DSC/TGA reflect polymer stability and mobility; GPC reflects oligomer and bulk degradation risk. We define tolerance windows for FTIR oxidation index/correlation factor, DSC glass/melt transitions and oxidation induction times, TGA degradation temperatures and compositional percentages, and GPC molecular weight moments and polydispersity indices. The framework incorporates prior biocompatibility and clinical data to enable risk-based customization of testing depth and acceptance windows. Tests conducted within the framework are additive and complementary; each must independently demonstrate equivalence within predefined limits. Application is demonstrated for common medical polymer families and manufacturing processes, with case studies illustrating both successful and non-equivalent outcomes. **Conclusions:** We present a risk-based material equivalence framework providing a scientifically robust approach for change control, regulatory submissions, and internal decision-making. By enabling practitioners to pre-define and defend analytical test selection and acceptance criteria, the framework enhances consistency, transparency, and regulatory confidence. The adaptable structure supports relevance across diverse materials, manufacturing processes, and device types. Future work will validate the predictive value of the material equivalence framework against biological endpoints and nontargeted chemical characterization. We welcome collaboration from industry and notified body colleagues to support further harmonization, reduce unnecessary testing, and support safe yet efficient innovation in medical devices.

PS 3738 Assessing the application of *In vitro* Irritation in a diverse range of Medical Devices

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Background and Purpose: Historically, medical devices have been assessed for skin irritation through the current *in vivo* animal rabbit model. The 2021 revision of ISO 10993-23 introduced the *in vitro* protocol for irritation testing of medical devices based on the OECD 439(2013) Reconstructed Human Epidermis (RhE) model. This *in vitro* test has been widely accepted and validated for driving the use of alternative methods to reduce, refine and replace animal testing, in chemical and cosmetic industries. The Reconstructed Human Epidermis (RhE) is currently accepted within the ISO 10993 series as the *in vitro* irritation method for evaluating skin irritation and has been adapted for use in the testing of medical devices. Some of the common challenges faced with the introduction of new approach methodologies (NAMS) can be acceptance in different regulatory landscapes especially where diversity in data from different medical devices is lacking. Cardinal Health's product portfolio contains a wide range of Class I and Class II medical devices such as surgical gloves, wound dressings, enteral feeding devices, urology catheters, among others. These products are comprised of a wide range of raw material constituents making suitable candidates to address the medical devices diversification concerns from regulatory agencies. The purpose of this project is to demonstrate the feasibility of Reconstructed Human Epidermis (RhE) for skin irritation testing across a wide range of products to fulfil requirements of ISO 10993 guidelines for biocompatibility evaluation. **Methods:** Internal Cardinal Health Biocompatibility data was reviewed over the past five years for existing *in vivo* testing data across the overall product port-

folio. Within a subsection of these different product families, around 10-20 devices were selected for *in vitro* evaluation bases on their materials of construction and for which existing *in vivo* animal data is available. These devices were tested in both polar and non-polar solvent using the Reconstructed Human Epidermis (RhE) model as outlined in ISO 10993-23. Data analysis against existing *in vivo* information was also conducted. **Results:** In this work we discuss the outcome of the Reconstructed Human Epidermis (RhE) test on a diverse range (10-20) different products comprising of varying materials and varying categories of patient contact and duration. Favourable correlations were observed between the pre-existing *in vivo* testing and the *in vitro* Reconstructed Human Epidermis test. Post-commercial device changes were also studied to determine the impact of the testing and the suitable integration of the test into the overall biocompatibility risk assessment approach. **Conclusions:** The proposed approach provides a framework to support the acceptance of NAMS by regulatory agencies and notified bodies. This work will show the applicability of the *in vitro* irritation test across a diverse range of medical devices. Furthermore, understanding how material formulations prior to biocompatibility testing will most likely support the 3R concept of Reduce, Remove and Replacement of animal testing in line with other global industries costs and help in the time to market for medical device manufacturers.

PS 3739 Data Deconvolution and High-Resolution Mass Spectrometry in Medical Device Chemical Characterization: Case Studies on TRA Impact

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Background and Purpose: A chemical characterization study of a medical device per ISO 10993-18 provides invaluable data for the toxicological risk assessment (TRA). It is crucial that high confidence and thorough extractable data be provided by a laboratory to the toxicologist for assessment. Instrumental and interpretative approaches to the extractable data have evolved since the inception of Part 18 and since the 2020 ISO revision. High resolution mass spectrometry with data deconvolution must become a core expectation for chemical characterization of medical devices. **Methods:** Extractable data from chemical characterization studies on diverse medical devices were assessed both with and without high-resolution mass spectrometry and data deconvolution. Comparisons were made, with discussion of the impact to the TRA from instrumental and interpretative approaches. **Results:** Case studies in the application of data deconvolution and high-resolution mass spectrometry for ISO 10993-18 chemical characterization studies will be presented, illustrating the impact to a TRA and ultimately to patient safety. **Conclusions:** Data deconvolution of high-resolution mass spectral data is foundational for modern chemical characterization studies to increase identification confidence and extractable profile coverage, ensuring accuracy in the TRA's assessment of patient safety.

PS 3740 Avoiding False Positives in Medical Device Risk Assessment: Differentiating Real MDI from Analytical Artifact

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Background and Purpose: Polyurethanes, including those used for long-term implantable medical devices, are synthesized by reacting a polyol with an isocyanate such as methylene diphenyl diisocyanate (MDI; CASRN 101-68-8). MDI is of toxicological concern due to its classification as both a skin and respiratory irritant and sensitizer. However, MDI can be artificially generated during extraction studies of polyurethane-containing medical devices. This is due to gas chromatography inlet temperatures reaching temperatures above those where thermoplastic polyurethanes are fully stable. Therefore, it is important to parse detection of MDI as a clinically relevant leachable from an analytical artifact. Standard extractables and leachables screening studies are not sufficient for accurate analysis of MDI from polyurethane-containing medical devices, and herein we propose a simple additional study that can distinguish between extractable MDI requiring toxicological risk assessment and a false positive. In this study, high levels of MDI were initially observed by standard extractables screening methods (specifically, GC-MS) and a targeted derivatization method was implemented to investigate the results. **Methods:** Acetonitrile and hexane device extracts were analyzed for MDI content. An aliquot of each extract was spiked with a solution of a dialkylamine derivatization agent (e.g., dibutylamine) in dichloromethane. The resulting solution was analyzed by LC-MS with a calibration curve generated from MDI standards prepared in the same manner. **Results:** No MDI was observed above the limit of quantitation using the derivatization LC-MS method. This indicates that no free MDI was present in the device extracts and supports the hypothesis that MDI observed by the standard GC-MS screening method are the result of thermal degradation during the analysis. **Conclusions:** This study demonstrates that standard screening of extractables and leachables from polyurethane-containing medical devices by GC-MS may artificially generate MDI, leading to potential false positives in the study report that require toxicological risk assessment. The addition of an LC-MS study with appropriate derivatization provides a simple way to distinguish between real MDI and artificially generated MDI. The addition of this work to

standard extractables and leachables screening studies will help provide a more realistic understanding of clinically-relevant device extractables, and prevent an overly conservative assessment of toxicological risk.

PS 3741 Chemometric Approaches to ISO 10993-18 Workflows - Accelerating Risk-Based Approaches for Rapid Chemical Characterization

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Background and Purpose: Chemical characterization studies per ISO 10993-18:2020 are critical for ensuring patient safety by identifying chemical constituents released from medical devices. While applying ISO 10993-18 to the final finished form remains the gold standard, this approach can be overly burdensome when addressing manufacturing changes, sterilization modalities, or alternative suppliers. Some proposals suggest shifting characterization toward bulk property evaluations; however, this overlooks small molecules that are toxicologically relevant, making bulk property analysis an inadequate substitute. A more effective solution is to develop a rapid, risk-based chemical characterization approach that leverages advanced data processing strategies while maintaining ISO 10993-18 principles. In this context, a priori compositional profiling serves as one tool, not a replacement, to accelerate workflows and improve decision-making without compromising patient safety. **Methods:** Utilizing ISO 10993-18 methodologies for volatile, semi-volatile, and non-volatile organic compound analysis, we developed a streamlined data processing workflow focused on small molecules. The approach prioritized extractables and impurities present in the proposed material but absent in the existing material or their estimated concentrations are higher in the proposed material compared to the existing material, enabling rapid identification of toxicologically relevant compounds. We compared outcomes from this workflow against traditional ISO 10993-18 extractables studies, standard information-gathering practices, and bulk property-based evaluations to assess efficiency and scientific robustness. **Results:** Information-gathering alone revealed gaps in detecting relevant differences between materials, and strict reliance on supplier documentation for risk assessment proved insufficient. Equivalence evaluations across polymers, sterilization modalities, and chemical impurities demonstrated that bulk property testing failed to identify changes in small molecules. In contrast, chemometric approaches aligned to ISO 10993-18 workflows successfully detected differences in small molecule extractables and impurities, confirming superior sensitivity, accuracy and specificity for a toxicological risk assessment. **Conclusions:** This work introduces a risk-based chemical characterization strategy that accelerates ISO 10993-18 workflows through advanced data processing while preserving toxicological relevance. By prioritizing small molecule identification and using information gathering as a supporting tool, the approach enables faster, scientifically robust evaluations for equivalency without compromising patient safety.

PS 3742 Survey of Neurotoxicants Released from Medical Devices

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Background and Purpose: Medical devices are made of a diverse range of materials (i.e. plastics, metals, and polymers) which may release various compounds into the patient's body during clinical use that may act as neurotoxicants, adversely affecting the central and peripheral nervous systems and sensory organs. Neurotoxic effects range from reversible symptoms like narcosis, nausea, and dizziness to irreversible outcomes such as neurodegenerative disorders. ISO/FDIS 10993-1:2025 emphasizes medical devices contacting the central or peripheral nervous system or cerebrospinal fluid must undergo neurotoxicity evaluation; including consideration of local (effects near the device) and systemic (neurological effects throughout the body) neurotoxicity. Currently, no comprehensive reference list exists for neurotoxicants associated with medical device extractables. By comparing identified neurotoxicants against robust, published datasets of medical device extractables, we aim to create and classify a consolidated reference list of neurotoxic substances identified from medical device materials. **Methods:** Craenen *et al.* (2025) recently published reference list of industrial chemicals classified as neurotoxic in accordance with classifications listed in Classification, Labelling, and Packaging (CLP) for hazard classification. We screened this reference list of neurotoxic substances against datasets of chemicals established to be released from medical devices recently published by Builee *et al.* (2025), and Borton *et al.* (2025). Next, the identified chemicals were categorized according to Specific Target Organ Toxicity Single Exposure (STOT SE) and STOT Repeated Exposure (STOT RE), as defined in CLP Annex V and the Globally Harmonized System of Classification and Labelling of Chemicals (GHS). GHS category 1 chemicals produce and/or presumed to have significant toxicity in humans based on evidence from studies in experimental animals following a single and/or repeated exposure. Similarly, GHS category 2 chemicals have the potential to produce significant toxicity in humans, solely based on evidence from studies in experimental animals following a single and/or repeated exposure. Finally, GHS category 3 chemicals may produce transient target organ effects only after a single exposure. **Results:** From 113 neurotoxicants identified by Craenen *et al.* (2025), 60 unique chemicals were found in at least one of the two medical device extractables

datasets. A majority of the identified compounds (53 chemicals) were classified as STOT SE3, causing narcotic effects and respiratory tract irritation; and have no fixed pre-defined limits for the exposure guidance values (under GHS). Two compounds, resorcinol and cyfluthrin, were classified as STOT SE1, and are associated with oral (LOAEL $\leq$ 300 mg/kg-bw) or gas inhalation (LOAEC $\leq$ 2,500 ppm per volume (ppmV)/4 hours) exposure ranges in rats. In addition, three neurotoxicants (ethylene oxide, bifenthrin, and indoxacarb) were classified as STOT RE1, indicating LOAELs/LOAECs of $\leq$ 10 mg/kg bw/day for oral exposure and $\leq$ 50 ppmV/6 hours/day for gas inhalation exposure in rats. Two neurotoxicants (cypermethrin and mesotrione) were identified as a STOT RE2 classification, with LOAEL/LOAEC ranges of 10 to $\leq$ 100 mg/kg bw/day for oral exposure and $>$ 50 to $\leq$ 250 ppmV/6 hours/day for gas inhalation exposure in rats. **Conclusions:** Our findings establish a consolidated reference list of neurotoxicants associated with medical device extractables by screening harmonized neurotoxicity classifications against two robust extractables datasets. Among the 60 unique chemicals identified in medical device materials, the majority were classified as STOT SE3, indicating transient neurological effects. A smaller subset exhibited higher toxicity classifications (STOT SE1, STOT RE1, and STOT RE2) following single or repeated exposure. Further delineation of the type and source of these chemicals will be presented in future research. This reference list helps proactive screening of neurotoxic compounds and supporting manufacturers in meeting evolving standards, particularly for devices that contact nervous system tissues or cerebrospinal fluid, as required by ISO FDIS 10993-1:2025.

PS 3743 Comparative Application of ISO 18562-1:2017 and ISO 18562-1:2024 in Toxicological Risk Assessment of VOS Emissions from a Breathing Gas Pathway Device

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Background and Purpose: Authoritative bodies will soon require premarket submissions for medical devices to follow updated ISO standards. To ensure compliance, toxicological risk assessment approaches for volatile organic substances (VOS) emitted from breathing gas pathway devices must align with ISO 10993-17:2023 and ISO 18562-1:2024. ISO 10993-17:2023 defines principles for deriving tolerable intake (TI) and tolerable exposure (TE) values for chemical constituents of medical devices, while ISO 18562-1:2024 outlines a dedicated framework for evaluating the biocompatibility of breathing gas pathway devices and introduces updated exposure modeling, expanded patient population categories, and improved alignment with ISO 10993-17:2023. This assessment emphasizes ISO 18562-1:2024, comparing its framework directly with ISO 18562-1:2017 to evaluate the impact of the updates on toxicological risk assessment for breathing gas pathway devices. **Methods:** A breathing gas pathway device previously evaluated under ISO 10993-17:2023 and ISO 18562-1:2017 was re-assessed using the ISO 18562-1:2024 framework. A case study on a limited exposure ($\leq$ 24 h) anesthesia workstation, highlighting three representative VOS - naphthalene, diphenyl sulfone, and 2,5-bis(tert-butylperoxy)-2,5-dimethylhexane - was used to show outputs across population groups and assessment approaches. A comparative analysis was then conducted to examine changes in inhalation exposure modeling and TE derivation between the standards. **Results:** Re-evaluation of the device biocompatibility under ISO 18562-1:2024 resulted in altered TE values for select VOS compared to assessment under ISO 18562-1:2017 due to revised breathing parameters and updates to population subgroup definitions, including the addition of new pediatric subgroups. Effects on TE values were process-dependent, and impacts on toxicological risk assessment conclusions were primarily driven by updated default daily breathing volumes. Across the three VOS, representative of differing TE derivation processes, analysis showed that when assessment was based on established limits from authoritative bodies, no notable change in margin-of-safety (MoS) values occurred, whereas when TE values were derived from select toxicological endpoint data or the threshold of toxicological concern (TTC) was applied, MoS values increased in some patient groups but decreased in others. **Conclusions:** Integration of ISO 18562-1:2024 within the ISO 10993-17:2023 framework enhances consistency across toxicological evaluations for breathing gas pathway devices and influences how TEs affect risk assessment outcomes, as demonstrated through a comparative case study of select VOS emitted from an anesthesia workstation. These findings underscore the value of early adoption to strengthen patient protection and support global regulatory readiness through more precise, population-specific risk characterization. Results also suggest that existing assessments may warrant re-evaluation under ISO 18562-1:2024 to best substantiate biocompatibility.

PS 3744 VVOCs in ISO 18562-1:2024: Risk-Based Modelling of Patient Exposure from Breathing Gas Pathway Devices

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Background and Purpose: Breathing gas pathway medical device biocompatibility is covered in the ISO 18562 series of standards, which recently underwent a revision. ISO 18562-1:2017 had VOCs (volatile organic compounds) in scope for airpath assessment, encompassing volatile organic compounds with a boiling point between 50°C to 260°C. ISO 18562-1:2024 redefines VOCs (50-250°C), and adds assessment of VVOCs (very volatile organic compounds) (0-50°C) and SVOCs (semi-volatile

organic compounds) ($>$ 250°C). As regulatory expectations expand to include VVOCs, there is uncertainty regarding whether these highly volatile substances persist long enough to contribute meaningfully to patient exposure. The additional time and significantly increased cost of testing for VVOCs may be disproportionate to the risk posed by VVOCs. Therefore, the likelihood of VVOCs being delivered to medical device patients is explored in this work. Specifically, this study evaluates whether the inclusion of VVOCs in start-of-life testing per ISO 18562-1:2024 is scientifically justified when considering realistic emission and diffusion dynamics. Potential sources of VVOCs are from manufacturing, materials of construction, and ambient contamination. Medical devices are constructed out of materials that are stable over the intended lifetime, and ambient contamination is out of scope of ISO 18562-3:2024. Therefore, the primary potential source of VVOCs are from manufacturing. **Methods:** A model based on molecular weight and vapour pressure was used to calculate the rate of evaporation of various VVOCs from a device, and found emission rates to be on the order of g/m²-s, which implies rapid off-gassing. Given the high vapour pressures and low boiling points, VVOCs are likely to be completely lost from components before they are assembled and packaged. However, for conservatism, the exaggerated case is assumed that all of the VVOCs emitted would be retained in the device until the device is packaged into an LDPE or HDPE (low/high density polyethylene) bag, and thus the entire quantity of VVOCs is present from $t = 0$. The release of VVOCs from inside the bag to the environment was considered as a diffusion problem, with VVOCs inside diffusing through the PE barrier and being released into open air (treated as an infinite clean sink with concentration = 0). The time taken to establish a steady-state flux across the membrane was calculated based on Piringer's correlation for the diffusion coefficient. Under steady-state flux, a model was derived showing exponential decay in the concentration of VVOCs in the bag, assuming an initial concentration of VVOCs present in the bag. **Results:** The findings showed that the total VVOCs concentration, even if attributed to a single VVOC, was either below the tolerable intake and required no further evaluation, or the concentration would decay to tolerable levels before the device reaches the patient. **Conclusions:** The modelling supports that VVOCs do not pose a meaningful patient risk at start-of-life. A weight-of-evidence approach integrating modelling and post-market data indicates that analytical assessment alone is sufficient for most devices under ISO 18562-1:2024.

PS 3745 How the ASTM F2382-24 Update Is Changing PTT Test Evaluation for Medical Devices

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Background and Purpose: Partial Thromboplastin Time (PTT) test, Platelet Leukocyte Count (P&L), SC5b-9 Complement Activation test, and hemolysis assays are common *in vitro* tests to assess hemocompatibility of medical devices per ISO 10993-4. The PTT test, as described in ASTM F2382, is a well-recognized test method for screening circulating blood-contacting device materials for their ability to induce blood coagulation via the intrinsic coagulation pathway. The ASTM F2382-18 revision in 2024 included assay validity and test evaluation sections to align with the ASTM F2888 standard for assessing thrombogenicity through P&L counts. The test article is considered to pass the PTT test if there is no statistical difference in the average clotting time between the test article and the negative control (untreated plasma) or the negative reference material control (high density polyethylene HDPE), and a legally marketed comparator device (LMCD) is often assessed alongside the test article to support devices that do not meet this pass criteria. While this acceptance criterion remains in the 2024 version, the revised guidelines also introduce an alternative pass criterion when the test article's average clotting time is $>$ 80% relative to the negative control which is also the acceptance criterion for HDPE. This study presents a comprehensive data mining analysis of PTT test results across a wide portfolio of medical devices to assess the impact of the revision on pass/fail rates and whether including an LMCD is still worthwhile. **Methods:** Our analysis encompassed over five hundred PTT test results which were conducted at two test facilities from 2021-2025. The dataset comprised of roughly equal number of studies conducted under either the previous or the current ASTM guidelines. The pass rates for devices evaluated in accordance with either the 2018 or 2024 guidelines were determined. The percentage of tests performed together with an LMCD was also calculated. Results previously assessed under ASTM F2382-18 guidelines were re-evaluated under the 2024 criteria to determine any change in test outcome. A few case studies are included to examine any change in test evaluation outcome per the revised guidelines and demonstrate the continued importance of having an LMCD in data interpretation. **Results:** A total of 265 and 245 PTT studies, conducted under ASTM F2382-18 and -24 respectively, were included in this analysis. About equal number of PTT studies were conducted with test article (TA) only vs. TA and LMCD. Pass rates for tests under the 2018 and 2024 ASTM guidelines were 93% and 97%, respectively, reflecting a 4% increase in passing evaluations potentially due to the change in pass criteria. To evaluate the true impact in pass rate, all 55 Boston Scientific-sponsored studies conducted under the 2018 ASTM guidelines were re-evaluated using the updated guidelines. Under the 2018 guidelines, 82% (45/55) were given a final evaluation of "pass", while 10/55 did not meet the pass criteria. However, when the results of these 10 studies that did not meet the 2018 pass criteria were assessed using the updated 2024 ASTM guidelines, 6/10 met the new alternate pass criteria, resulting in 93% (51/55) passing the assay under the new

guidelines. In the 4 studies that TA did not meet the 2024 criteria, 3 had LMCD data in parallel and 2 had results comparable to an LMCD. **Conclusions:** The alternative pass criterion introduced in ASTM F2382-24 aligns with the Platelet and Leukocyte ASTM F2888 test methods, and our analysis results support that the updated guidelines **may** reduce the need to rely on an LMCD by lowering the threshold for what is considered an acceptable decrease in plasma clotting time for medical devices. Overall, ASTM F2382-24 reflects a more aligned and reasonable approach to screen for material-related thrombogenicity. The observed recent increase in PTT passing rate, along with additional cost and availability consideration, can guide more effective use of running testing alongside an LMCD.

PS 3746 Modernizing Menstrual Care: Key Insights from FDA's Updated Guidance

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Background and Purpose: The Center for Devices & Radiological Health (CDRH) of the U.S. Food and Drug Administration (FDA) ensures the safety and effectiveness of menstrual products such as tampons, pads, cups, and pregnancy diagnostic devices in the U.S. In October 2025, the FDA released a draft guidance titled, "Menstrual Products - Performance Testing and Labeling Recommendations." This document includes substantial updates to FDA's previous guidance issued over 20 years prior ("Menstrual Tampons and Pads: Information for Premarket Notification Submissions (510(k)s)," July 2005). Thus, it is important to understand the implications that the draft guidance may have for biological safety evaluation and the future development and marketing of these products. The objective of this work is to compare the recommendations for biological safety evaluation between the proposed draft and previous guidance. Two case studies are presented to illustrate the differences between guidances and the potential impact on the biological evaluation methods needed for menstrual products. **Methods:** A review of current international standards and regulatory guidance relevant to menstrual products was conducted to understand the state of the art for biocompatibility evaluation of these devices. The documents reviewed included the International Organization of Standardization (ISO) standards for evaluating the biocompatibility of medical devices (ISO 10993 series), FDA's guidance on ISO 10993-1 (September 2023), FDA's guidance on menstrual tampons and pads (July 2005), and FDA's draft guidance on menstrual products (October 2025). The review compared each document in key aspects of biological safety evaluation, including contact categorization, applicable biological endpoints, and methods for addressing the endpoints, including testing and risk assessment. Case studies were then presented to illustrate the differences between FDA's proposed and previous biological safety expectations for menstrual products. **Results:** The draft guidance clarifies FDA's expectations for safety evaluation and marketing of menstrual products. These details include recommendations for menstrual cups, which were not addressed in the 2005 guidance, clarification of patient contact type/duration for different menstrual products, recommendations for ingredient disclosure, contaminant evaluation and proposed test methods, and clarifications on non-clinical bench testing of tampons. Importantly, recommendations for the biocompatibility evaluation have been expanded. Case 1: A biocompatibility evaluation plan (BEP) for a diagnostic menstrual pad requiring a 510(k) submission that was marketed prior to the 2025 draft guidance was developed. This BEP was based on the 2005 guidance and assigned a different type of patient contact than suggested in the 2025 draft guidance. Consequently, a subset of evaluations was recommended compared to expectations proposed in the 2025 draft guidance. Case 2: A BEP for a reusable menstrual cup was developed based on the state of the art prior to the 2025 draft guidance (i.e., when menstrual cups were not described in FDA's 2005 guidance). The biocompatibility testing and evaluations in this BEP were compared to those proposed in the 2025 draft guidance. Discussion on whether the prescribed testing may address phthalates and PFAS materials that have been identified in reusable menstrual products was also provided. **Conclusions:** The 2025 draft guidance clarifies FDA's current thinking on the safety evaluation and labeling of menstrual products, addressing gaps left by previous guidance documents and international standards. By expanding recommendations for biocompatibility testing, ingredient disclosure, and contaminant evaluation, and expanding the scope of guidance to include other menstrual products, the guidance reflects evolving scientific understanding and public health concerns. These changes may enhance regulatory predictability and support manufacturers in developing safer, more transparent products. However, these changes may also expand the testing burden and may still not fully address all patient health risks. Expert biological safety evaluation remains essential to complement the forthcoming guidance in promoting biological safety for menstrual products.

PS 3747 Comparative Review of Carcinogenicity Testing Guidelines in Context of Medical Device Risk Assessment

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Background and Purpose: ISO 10993-3 recommends carcinogenicity studies aligned with OECD 451 guidelines, and combined chronic toxicity/carcinogenicity studies aligned with OECD 453. However, if there are sufficient toxicity data, ISO 10993-3 recommends a risk assessment using these data rather than conducting additional carcinogenicity tests. It is common to consider studies conducted under different testing guidelines in the same chemical risk assessment; thus, it is important to understand similarities and differences in requirements of other frequently followed testing guidelines, such as US EPA TG 870.4300, US EPA TG 870.4200, GB/T 15670.28-2017, and GB/T 15670.27-2017. We compare study design requirements in the US EPA and Chinese national standard (GB/T) guidelines to OECD requirements to determine whether these studies could be considered in a risk assessment that complies with ISO 10993-3. **Methods:** We reviewed and compared carcinogenicity testing guidelines in OECD 451, US EPA TG 870.4200, and GB/T 15670.27-2017, and did the same for combined chronic toxicity/ carcinogenicity study guidelines in OECD 453, US EPA TG 870.4300, and GB/T 15670.28-2017. We focused on key study parameters, including the preferred animal model, study size, exposure and observation duration, dose selection, and the endpoints evaluated. We also considered rubrics regarding how study results are evaluated and confounding factors are controlled, acceptance criteria for negative findings, requirements for dose-dependent responses, and criteria for early study termination due to elevated mortality. **Results:** In general, testing guidelines by OECD, US EPA, and GB/T are similar with respect to requirements for study size, exposure duration, dose selection, and the handling of test and control animals. A few noteworthy differences include: 1. US EPA requires carcinogenicity testing in at least two rodent species to better identify species-specific responses, while OECD and GB/T recommend only one species. 2. OECD 451 and 453, and US EPA TG 870.4200 and 870.4300 recommend comparing neoplastic findings in the study with historical controls from the same strain of animal kept in the same laboratory facility. 3. Both the US EPA and GB/T require the dose-response relationship to be included when evaluating a toxicity response. OECD only requires a statistical summary of the findings. 4. Both US EPA and GB/T include a maximum percentage mortality: ≤10% loss per group due to autolysis, cannibalism, and management, and ≥50% survival at 15 months for mice and 18 months for rats. This is to avoid the study being confounded by an elevated mortality rate. **Conclusions:** There are a large number of similarities with respect to key study design parameters and how results should be evaluated in the OECD, US EPA, and GB/T guidelines. This indicates that risk assessments based on studies conducted following any of these study guidelines should be similarly robust and acceptable for an OECD carcinogenicity evaluation.

PS 3748 Varied Global Regulatory Requirements in Medical devices for a Material Formulation Change - A Case Study

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Background and Purpose: ISO-10993-1 standard provides requirements on the biocompatibility evaluation for medical devices when a material formulation change occurs. For such a change, the risk assessment should consider what is known about the proposed constituent, base material constituents and potential chemical interactions amongst them. Since such information is not easily obtained due to its proprietary nature, standard biological endpoint testing provides a path to evaluate the change. This case study describes the biocompatibility evaluation for a formulation change to a resin used on numerous device components. The resin was re-formulated by replacement of organic additives at a maximum of four ten-thousands of a percent of the formulation, all other constituents remaining same. A modification to the compounding of the resin formulation also occurred. This change was across eight different formulations with the same base resin but differing additives. The proposed new materials would be used in externally communicating components in contact with tissue, bone, or dentin for less than 24 hours, "Limited" as defined in ISO 10993-1. While a vast majority of the devices using these resins would be gamma sterilized, some would exclusively use ethylene oxide. **Methods:** Test articles were pellets representative of four test formulations. These formulations were designed with a "Kitchen Sink" approach. Each test formulation was prepared with some of the constituents including base resin, colors, fillers and additives at the worst-case composition. Collectively, the four test formulations represented the worst-case in terms of composition for each of the constituents, in the eight formulations undergoing the change. Biological endpoint testing per ISO-10993-1 for a "Limited" medical device namely, cytotoxicity, acute systemic toxicity, irritation, sensitization and pyrogenicity was performed. Test articles were irradiated with gamma as the worst-case sterilization modality in terms of impact on polymeric materials, in comparison to ethylene oxide. **Results:** The test formulations favorably met the biological endpoint testing for a "Limited" device and the "Kitchen Sink" approach was acceptable to regulatory agencies. However, one notified body conveyed a deficiency, that toxicological equivalence to the current material was not demonstrated, since the chemical interaction of the constituents in the designed test formulations departed from the proposed resin formulations. In the absence of extractables/ leachables data

to show equivalence to current resin, an alternative strategy was adopted for the response. With vendor consent, the chemical composition for each proposed formulation was shared confidentially. Comparison of the constituent composition of the proposed resins with the test formulations illustrated that the latter approximated closely and underlined the new additive as a minor constituent. The test formulations had been designed to include both ends of the composition spectrum for proposed resins. The worst-case amount of a constituent and the best-case, which could be absence of a constituent. Since different resins had varying constituents, this gave assurance that all possible chemical and toxicological interactions of the proposed resins were captured in the test formulations. Favorable USP Physicochemical data, supported there was not a toxicological concern from heavy metals and non-volatile residue. Predicate use of the proposed additive in similar base resin formulations using both sterilization modalities of gamma and ethylene oxide was highlighted. This Weight of Evidence approach was accepted, the new formulation was considered toxicologically equivalent to the current formulation and approval was granted. **Conclusions:** While ISO-10993-1 provides a standardized framework for biological evaluation of medical devices for material related changes, variations in regulatory requirements exist. Partnering with a vendor on "proprietary" material information or via a "Master File" as recommended in Annex B of ISO-10993-1, helps characterize the chemical and toxicological makeup of proposed formulations. As it is not practical to test all formulations, using representatives is acceptable. A judicious design of test formulations assuring all possible chemical and toxicological interactions are addressed, can help obtain universal regulatory approval.

PS 3749 Evaluation of Degradation of Polyurethane Oligomers to MDI or MDA: Cohort of Concern or No Concern?

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Background and Purpose: Methylenebisphenyl diisocyanate (MDI) is an important component of poly(ether)urethanes (PEU). Exposure to MDI induced lung adenoma in male rats and is presumed to pose a similar risk to humans. MDI hydrolyzes in water to 4,4'-methylene dianiline (MDA), which is associated with liver and thyroid toxicity. As such, MDI is considered a cohort of concern compound per ISO 21726:2019 due to its polycyclic amine structure. Small MDI-containing oligomers, which may be present as byproducts of PEU synthesis, have the potential to leach from a device into the body over time. As patients may be exposed to these oligomers through medical devices, the approach from a toxicological perspective assumes that these oligomers degrade to MDI or MDA and thus must be assessed toxicologically as the more reactive monomers. This results in increased burden for both the chemist and toxicologist to accurately identify and evaluate these species. Therefore, evaluation of degradation of these compounds in a laboratory setting is needed to better understand this degradation potential in a clinical setting to ensure safety assessments adequately evaluate patient risk. **Methods:** To determine whether MDI-containing oligomers can degrade into MDA or MDI, a literature search was performed. A manuscript by Chaffin et al (2014) showed that MDI/MDA monomers were formed from MDI-containing oligomers at 70 °C, but not at 50 °C, which is well above any reasonable exposure temperature during handling, transport, or *in vivo*. However, this study did not address chemical mechanisms of degradation that could lead to generation of MDI or MDA. Therefore, we synthesized model MDI-containing oligomers and extracted PEU 80A to isolate MDI-containing oligomers and subjected these model compounds and extracts to acid, base, UV, and hydrogen peroxide to determine whether these conditions could induce formation of MDI or MDA from MDI-containing oligomers. Samples were analyzed using LC-MS and GC-MS to monitor loss of oligomers and formation of MDI or MDA. **Results:** Treatment with acidified or alkaline methanol (1% HCl or 1% NaOH) did not result in production of MDI or MDA from PEU extracts or MDI-containing oligomers. Furthermore, no significant degradation of MDI-containing oligomers was observed under these conditions. Consistent with these results, exposure of PEU or MDI-containing oligomers to UV or hydrogen peroxide with cobalt chloride did not result in production of MDI or MDA despite significant degradation of MDI-containing oligomers. These results were consistent with previous studies showing that oxidation affects the methylene group of MDI or the polyether chain, rather than the urethane linkage. This information provides better understanding of the requirements needed to degrade these oligomers, ensuring that clinically relevant compounds are toxicologically assessed. **Conclusions:** Toxicological risk assessments are intended to provide a conservative estimate of risk to patient safety. In the case of MDI-containing oligomers, the most conservative approach assumes that MDI or MDA can form and evaluates that potential risk to patients. However, these oligomers are present in a distribution that includes 1-3 MDI subunits and 3-20 polyether subunits. By assuming the reported amounts are the monomers, data processing, quantitation, and risk assessment are complicated due to the need to treat these oligomers as cohort of concern compounds. The results of this study demonstrated that MDI-containing oligomers do not degrade to MDI or MDA under harsh conditions. Combined with previous results showing that neither MDI nor MDA are formed at 50 °C or lower, it is highly unlikely that MDI or MDA would form under physiological conditions. Therefore, the tolerable intake limits for these oligomers may be higher than those of MDI or MDA, allowing them to be removed from the cohort of concern categorization and be screened out using the TSL when applicable.

PS 3750 LS-100 and LS-200: Novel, hemocompatible nanoparticles with unexpected pharmacological properties

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Background and Purpose: Peripheral artery disease (PAD) is a vascular disease associated with atherosclerotic and thromboembolic obstruction in the lower limbs. While obstructed arteries can be cleared using angioplasty and stenting, blockages can recur. Two common mechanisms for reocclusion include neointimal hyperplasia (NIH) and thrombogenesis. Novel nanoparticle (pNP) formulations LS-100 and LS-200 are designed to deliver drugs that address these mechanisms. Neither formulation induces significant hemolysis and LS-200 prolongs activated partial thromboplastin time. However, their hemocompatibility is unknown. **Methods:** Canine blood was obtained with an IACUC-approved protocol. Porcine blood was obtained commercially. Activated partial thromboplastin time (aPTT) was measured using freshly collected citrated canine blood from healthy donors. Platelet activation in pNP-exposed canine blood was measured using PDGF-BB and thromboxane B2 ELISA. Hemolysis assays were conducted in both canine and porcine EDTA anticoagulated blood exposed to LS-100 and LS-200. Endothelial viability and function were assessed using ATP and NO assays. A novel physiologic flow study measured thrombus formation on materials of differing thrombogenic potential inserted into loops containing pNP-exposed porcine blood under flow conditions. **Results:** Hemolysis did not exceed 5% in LS-100 exposed blood. Hemolysis remained below 6% in canine blood and below 2% in porcine blood exposed to LS-200. aPTT was unchanged in LS-100 exposed blood but significantly prolonged in LS-200 exposed blood ($p < 0.05$). Platelet activation markers were unchanged with LS-200. The physiologic flow loop study revealed a significant decrease in platelet and leukocyte counts in blood exposed to highly thrombogenic materials ($p < 0.05$), however, exposure to LS-200 demonstrated platelet and leukocyte counts comparable to unexposed blood. Material thrombus coverage and thrombus dry weight was significantly decreased in LS-200 exposed blood containing thrombogenic test material compared to thrombogenic material without LS-200 treatment ($p < 0.05$). ATP production was unaffected below 0.75 mg/mL of LS-200 pNP and below 1.5 mg/mL with LS-100 ($p < 0.05$). NO production did not significantly decrease with either formulation. **Conclusions:** Overall, LS-200 decreased thrombus formation on thrombogenic materials and maintained hemocompatibility. Measurements of immunogenicity, mutagenicity, and other key aspects of biocompatibility are required. Given the bioactivity of LS-200 absent drug cargo, future studies will assess LS-200 as a hydrophilic coating for cardiovascular medical devices. Early LS-100 data suggests inert behavior in the blood compartment, but additional analysis of hemocompatibility including platelet activation and thrombogenicity are required.

PS 3751 PFAS in Chronically Implanted Cardiac Leads

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are a large group of environmentally persistent chemicals, some of which are linked to adverse health effects and are now under regulatory scrutiny, especially in medical devices. Medical-grade PFAS materials like PTFE and ETFE are valued for their chemical inertness and have shown consistent safety in biocompatibility studies. Yet, toxicological concerns remain about residual low-molecular-weight PFAS that might be present after manufacturing, particularly regarding potential bioaccumulation in the patient. Proposed EU regulations could either prohibit PFAS in medical devices altogether or impose strict limits, such as capping targeted water-soluble PFAS at 25 ppb and total semi-quantitated water-soluble PFAS at 250 ppb. Traditional chemical characterization methods generally employ instruments with high mass accuracy but lower sensitivity, resulting in quantitation limits around 20 ppb. While this complies with the requirements of ISO 10993-18:2020, water-soluble PFAS compounds have the potential to bioaccumulate. Thus, even at relatively low concentrations, this potential accumulation in the human body over time may suggest that more stringent limits for PFAS in medical devices are necessary. This study used highly sensitive triple quadrupole LC/MS to screen for trace PFAS in new and explanted cardiac leads, to evaluate if PFAS in these devices pose safety risks over their clinical lifetime. **Methods:** New and 20+ year explanted cardiac leads containing PTFE and ETFE were extracted based on ISO 10993-18:2020 methods to evaluate the patient-contacting portions of the devices. Taking into account the surface area and total solvent volume, the Analytical Evaluation Threshold (AET) for the extracted devices was determined to be 11.0 ppb. Triplicate exaggerated extractions of final, finished medical devices were performed with methanol and analyzed for PFAS using sensitive Sciex 7500 LC/MS/MS triple quad, detecting levels as low as 0.00250 ppb. PTFE and ETFE components from additional leads underwent separate extractions, first with methanol and later with dimethylformamide (DMF), to evaluate the maximum potential concentrations of short-chain PFAS in non-patient-contacting components. Untargeted analysis of the extracts was performed first to identify the compounds present in the extracts. Then targeted analysis was performed on all the detected PFAS and semi-quantitated using perfluorobutanesulfonic acid (PFBS) as the surrogate standard. A toxicological risk assessment was conducted to evaluate whether the PFAS detected in PTFE/ETFE containing medical device extracts posed any safety risk to patients. **Results:** Approximately sixteen water-soluble PFAS were identified above

the limit of detection, 0.0025 ppb, in both new and 20+ year explanted cardiac leads. The semi-quantitative results showed an approximate 88% reduction in total PFAS in the explanted leads compared to new leads. This decrease was also observed in methanol extracts of PTFE components, while ETFE components showed increased PFAS levels after long-term implantation. DMF extraction, used to assess worst-case PFAS presence, revealed that most PFAS in PTFE and ETFE were oligomers, and explanted ETFE contained over 600% more PFAS than new ETFE, likely due to increased surface area or exposure of non-intended patient contacting surfaces.

Conclusions: This study provides one of the most sensitive assessments to date of water-soluble PFAS in new and long-term implanted medical devices containing PTFE and ETFE, using advanced LC-MS/MS methods. PFAS levels in new devices were well below proposed regulatory thresholds, and concentrations in explanted leads decreased by an approximate 88% after 20 years; PTFE components showed an approximate 66% reduction, while ETFE components had increased PFAS, likely due to surface changes or contamination. Importantly, nearly all PFAS detected were at trace levels, with only one potentially regulated compound present in explanted leads and no evidence of significant patient exposure. As all PFAS values in the full devices were below the AET, the limited presence of water-soluble PFAS supports the continued safe use of PTFE and ETFE in medical devices and provides important data for regulatory considerations.

PS 3752 Assessing PFAS Biological Risks from Medical Devices Under ISO 10993-1 - Material Properties and Chemical-Specific Effects on Leachability, Systemic Exposure, Bioaccumulation, and Toxicity

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Background and Purpose: New requirements are being introduced into ISO 10993-1 for assessing biological safety of medical devices with respect to substances having a potential for bioaccumulation. Perfluoroalkyl substances (PFAS) represent a broad family of substances having significant resistance to chemical/biological degradation, and as a result regulatory restrictions have been proposed for phasing out subclasses of PFAS used in various countries. In particular, recent updates to the biocompatibility guideline, ISO 10993-1 require a recategorization of medical devices containing substances that may bioaccumulate, so that devices intended for less than 30 days contact are reassigned into the long-term contact category (≥ 30 days). This default strategy significantly increases testing rigor and necessitates a very low Analytical Evaluation Threshold for extractables testing of medical devices, requiring more stringent limits of quantitation for chemical analysis that could be excessive in many cases, given the clinical relevance of estimated levels of exposure from short term use applications. **Methods:** Notably, per- and polyfluoroalkyl substances (PFAS) are utilized across diverse medical device applications, including polytetrafluoroethylene (PTFE) in vascular grafts (low-exposure, long-term implant), fluorinated ethylene propylene (FEP) for catheter guide wires (surface coating), poly(ethylene-tetrafluoroethylene) (ETFE) for electrical insulators in pacemakers and implantable pulse generators, polyvinylidene fluoride (PVDF) in dialysis membranes (high-surface-area contact), hexafluoropropylene oxide dimer acid (HFPO-DA) as a processing agent to enhance polymerization (rinsed/removed to minimize residuals), and perfluorobutane sulfonate (PFBS) and other short chain perfluorinated compounds in coatings which are designed to reduce friction. **Results:** A toxicological strategy is outlined which emphasizes a consideration of chemical structure, leaching potential, and biological exposure modeling. Examples are provided to contrast the risk profiles of highly stable polymeric PFAS (e.g., PTFE in vascular grafts) which exhibit negligible leaching and clinical patient exposure, with those of highly mobile, short-chain substances (e.g., PFBS as a replacement for longer chain PFOS due to a shorter biological half-life). The primary emphasis is that different PFAS entities possess fundamentally different physico-chemical properties and toxicokinetic profiles. While high-molecular-weight polymers like PTFE have negligible leaching, low-molecular-weight PFAS like PFBS and perfluorooctanoic acid (PFOA) may contribute significantly greater chemical exposure and bioaccumulation. In addition, one must account for the estimated cumulative dose over the device's duration of use (short term versus longer term) to determine toxicological significance. In some cases, specific routes of exposure (e.g., contact with intact skin or low-leaching fluorinated polymers comprising an inhalation pathway device) may yield inherently low exposure profiles, and do not merit recategorization into the long-term contact category (≥ 30 days). **Conclusions:** In conclusion, assessing PFAS biological risk requires a route-specific evaluation of exposure, consideration of material-specific release potential, and chemical-specific toxicological assessment. The toxicological strategy framework should include an assessment of material integrity/inertness and release mechanisms, estimation of clinical exposure and toxicokinetics of systemic absorption, distribution, metabolism, and elimination, and a comparison to tolerable intakes, acknowledging that toxicity and bioaccumulation potential differ across the broad class of PFAS chemicals.

PS 3753 Prevalence and Toxicity Evaluation of the Types of PFAS Found in Medical Device Extractables and Leachables Datasets

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Background and Purpose: Medical devices (MDs) have the potential to release a wide range of chemical constituents during patient use. Several common components of MDs, such as seals, valves, stoppers, packaging, lubricants, and coatings, are constructed from materials consisting of per- and polyfluoroalkyl substances (PFAS). While PFAS regulations have been increasing rapidly across the world, most do not make the distinction between fluoropolymers and non-fluoropolymers. This distinction is important, as fluoropolymers have very different chemical, physical, and biological properties compared to non-fluoropolymers (Henry *et al.*, 2018). Additionally, regulatory agencies across the world have differing definitions of PFAS, ranging from a few select PFAS (e.g., perfluorooctanoic acid [PFOA]) to the broad European Union (EU) definition of "fluorinated substances that contain at least one fully fluorinated methyl or methylene carbon atom (without any H/Cl/Br/I atom attached to it)" (OECD, 2021). There is a clear need for identification of the types of PFAS that are in MD extractable profiles. To address this need, we leveraged two large datasets of MD extractable profiles and analyzed the types of PFAS from approximately 19,000 chemicals. Our objectives are: 1) to determine the types of PFAS found using different regulatory definitions; and 2) to determine whether the broad types of these PFAS are associated with reproductive/developmental toxicity, genotoxicity, and carcinogenicity. **Methods:** Published lists of MD constituents from hundreds of extractable and leachable studies (Borton and Coleman, 2025; Builee *et al.*, 2025) were compiled and filtered to contain only unique fluorinated compounds. The fluorinated constituents were classified as PFAS using various regulatory definitions, including that of the EU, United States (US) Toxic Substances Control Act (TSCA), and the Canadian Environmental Protection Act (CEPA) list. The PFAS was further categorized as polymers and non-polymers based on categories described in Henry *et al.* (2018). A comparative analysis was conducted on the fluorinated compounds to assess the percentage of concordance between the list of PFAS in MD extractable profiles according to different regulatory definitions. Literature searches were conducted for chemical-specific toxicity data for the PFAS categories via the data aggregator ToxPlanet (*i.e.*, a federated search engine that extracts content from 500+ websites and databases, such as CompTox and the European Chemicals Agency [ECHA] CHEM). **Results:** We found that approximately 2.6% (N = 488) of the 19,075 chemicals extracted from MDs are fluorinated. Of those 2.6% fluorinated chemicals, approximately 48.7% were considered PFAS according to the EU definition, 24.6% were considered PFAS according to the TSCA definition, and only 1.6% were considered PFAS according to the Schedule 1 substances list published by CEPA. Among the three PFAS definitions, 242 (1.3% of the MD constituents) were found to be unique PFAS - 2 were polymers and 240 were non-polymers. Further, 11% of the fluorinated chemicals had chemical-specific toxicological data available according to the CompTox chemicals dashboard, and only 6% had an oral and/or inhalation point of departure (POD). **Conclusions:** Our results suggest that approximately 1.3% of chemicals extracted from MD extractable and leachable profiles are PFAS; therefore, the likelihood of patient exposure to PFAS through medical devices is low. However, extractable and leachable studies are only conducted on patient-contacting components, whereas most PFAS regulations impacting medical devices are determined based on the entire device (*i.e.*, patient- and non-patient-contacting components). PFAS detected in MD extractables are likely to originate from both patient-contacting components and processing aids. Notably, only two PFAS polymers were identified in this dataset. Since fluorinated side chain polymers are known to break down into non-polymer constituents, this finding does not indicate a lack of PFAS polymers in MDs. Further, fluoropolymers, such as polytetrafluoroethylene (PTFE), were not found in this dataset. There is a paucity of data for PFAS in MD extractables; however, available data show that PFAS chemicals have a wide range of toxicity.

PS 3754 A Tiered Safety Strategy for a Novel Personal Care Product Category - Whole Body deodorant: From *in Vitro* Models to Clinical Validation

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Background and Purpose: The personal care landscape has rapidly evolved with the emergence of a distinct product category: deodorant for whole body application including sensitive intimate regions. Unlike conventional underarm deodorants, which are often spray-based for localized use, these novel products are typically lightweight creams designed for extensive application across diverse body areas prone to sweating and odor. Crucially, many of these target sites, particularly the intimate and intertriginous zones, exhibit unique physiological characteristics such as increased skin permeability due to thinner stratum corneum, higher moisture content, and the potential for prolonged occlusion and/or direct contact with mucous membranes. This expanded use, coupled with the formulations enabling widespread application over a significantly larger total body surface area, introduces specific toxicological considerations, including higher potential systemic exposure due to the large surface area and an increased risk of local intolerance on sensitive skin and mucosal sites. Consequently, a dedicated and robust safety assessment strategy is

crucial to ensure consumer safety for this unique product type. **Methods:** A specific safety assessment strategy was developed to evaluate systemic toxicity and local tolerance, tailored specifically for the unique characteristics of this novel product category. To assess systemic toxicity, a conservative whole-body exposure assessment was employed for all ingredients, using the SCCS body lotion quantity to calculate the Margin of Safety (MoS). This approach maximizes theoretical exposure, thus accounting for potential product misuse. For local tolerance, a tiered testing strategy was executed on the formulation. It began with an initial screening that included assessment of the formulation's physicochemical properties (i.e., pH) and a thorough safety review of each ingredient using published literature and historical data. This was followed by New Approach Methodologies (NAM) based-*in vitro* assays to assess skin irritation (Reconstructed Human Epidermis model) and, critically, potential vaginal mucosal irritation using a Reconstructed Human Vaginal Epithelium model. Following the *in vitro* evaluation, consumer tolerance was confirmed clinically via a Human Repeat Insult Patch Test (HRIPT) and an in-use tolerance study conducted under both dermatological and gynecological supervision. **Results:** We have substantiated the safety of a novel deodorant formulation for its intended use on the whole body and external intimate area using this specific safety assessment approach. The conservative whole-body assessment confirmed all raw materials have a sufficient MoS (>100 per the SCCS Notes of Guidance). In the initial local tolerance screening, the formulation's pH was found to be compatible with the physiological environment of its intended application sites, specifically the external intimate area, and the safety review of its ingredients raised no concerns. Subsequently, the formulation was predicted as non-irritating in *in vitro* reconstructed human skin and vaginal epithelium models. Clinically, the product was proven to be non-irritating and non-sensitizing in a HRIPT. An in-use study conducted under dermatological and gynecological supervision confirmed the product demonstrated excellent tolerance, with no adverse reactions observed. **Conclusions:** The safety assessment strategy presented successfully demonstrated that the novel whole-body deodorant is safe and well-tolerated for its intended use. The integration of a conservative, whole-body systemic safety assessment with a tiered, targeted local tolerance evaluation - notably including a reconstructed vaginal epithelium model and clinical oversight by both dermatologist and gynecologist - proved to be a robust and highly relevant approach. This work establishes a benchmark for the safety validation of innovative cosmetic products for sensitive applications, underscoring the power of a modern testing strategy rooted in Next Generation Risk Assessment (NGRA) principles to ensure consumer safety without animal testing.

PS 3755 Untargeted Exposomic Analysis of CBD Oil Tinctures and Personal Care Products

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Background and Purpose: Public demand for cannabidiol (CBD) products has expanded dramatically in recent years. Following the 2018 Agricultural Improvement Act, which legalized hemp-derived products containing <0.3% THC, CBD oils and personal care products have proliferated across the United States. However, federal regulation is lacking, and state-level oversight remains inconsistent. Given the diverse cultivation and processing methods, CBD products may contain a wide range of environmental contaminants and synthetic chemicals in addition to phytocannabinoids, raising significant public health concerns. This study applied an untargeted exposomic approach using high-resolution mass spectrometry (HRMS) to comprehensively profile environmental contaminants and phytocannabinoids in CBD oil tinctures and CBD-infused personal care products. **Methods:** Samples were analyzed using inductively coupled plasma mass spectrometry (ICP-MS) for metals and metalloids, and both gas and liquid chromatography coupled to HRMS (GC-HRMS and LC-HRMS) for organic contaminants and phytocannabinoids. **Results:** Natural CBD oil tinctures contained a broad spectrum of phytocannabinoids in both acid and neutral forms, including C4- and C5-CBD, CBDA, CBGA, CBNVA, CBTv, CBGM, and sesquicannabigerolic acid, whereas synthetically derived products (e.g., delta-8 formulations) showed narrower profiles. All oil tinctures contained metal contaminants, with 22 distinct essential and non-essential elements detected. Most essential metals were below 10 ppb, with Mn, Zn, and Cu showing the highest levels. Mn concentrations in some samples exceeded both the EPA secondary Maximum Contaminant Level (MCL) and lifetime health advisory level, suggesting potential neurotoxicity risk. Non-essential metals were generally below regulatory limits, except Tl, which exceeded the EPA MCL in one sample. Ba was the most abundant non-essential metal, and naturally derived tinctures showed higher overall concentrations than the synthetic product. The heavy metals, As, Cd, Hg, and Pb, were present in all samples but below enforceable MCLs; however, this remains concerning since there are no safe levels for As and Pb. GC-HRMS identified 17 non-cannabinoid organic compounds, including naturally occurring and synthetic chemicals such as *m*-cresol. LC-HRMS analysis annotated potential contaminants such as butyl paraben, 4-tert-octylphenol, and the pesticide linuron. Ongoing lab analyses are being performed on 50 personal care products that included naturally derived CBD, with the vendor-disclosed CBD content ranging from 25 mg to 1000 mg. **Conclusions:** This multi-platform, exposomics approach provides an unbiased, systems-level framework to address the chemical complexity and diversity of CBD consumer products, which contains hundreds of phytochemicals alongside potential contaminants. Our findings highlight significant variability in metal and organic contaminant profiles,

emphasizing the need for quality control and regulatory oversight, particularly for plant-derived CBD products that remain vulnerable to agricultural and processing-related contamination. This work was supported by ULRI Discoveries in Safety Grants.

PS 3756 Personal care product ingredient safety disparities based on demographic marketing: opportunities for alternatives assessment and consumer education

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Background and Purpose: Studies have demonstrated that PCP use is associated with changes in biomarkers of exposure, as well as with health outcomes like hormone-dependent cancers and uterine fibroids. These effects may be more pronounced for women of color, which has been attributed to the use of certain products like hair straighteners, skin lightening creams, and fragrances. However, differences in product formulations within product categories may also contribute to these disparities. We used the Environmental Working Group (EWG) Skin Deep personal care products database to evaluate differences in PCP formulations based on demographic marketing. **Methods:** Using online discussion forums and in store product scanning, we identified 4,011 products marketed to Black women based on product or brand name, use instructions, and advertising language. We added any product that did not yet have a Skin Deep product hazard score to the Skin Deep database. Skin Deep hazard scores are based on data from authoritative bodies (ex. ECHA, U.S. EPA, OECD), industry reports (ex. CIR, RIFM), and open literature reviews. The data for each ingredient are grouped into 11 endpoint buckets: allergies/immunotoxicity, cellular/biochemical changes, cancer/mutagenicity, ecotoxicity, endocrine disruption potential, irritation, neurotoxicity, occupational hazards, organ system toxicity, persistence/bioaccumulation, and reproductive/developmental toxicity. Each data point within an endpoint bucket is scored from 0 to 100 according to severity and weight of evidence. The highest score from each endpoint is combined and weighted to calculate overall raw ingredient scores, which are combined by endpoint to calculate the raw product score. Raw ingredient and product scores are scaled 1-10 with lower ratings representing lower hazard based on predetermined score cutoffs established by deciles. Products are also tagged by category to allow direct comparison for products in the same category. We evaluated product and ingredient hazard scores to determine whether formulations, not product category alone, may be driving exposure and health disparities. **Results:** When comparing the Skin Deep hazard scores of 4,011 products marketed to Black women to the remaining 113,959 products without demographic marketing, we found that PCPs marketed to Black women had higher Skin Deep hazard scores. Certain color cosmetics and hair treatment/styling products seemed to drive the hazard score disparity. Of the 4,011 products, 71% were hair products and 6% were makeup. While the prevalence of some higher hazard ingredients in products marketed to Black women decreased since our 2016 analysis, we noted the presence of ingredients the market has started shifting away from including classes of preservatives (isothiazolinones, parabens, and FRPs), cyclic siloxanes, talc, and some fragrance ingredients. **Conclusions:** Because alternatives for many of these ingredients are already well-established in the PCP sector, a shift towards lower hazard formulations should be possible for many of these products. However, while small sample sizes for brand names and some product types may limit the generalizability of the results, this secondary analysis suggests that products marketed to Black women may not be reformulated to avoid hazardous ingredients at the same rate as the rest of the market. This potential lag time in reformulation indicates that 1) third party certifications can address exposure disparities by intentionally partnering with brands marketing to Black women and 2) consumer education initiatives like Skin Deep can be leveraged to reduce exposure disparities while the market catches up.

PS 3757 Evaluation of the Safety of Raspberry Ketone and Its Skin Compatibility in Cosmetics through New Approach Methodologies (NAM) and Mode of Action Mechanism Analysis

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Background and Purpose: In cosmetics, raspberry ketone has been used as skin conditioning or fragrance ingredient. The use of raspberry ketone as a multifunctional antimicrobial ingredient in cosmetics is new. The maximum recommended use concentration of raspberry ketone in cosmetics is 1% wt. In this research, raspberry ketone is evaluated for its safety as a cosmetic ingredient, particularly focusing on its potential to cause chemical leukoderma, a skin depigmentation disorder, by comparing it to rhododendrol, a structurally similar compound known to cause this condition. The in-use safety of raspberry ketone and its skin compatibility have been further supported by utilizing NAM and mechanism of action (MoA) analysis. **Methods:** Certain phenolic compounds are known to cause contact leukoderma. Based on the currently available information, the action mechanism of leukoderma appearance by phenolic compounds in human involves the following sequences: (1) tyrosinase-mediated oxidation of susceptible phenolic compounds into toxic metabolites, leading to protein-SH unfolding and accumulation in the endoplasmic reticulum (ER) and/or activation of the apoptotic pathway that contributes to the

direct and specific melanocyte cytotoxicity, (2) clinical or pathological symptoms of contact dermatitis, and (3) appearance of leukoderma or localized white depigmented skin. **Results:** The experimental results are summarized as following: (1) the gas chromatography analysis showed no detectable level of rhododendrol in raspberry ketone with a detection limit of 50 ppm, (2) the comparative *in vitro* cytotoxicity and melanin quantification assays showed raspberry ketone is less cytotoxic and has relatively low potency to cause chemical leukoderma when compared to other phenolic substances that are safely used in cosmetics and also to rhododendrol, and (3) no symptoms of contact dermatitis, irritation or sensitization, have been observed at 1%, 5%, 10% and 12% raspberry ketone in multiple and repeated-exposure human patch tests (human repeat insult patch tests and 48-hr human patch tests in different geographical regions). **Conclusions:** The above results provide an adequate weight of evidence (WoE) that 1% raspberry ketone in cosmetics is skin compatible and does not represent any leukoderma risk under the current practice of use and foreseeable misuse conditions.

PS 3758 Proteomic analysis of the effects of the novel refrigerant R-1132(E) on rabbit kidney and liver from a 28-day inhalation study

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Background and Purpose: Climate change driven by global warming continues to draw worldwide concern, prompting the search for refrigerants with minimal climate impact. R-1132(E), trans-1,2-Difluoroethylene, whose global warming potential (GWP) is <1, has emerged as a promising candidate. R-1132(E) belongs to a chemical family, called haloalkenes, which includes R-1234yf (2,3,3,3-Tetrafluoropropene) and HFO-1123 (1,1,2-Trifluoroethene). Previous animal studies on R-1234yf and HFO-1123 showed the species-specific responses in rabbits. R-1132(E) also showed similar responses in rabbit inhalation studies. In this study, we evaluated the effects of R-1132(E) exposure on the liver, a key organ for detoxification, and on the kidney, which mediates excretion, using proteomic profiling. **Methods:** Control (filtered air) and R-1132(E) atmospheres (500, 1000 and 2000 ppm) were administered as a 6-hour/day, whole-body inhalation exposures on a 5-day per week basis for 4 weeks. At the time of the scheduled euthanasia, a sample of kidney and liver tissue was collected from each animal per group and snap frozen in liquid nitrogen. The tissues from control and 2000 ppm group were used in further analysis. The powdered organs were dissolved in SDS solution and homogenized to extract proteins. The extracts were subjected to reduction, alkylation, and trypsin digestion to generate peptide solutions, which were analyzed by nanoLC-MS/MS. Statistical analysis of the proteomic data was performed using the R package DEqMS. **Results:** In the liver, pathway enrichment analysis revealed alterations related to inflammatory responses and transporters. Focusing on liver function-related proteins, various CYP families showed changes, with particularly pronounced upregulation in CYP2C; this is consistent with results from a separate *in vitro* study. In the exposure group, inflammatory markers centered on IL16 and NFκB1 were activated, and increases in IL16 and ICAM1, an adhesion molecule involved in leukocyte recruitment, suggested recruitment of macrophages and T cells due to inflammation. In the kidney, even more pronounced changes were observed. GO enrichment analysis and gene set enrichment analysis (GSEA) detected downregulation of transporter-related terms (e.g., monoatomic ion transmembrane transport, organic anion transport) in the R-1132(E) exposed group. Pathway-level GSEA also indicated upregulation of inflammation-related categories. At the level of individual kidney function-related proteins, the R-1132(E) exposed group showed broad functional decreases encompassing AQP2 (water channel), the CLDN family (tight junctions), the CA family (acid handling), Na⁺, K⁺, and phosphate reabsorption transporters, and Na⁺/K⁺-ATPase. As in the liver, inflammatory markers centered on IL16 and NFκB1 were activated in the R-1132(E) exposed group. **Conclusions:** Proteomic analysis of liver and kidney from R-1132(E) exposed rabbits revealed changes in the hepatic CYP2C family, transporter alterations, and activation of inflammatory markers in the liver. The kidney exhibited even more prominent changes, including widespread decreases in transporter function, suggesting potential disturbances of urinary and serum electrolyte balance. These findings indicate that inhalation of R-1132(E) may particularly impact renal function in rabbits. However, due to interspecies differences, these results cannot be directly extrapolated to humans. Further evaluation using human cells is warranted.

PS 3759 Normal, But Not Normal, Review of Some Vehicle-Related Histopathology Findings in the Concurrent Control Animals in the General Toxicity Studies

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Background and Purpose: Concurrent control groups are essential in general toxicity studies, and are typically composed of animals administered with the vehicle. The vehicle is usually the same solvent used to dissolve the test article, such as saline,

the most abundantly used vehicle. However, some solvents, including surfactants or excipients, may be employed to meet the solubility and absorption requirements of pharmaceutical formulations. This report describes toxicity findings from three less common vehicles used in the general toxicity studies, highlighting the need for toxicologic pathologists and toxicologists to understand that the concurrent control group may not always represent a completely normal baseline. **Methods:** Histopathology data for three vehicles, dimethylacetamide (DMAC), caprylocaproyl macrogol-8 glyceride (Labrasol), and β-cyclodextrin, used in the control groups from preclinical general toxicity studies were reviewed at our facility. **Results:** Toxicities of DMAC as a vehicle, were observed in the liver, testes, and eyes of the Sprague-Dawley (SD) rats or Beagle dogs. Histopathological changes included centrilobular mixed cell infiltration and occasional single-cell necrosis of hepatocytes in the liver, seminiferous tubular degeneration and atrophy in the testes, accompanied by reduced sperm and presence of cellular debris in the epididymides and single-cell necrosis of the corneal epithelium with fibroplasia of the corneal stroma in the eyes. Toxicities of Labrasol, as a vehicle, was observed in the lungs of SD rats, including bronchiolo-alveolar mixed cell infiltration and obliterative bronchiolitis with foreign material. Mortality was observed and likely attributed to these lung changes. Lastly, β-cyclodextrin, used in the control group, was often observed to induce hepatocellular necrosis in the liver of ICR mice and tubular vacuolation in the kidneys of SD rats. **Conclusions:** Some vehicles such as DMAC, Labrasol, and β-cyclodextrin could exhibit some toxicities. Study pathologists and toxicologists may need to pay special attention to the histopathological findings in the concurrent control groups when these vehicles are used in the studies and differentiate them from test article-related findings in the testing groups.

PS 3760 Formalin-Fixed, Paraffin-Embedded (FFPE) Tissues for Retrospective Transgenic Mouse (rasH2) Genotyping

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Background and Purpose: Following the completion of a transgenic (rasH2) mouse (CByB6F1-Tg(HRAS)2Jic (rasH2 tg/wt)) carcinogenicity study, a *post hoc* confirmation of genotype was required. Standard genotyping methods using fresh tail snip or ear punch were not feasible, as necropsy had been completed. Available biological materials for potential genotyping included formalin-fixed tissues, small volumes of plasma, and formalin-fixed paraffin-embedded (FFPE) tissues. **Methods:** DNA was extracted from formalin-fixed (wet) or FFPE tissues using the Mag-Bind® FFPE DNA 96 Kit (Omega Bio-tek, Cat. # M6958). PCR amplification was performed using established primer pairs, and products were analyzed via 1.5% agarose gel electrophoresis to assess genotype based on the size and number of PCR products. **Results:** Study samples from FFPE liver and intestinal tissues (stored up to 232 days prior to paraffin embedding) were successfully analyzed and genotyped. PCR amplification using DNA extracted from formalin-fixed (wet) tissues was unsuccessful, likely a result of insufficient DNA yield from these tissues. As longer exposure to formalin can lead to increased DNA fragmentation reducing the availability of amplifiable DNA, the extended storage time of these tissues in formalin likely contributed to the insufficient yield. PCR amplification of DNA extracted from FFPE liver and intestinal tissues was successful in amplifying the 260 and 130 base pair amplicons that differentiate the transgenic and wild-type genotypes. The larger amplicon (498 base pair) did not amplify possibly due to DNA degradation from formalin exposure. **Conclusions:** The DNA extraction method developed for paraffin embedded tissues was successful regardless of formalin fixation time. All tested samples were successfully genotyped as wild-type or heterozygous rasH2 using FFPE liver and intestinal tissues.

PS 3761 Refining Acute Oral and Inhalation Toxicity Testing: Current Practices and Opportunities

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Background and Purpose: Acute toxicity studies are conducted in animals for classification and labelling purposes, and human health risk assessment and management of chemicals and agrochemicals. Currently there are three Organisation for Economic Co-operation and Development (OECD) test guidelines each for acute oral (TGs 420, 423 and 425) and acute inhalation (TGs 403, 433 and 436) toxicity studies. TGs 423, 425, 403 and 436 use lethality as an endpoint, while TG 420 and TG 433 require fewer animals and replace death with a more refined endpoint based on 'evident toxicity', defined as clear signs that exposure to a higher dose would result in severe toxicity or death. However, it is unclear how widely these refined methods are being used, and the subjective nature of 'evident toxicity' may be preventing wider use. Previously, the UK National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and the European Partnership for Alternative Approaches to Animal Testing (EPAA) collaborated to provide recommendations on the recognition of 'evident toxicity', with the aim of making the endpoint more objective and transferable between laboratories. Guidance for the use of evident toxicity is included in TG 433 (adopted in 2017) for inhalation studies, but it is not included in the corresponding test guideline for acute oral studies (TG 420, adopted in 2001). This poster

investigates the current use of these refined methods and opportunities to promote their use in preference to the methods requiring lethality. In addition, the impact of deleting TG 403, LC50) is discussed, considering that the corresponding oral test (TG 401, LD50) was deleted in 2002. **Methods:** In 2025 the NC3Rs convened experts from industry, CROs, and consultancies across multiple regions (UK, USA, EU, India) to assess guideline use and barriers to refinement. **Results:** Responses indicated that the TGs with death as an endpoint are still widely used and the preference of guideline used is regional. The use of the guidelines were as follows, for acute oral studies TG 420 (14%), TG 423 (57%) and TG 425 (29%) and for acute inhalation studies TG 403 (33%), TG 433 (20%) and TG 436 (47%). Concerns over using 'evident toxicity' as an endpoint, particularly for inhalation (75% had concerns), included global acceptance of these guidelines, not achieving classification cut-offs and considered to use more animals and be more expensive than other guidelines when adapted as limit tests. Cost associated with the unfamiliarity of using 'evident toxicity' was also cited. There were also perceived geographical preferences of regulatory bodies. Concerns (47%) on the implications of deleting TG 403 (LC50), included a perception that some regulatory agencies require a point estimate of LC50, the potential to misclassify chemicals using 'evident toxicity' and the requirement of some regulators for risk assessment (TG 403 only) in addition to Global Harmonised System (GHS) for classification and labelling. The TG 403 also can be used for Concentration x Time studies (CxT) for some gases, vapours and aerosols. TG 403 has also been adapted to use as a limit test, potentially reducing number of animals used and cost. **Conclusions:** The goal is to encourage the use of the more refined methods and to move away from studies using death as an endpoint. Based on the responses from this expert group, less than 20% are currently using these refined tests, so next steps include: 1. convening a larger group of experts and regulators to discuss perceived differences and to confirm that TG 420 and 433 are accepted globally. 2. Provide additional guidance on the use of 'evident toxicity' as an endpoint to increase familiarity and confidence that chemicals will not be misclassified, including the addition of this guidance in the oral guideline (TG 420). 3. Confirm that range estimates of LD50/LC50 can be used to calculate exposure levels and risk assessments instead of point estimates. 4. Investigate if the TGs using 'evident toxicity' can be flexible and adapted, for example used as limit tests or as studies covering shorter durations.

PS 3762 Forensic Sampling and Risk Assessment of Downwind Chlorine Plumes on Paper Products Following a 2024 Chemical Storage Fire

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Background and Purpose: In 2024, a large chemical fire occurred at a chemical storage facility in Georgia, releasing a visible plume of chlorine-containing decomposition byproducts. As many as 17,000 people were evacuated and 90,000 were advised to shelter in place due to the plume. A paper products manufacturing company located several miles downwind raised concerns regarding potential contamination of their products from the chemical-containing smoke. This concern involved the potential deposition of combustion byproducts onto paper products and that chlorinated chemicals may be adsorbed by various paper products. A team conducted rapid sampling and forensic analysis across the facility and product lines. **Methods:** Surface wipe samples were collected following ASTM D6661-17 and water samples (from the retention ponds at the impacted campus) were collected to assess the presence of polycyclic aromatic hydrocarbons (PAHs) and dioxins, which are combustion byproducts of concern in most fires. X-ray fluorescence (XRF) measurements were taken to assess the presence of elemental chlorine in paper products, where elevated levels may indicate adsorption of chlorine-containing chemicals into paper products. Odor sampling was conducted using samples of paper products exposed during the plume to assess for the presence of any unusual odors, which would qualitatively indicate that they may have been contaminated during the fire and would be unfit for their intended use. Samples were collected from the facility exposed to the plume from the fire and from a control facility that handled the same paper products nearly 100 miles away. In total, over 650 samples and measurements were collected across two facilities. **Results:** One or more of five PAHs (phenanthrene, pyrene, indeno(1,2,3-cd)pyrene, benzo(ghi)perylene, and anthracene) were detected in 52 of the 253 wipe samples analyzed at both the control and exposed facilities. Phenanthrene, the most frequently detected PAH, was reported in 51 of these 52 surface wipe sample detections, with more positive detections identified at the control facility than the exposed facility. Phenanthrene concentrations, when detected, ranged from the detection limit (0.0005 micrograms per cubic centimeter [$\mu\text{g}/\text{cm}^3$]) to 0.0014 $\mu\text{g}/\text{cm}^2$. The detected phenanthrene concentrations were within an order of magnitude of the concentrations of the other four PAHs (pyrene, indeno(1,2,3-cd)pyrene, benzo(ghi)perylene, and anthracene). All four PAHs were detected in samples only once in 253 wipe samples (approximately 1% of samples). Benzo(ghi)perylene, anthracene, and indeno(1,2,3-cd)pyrene were detected at the exposed facility, while pyrene was detected at the control facility. An analysis was conducted for 17 dioxin congeners, including 2,3,7,8 -TCDD. Ten surface wipe samples were collected from various paper products and were reported to have no detectable concentrations of dioxins. Water samples were collected from the retention ponds at the impacted facility and analyzed for the presence of chlorine and PAHs. Twenty of the 27 samples tested positive for chloride, while 10 tested positive for PAHs. The data provided by the XRF analyzer indicated that elemental chlorine was present in all of the paper products at both the exposed and control facilities. Concentrations ranged

from 154 to 11,296 parts per million (ppm) on paper products. It was determined that during the paper-making process, municipal drinking water containing residual chlorine is utilized to mix fiber and additives to manufacture paper. Some of the additives are comprised of various chlorine-containing chemicals, which are added for biocidal and disinfectant purposes. Therefore, the presence of elemental chlorine in the intermediate and finished products was expected and cannot be attributed to chlorine residue from the fire. Odor sampling was conducted using young female scientists (who are known to be the most sensitive to odors) and no unusual odors were detected in the treated or control paper products. **Conclusions:** Data indicate the downwind facility's products were not measurably affected by the fire. PAHs were infrequent and low, and no dioxins were detected. Paper products exposed to the plume did not differ from those at the control site. This outcome aligns with the expectation that only very large, ground-level fires typically generate measurable off-site deposition above background levels.

PS 3763 Rapid Assessment of Skin Integrity Using 1-Hour Tritiated Water (%Absorption) in Regulatory OECD TG 428 Dermal Absorption Studies

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Background and Purpose: The skin integrity assay used in regulatory dermal absorption tests is designed to identify damaged and intact skin. Reliable measurement of skin integrity is a critical step for safety screening of topical and transdermal compounds. The conventional integrity assessment, involving a 2-6 hour absorption phase followed by a 5-minute scintillation counting step, is time-consuming, limits laboratory throughput, and may result in reducing the skin barrier function before test preparation application. Although shorter absorption metrics offer a potential to accelerate testing, their predictive accuracy, classification fidelity, and agreement with standard permeability endpoints must be rigorously validated before implementation. The primary objective of this study was to develop and validate a statistical model capable of predicting the 1-minute permeability coefficient (kp_1 ; cm/h) from early tritiated water (T_2O) absorption data obtained at 1 hour ($\%M_1H_1$) and 2 hours ($\%M_2H_2$) post dose. A secondary objective was to determine whether the 1-minute scintillation count time (kp_1) could reliably substitute for the conventional 5-minute measurement (kp_5), thereby reducing skin integrity duration by 50% without compromising accuracy or data integrity. **Methods:** Historical data collected from GLP studies conducted using a total of 2054 skin samples from the human abdominal or breast were selected. The permeability coefficient (kp) of T_2O was performed in accordance with OECD TG 428 recommendations. T_2O was applied onto the skin, and receptor fluid samples were collected at 1 and 2 hours post dose to yield % absorption values. The kps were calculated from both 1-minute (kp_1) and 5-minute (kp_5) scintillation counts. Robust MM-estimator regression modelled kp_1 as a function of $\%M_1H_1$ (Model A1) and $\%M_2H_2$ (Model A2), and regression inversion identified the % absorption thresholds corresponding to the cutoff kp of 4.5×10^{-3} cm/h (Meidan and Roper 2008). Discrimination was evaluated by ROC analysis. Classification metrics quantified sensitivity, specificity, predictive values, accuracy, F_1 -score, Matthew's correlation coefficient, and Youden's index. Finally, the agreement between kp_1 and kp_5 was examined by Bland-Altman analysis, paired t-test, Two One-Sided Tests for equivalence (TOST), and effect-size estimation. **Results:** The 1-hour absorption model (Model A1) explained 87% of the variability in kp_1 ($R^2 = 0.8705$), whereas the 2-hour absorption model explained variance of 97% of the variability in kp_1 with a strong linear relationship ($p < 0.001$). Inverting the regression equations of both models defined % absorption thresholds of 1.289% for 1-hour and 3.192% for up to 2-hours corresponding to the kp of 4.5×10^{-3} cm/h. Using these threshold values, ROC analysis showed excellent discrimination ($\text{AUC}_h = 0.9735$; $\text{AUC}_h = 0.9953$) with the 1-hour surrogate achieving 96.6% overall accuracy (sensitivity 70.5%, specificity 98.7%), while the 2-hour surrogate reached 98.3% accuracy (sensitivity 87.2%, specificity 99.3%). Agreement between 1-minute (kp_1) and 5-minute (kp_5) scintillation counts was confirmed. Bland-Altman analysis indicated negligible bias (mean difference = -1.29×10^{-5} cm/h), and equivalence testing (TOST) supported interchangeability within $\pm 5\%$ of the mean kp of 0.002838. Effect size was minimal (Hedges' $g = -0.113$), confirming that kp_1 and kp_5 are statistically equivalent, indicating a negligible difference in kp estimation. **Conclusions:** The 1-hour % absorption measurement combined with a 1-minute scintillation count provides a rapid reliable and accurate alternative for evaluating the skin integrity using a T_2O barrier integrity test. The resulting kp_1 values showed statistical equivalence to 5-minute conventional scintillation counts, demonstrating high accuracy, discrimination, and calibration. These findings support the implementation of short absorption time metrics as a validated approach for skin integrity assessments in regulatory dermal absorption studies.

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Background and Purpose: Ultraviolet (UV) filters, which are active ingredients in the United States, are essential in sunscreen formulations, providing critical protection against acute and chronic skin damage from UV exposure. However, increasing scrutiny over their human and environmental safety has prompted requests for additional data. The U.S. Food and Drug Administration (FDA), have requested data from traditional toxicological animal studies to address safety concerns. Although rodent studies for endpoints like carcinogenicity have historically been employed to evaluate risk, these models often lack human relevance and are being reconsidered globally in favor of more targeted mechanistic, non-animal approaches. A comprehensive review of existing toxicity data for the six most commonly used UV filters, avobenzone, ensulizole, homosalate, octinoxate, octisalate, and octocrylene, was conducted and then a human risk assessment to evaluate their safety as currently used in sunscreen formulations was performed. **Methods:** Data were compiled from available *in silico*, *in vitro*, and *in vivo* studies, identified in the literature from regulatory and peer-reviewed sources, including dossiers submitted to the European Chemicals Agency (ECHA). Each UV filter was then assessed for pharmacokinetics, acute and repeat-dose toxicity, genotoxicity and photogenotoxicity, developmental and reproductive toxicity (DART), immunotoxicity, neurotoxicity, endocrine activity, and local effects such as skin irritation, sensitization, and photocarcinogenicity. The data were used in margin of safety (MoS) calculations, using conservatively estimated systemic doses, with MoS values >100 generally interpreted to be acceptable and protective for non-genotoxic and non-carcinogenic effects. Additionally, an approach evaluating carcinogenic modes of action (MOA) combined with systemic exposure data to supplement the existing safety assessments was performed to evaluate the carcinogenic potential of the UV filters. **Results:** The six evaluated UV filters have no structural alerts for DNA reactivity and are non-genotoxic in standard *in vitro/in vivo* studies. For each UV filter, the MoS were >100 when assessing the maximum allowed concentration of each UV filter and realistic human exposures, calculated for both daily (facial) and recreational (whole body) use. MOA analyses revealed no biologically plausible carcinogenic pathways, and systemic exposure levels in humans were consistently below thresholds associated with biological activity. **Conclusions:** This approach aligns with evolving international regulatory paradigms, which advocate for the replacement of long-term rodent studies with more human-relevant, mechanistic approaches. Despite some limitations in historical data, the overall evidence supports the continued safe use of these UV filters in sunscreen products. These findings reinforce the importance of modern, science-based risk assessment strategies in ensuring consumer safety while reducing reliance on animal testing.

PS 3765 Mercury disposition in Wistar rats after thimerosal exposure

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Background and Purpose: Thimerosal is a mercury-based preservative used in vaccines, cosmetics, and pharmaceuticals. It's known that the conversion of thimerosal to ethylmercury and thiosalicylate occurs rapidly. However, its disposition in the human body is not well understood. Thus, the aim of this study was to evaluate mercury accumulation in the organs of Wistar rats after the injection of thimerosal at different concentrations and to document possible histopathological damage in these tissues. **Methods:** Thirteen female Wistar rats were injected via the femoral vein with saline, 0.5, 1.25, 6.175, or 12.35 $\mu\text{mole} \cdot \text{mg}^{-1} \cdot \text{kg}^{-1}$ thimerosal. All rats were anesthetized with an intraperitoneal injection of 30 $\text{mg} \cdot \text{kg}^{-1}$ ketamine and 70 $\text{mg} \cdot \text{kg}^{-1}$ xylazine before the injection. Twenty-four hours after the injection, animals were euthanized, and blood, liver, kidneys, spleen, and brain were collected. Samples were lyophilized and the amount of Hg in each was determined using a direct mercury analyzer (DMA-80). Renal and hepatic tissue from each rat was fixed, processed, sectioned, and stained for histopathological analysis. **Results:** The results showed that Hg accumulated in organs after exposure to each concentration of thimerosal. The greatest accumulation was observed when rats were injected with 12.35 $\mu\text{mole} \cdot \text{mg}^{-1} \cdot \text{kg}^{-1}$ of thimerosal. Among the organs tested, the kidneys had the greatest accumulation of Hg after exposure to every dose of thimerosal. The histopathology analyses shown hemorrhages, protein inclusions and cytoplasmic degeneration in the renal cortex and outer stripe of the outer medulla in the animals treated with 6.175 or 12.35 $\mu\text{mole} \cdot \text{mg}^{-1} \cdot \text{kg}^{-1}$ thimerosal. **Conclusions:** Our findings demonstrate that this preservative induces dose-dependent mercury accumulation across multiple organs, with high doses causing acute renal injury. This highlights the importance of dose as a critical factor in the toxicokinetic and toxicodynamic properties of thimerosal.

PS 3766 A novel air sampler using a wet inertial impactor to collect welding fume particles in liquid media for analytical and toxicological studies

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Background and Purpose: In the United States, over 771,000 welders are employed and are routinely exposed to welding fumes. The particles in welding fumes range from 50 nm to 20 μm and a significant portion of particles are small enough to reach the alveolar regions, where they interact with epithelial cells and macrophages, inducing local oxidative stress and inflammation. The particles consist primarily of iron but also contain hazardous metals (e.g., manganese, chromium, etc.), which can generate reactive oxygen species (ROS) and cause cellular damage. Soluble metals may also translocate into systemic circulation, potentially contributing to cardiovascular and neurological toxicity. Epidemiological and toxicological studies link welding fume exposure to impaired lung function, chronic bronchitis, cardiovascular disease, neurotoxicity, and increased lung cancer risk. Current *in vitro* and *in vivo* toxicological studies typically collect welding fume particles on filters and subsequently extract them into suspension for exposing them to cells or animal models. However, this process is time-consuming, may result in sample loss, and often alters the particle properties. To address limitations, we developed a novel air sampler to collect welding fume particles directly into liquid media. This method preserves the particle composition and reactivity and enables more accurate analytical and toxicological assessments. We tested the developed sampler under laboratory conditions. **Methods:** The developed sampler utilized a wet inertial impactor that consisted of a nebulizer positioned upstream of the impactor nozzle to spray the deionized water (ASTM Type I). Particles collected on the impaction plate were immediately washed by a jet stream containing deionized water droplets and the resulting suspension flowed down to the collection tube (50 mL). The sampler had a cutoff diameter of 120 nm at a flow rate of 8 L/min and collected total suspended particles (TSP) larger than 120 nm. To test the sampler, welding fumes were generated using a custom-built welding station. Specifically, flux-cored arc welding was used. A 225-L exposure chamber was connected to the station. The developed air sampler was placed in the exposure chamber. Simultaneously, an open-face cassette equipped with a 37-mm polyvinyl chloride filter was positioned alongside the sampler to collect TSP at a flow rate of 1 L/min. Welding fumes were generated at three different concentrations (low, middle, and high) by adjusting welding intervals. Real-time fume concentrations were monitored using an optical particle sizer (OPS). After sampling for 20 min, metal content in both the suspension from the developed sampler and the filters was analyzed using field-portable X-ray fluorescence (FP-XRF). Linear regression analysis was used to evaluate the association between two sampling methods. **Results:** The average total mass concentrations measured using OPS were approximately 0.9, 1.5, and 2.8 $\text{mg} \cdot \text{m}^{-3}$ under low, middle, and high generation conditions, respectively. Most particles were smaller than 10 μm and the overall size distribution remained similar across all concentration levels, with a mode diameter smaller than 1 μm . FP-XRF analysis confirmed that Fe was the major metal component in the welding fumes. The Fe mass collected using the developed sampler was 0.14 mg, 0.51 mg, and 2.06 mg for the low, middle, and high concentration conditions, respectively, while the Fe mass collected on the filters was 0.21 mg, 0.50 mg, and 1.62 mg. Linear regression analysis from the two methods showed a strong linear relationship ($R^2 = 0.9$, p -value < 0.05), demonstrating that the sampler provided comparable fume collection performance to the conventional filter method. **Conclusions:** The developed sampler demonstrated collection performance comparable to the conventional filter method and showed good recovery results for the collected sample. By allowing direct collection into liquid media, this novel method can reduce sample process time and steps, minimize potential sample loss, and better preserve the particle characteristics for the toxicological assays. Since the collected samples are already in liquid form, subsequent analyses, including metal quantification, can also be simplified. Overall, the developed method provides a more convenient and versatile platform for analytical and toxicological studies in both laboratory and field settings.

PS 3767 Parental dietary exposure to an arsenic-contaminated natural diet induces neurobehavioural impairments in Zebrafish (*Danio rerio*) offspring

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Background and Purpose: Arsenic is a non-essential, highly toxic element and one of the most widespread environmental contaminants, entering aquatic ecosystems through both natural sources and human activities. Owing to its persistence, bioaccumulative potential, and ability to disrupt multiple physiological systems, arsenic has been designated a global priority pollutant. Chronic arsenic exposure is associated with severe health effects in mammals and aquatic organisms; however, current knowledge about arsenic toxicity is largely limited to its direct exposure effects, whereas much less is understood about the multigenerational consequences of parental exposure, particularly under environmentally realistic conditions during early developmental stages when arsenic is maternally transferred from adults to their offspring. Therefore, the main objective of this study was to investigate the neurobehavioural toxicity in larval offspring resulting from parental exposure to an arsenic-contaminated natural diet in zebrafish (*Danio rerio*). **Methods:** Adult zebrafish (F_0) were fed with oligochaete worms (*Lumbriculus variegatus*) with arsenic body burdens

of 0.25 (control), 34.69 (low), and 77.79 (high) $\mu\text{g/g}$ dry weight, respectively, for 60 days. Fish were then bred to generate F_1 embryos, which were reared in clean water and diet until 30 days post fertilization (dpf). In F_1 larvae, oxidative stress and apoptosis were evaluated at 5-dpf by fluorescence imaging, and immunostaining was performed at 24-hpf and 5-dpf to assess neurogenesis and neural signalling pathways. Behavioral assessments included photomotor response (5-dpf), thigmotaxis (15-dpf), social preference (21-dpf), and novel object recognition (30-dpf). Whole-transcriptome analysis in 5-dpf larvae was performed to identify molecular pathways associated with neurobehavioral alterations. **Results:** Parental dietary arsenic exposure resulted in a dose-dependent increase in reactive oxygen species and apoptosis in offspring. Neurodevelopmental impairments were also evident in arsenic lineages, including reduced hypothalamic neurogenesis and impaired development of dopaminergic and motor neurons. Larvae exhibited behavioral abnormalities such as reduced locomotor activity, heightened anxiety, and cognitive impairments. Transcriptomic analysis revealed significant dysregulation in pathways related to early neurodevelopment and synaptic functions. **Conclusions:** This study demonstrates, for the first time, that parental dietary exposure to environmentally relevant concentrations of arsenic via naturally contaminated oligochaete worms induces multigenerational neurodevelopmental and neurobehavioral impairments in F_1 larval Zebrafish mediated primarily by oxidative stress and disruptions in neurogenesis and neuro signaling pathways. These results emphasize the need for monitoring arsenic levels in benthic invertebrates and considering dietary exposure pathways, in addition to waterborne contamination, when assessing ecological risk and establishing regulatory guidelines for arsenic in aquatic environments.

PS 3768 Low Dose Heavy Metal Exposure Reprograms Central Metabolism in Human Fetal Lung Fibroblasts

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Background and Purpose: Fetal lung development is a critical process that establishes proper lung functioning in adulthood. Mesenchymal cells such as fibroblast provide essential structural and signaling support during this process. Environmental contamination by heavy metals, including cadmium (Cd) and arsenic (As), represents a significant risk factor that can disrupt metabolic networks essential for lung morphogenesis and may increase susceptibility to respiratory disease later in life. Previous research in lung epithelial cells showed that Cd and As inhibit *de novo* pyrimidine biosynthesis from glutamine, resulting in airway morphogenesis. However, whether this effect is cell type-specific remains unclear. Here, we aim to examine how Cd and As alter fibroblast-specific metabolic activity and identify molecular targets potentially vulnerable to heavy metal toxicity during development. **Methods:** We employed stable isotope metabolomics to determine the impact of Cd and As on metabolite levels and metabolic flux. Human fetal lung fibroblasts (HFL1) were pretreated with 0.1 μM Cd and As (as $\text{CdCl}_2 + \text{NaAsO}_2$) for 24 hours before adding the tracer. [¹⁵N,¹³C₃] Glutamine (2 mM) was added to cell culture media and cells were harvested for metabolomics at four time points (30 minutes, 60 minutes, 120 minutes, and overnight). Metabolites were extracted using ice-cold acetonitrile and water (2:1 v/v) and analyzed via dual liquid chromatography (C18 and HILIC) coupled with Orbitrap ID-X Tribrid mass spectrometry. Processed data were analyzed for metabolic flux and pathway enrichment using *Rodin*, a computational package for analyzing metabolomics data. **Results:** Low dose Cd+As exposure induced extensive remodeling of fibroblast metabolism. Twenty-six metabolic pathways were significantly altered relative to control ($P < 0.05$). Purine metabolism, including inosine and guanosine, and their nucleotide counterparts showed remarkable perturbation, along with several amino acid metabolic pathways, such as methionine and cysteine metabolism and glycine, serine, alanine, and threonine metabolism. Flux analysis revealed that multiple steps of the tricarboxylic acid cycle (TCA) were affected. We found increased synthesis of citrate and fumarate and decreased synthesis of succinate. Enhanced aspartate synthesis, likely via transamination, was also observed. **Conclusions:** Our study shows that low dose Cd+As exposure reprograms central carbon metabolism in fetal lung fibroblasts, revealing cell type-specific metabolic vulnerabilities to heavy metal toxicity during lung development.

PS 3769 Exposure to Hexavalent Chromium in Drinking Water Induces Sex-Dependent Essential Metal Dyshomeostasis in the Liver, Blood, and Brain of Guinea Pigs After 90 Days

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Background and Purpose: Hexavalent chromium [Cr(VI)] is a toxicant that enters the environment due to natural and man-made processes. Drinking water is at an elevated risk of Cr(VI) contamination due to industrial run-off settling in downstream drinking water basins. The liver is the main target organ of oral Cr(VI) exposure through contaminated drinking water, which has been found to induce hepatic inflammation and increase the incidence of liver disease and primary liver cancers in exposed individuals. Recent studies have also identified the liver as a major organ involved in the clearance of plasma amyloid beta (A β) from circulation, posing significant ramifications for the brain. Hepatic inflammation greatly reduces the ability of the liver to clear A β from circulation, allowing it to re-enter the brain and form A β plaques involved in the pathogenesis of Alzheimer's disease. Therefore, it is crucial to understand the role Cr(VI) plays in disrupting crosstalk along the liver-brain axis. One way to investigate crosstalk between these organs is to quantify essential metal levels, as essential metal dyshomeostasis has recently been identified as a potential biomarker for both liver disease and Alzheimer's disease. **Methods:** 10.5- to 11.5-month-old Hartley guinea pigs were separated into sex-matched control and Cr(VI)-exposed groups. Control animals received tap water, and exposed animals received 5 mg Cr(VI)/L in tap water. Sodium chromate was chosen as the representative Cr(VI) compound due to its high solubility in water. Water volume was measured twice daily to monitor water and Cr(VI) consumption, and body mass was recorded weekly. Chromium (Cr) and essential metal levels in the liver, brain, and blood were quantified using inductively coupled plasma mass spectrometry. Brains were micro-dissected to quantify metal levels in different brain regions, with special interest in the hippocampus and entorhinal cortex due to their implications in Alzheimer's disease. Normality of data was determined using a Shapiro-Wilk test. Normally distributed data was assessed using a two-tailed Student's t-test with Welch's correction, and non-normally distributed data was assessed using the Mann-Whitney U test. Significance was reported for p-values < 0.1 . **Results:** After 90 days, significant Cr accumulation was noted in the liver, blood, and hippocampus of exposed guinea pigs, with a notable sex-difference seen in the liver and blood. Considerable essential metal dyshomeostasis was observed in the liver and blood of exposed guinea pigs, with exposed females displaying significantly altered essential metal distribution compared to exposed males and controls. Minimal essential metal dyshomeostasis was seen in the whole brain, hippocampus, and entorhinal cortex. However, significantly decreased zinc levels were noted in the liver and whole brain of exposed female guinea pigs. This is particularly notable given that zinc is a co-factor of superoxide dismutase and glutathione peroxidase which are enzymes responsible for combating the oxidative stress induced by the intracellular reduction of Cr(VI). **Conclusions:** Exposed female guinea pigs appear to be more sensitive to oral Cr(VI) exposure than males, marking a notable sex difference. This was most apparent when assessing Cr accumulation and essential metal distribution in the liver and blood of exposed animals. Future studies will consider how pro-inflammatory cytokine production is impacted by oral Cr(VI) exposure, and how this drives disease severity and progression in the liver and brain. This work was supported in part by the Jewish Heritage Fund for Excellence Faculty Recruitment Grant Program (JLW), NIEHS R35ES032876 (JPWSr) and NIEHS T32ES011564 (JPWSr, MD, GG, SV, OO).

PS 3770 Validation of XRF Bone Lead Measurement in Black Vultures and Developing Next-Generation Portable XRF Instrumentation

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Background and Purpose: Chronic lead exposure poses ongoing toxicological risks to wildlife and humans. Bone integrates over 90% of total body lead and serves as a robust biomarker of cumulative exposure, but traditional measurements require destructive digestion and ICP-MS analysis. X-ray fluorescence (XRF) offers a non-destructive alternative, yet its performance for wildlife bone lead assessment and its ability to capture both cortical and trabecular bone markers require further evaluation. This work aimed to (1) validate portable and benchtop XRF systems for measuring bone lead in black vultures (*Coragyps atratus*), and (2) develop a next-generation Cd-109-based K-shell XRF (KXRF) system to advance cumulative exposure assessment. **Methods:** Femora and tarsometatarsi from 19 black vultures were analyzed using portable XRF (pXRF), benchtop XRF, and ICP-MS. *In vivo* and *ex vivo* measurements were compared across instruments, and intraclass correlation coefficients (ICC) were calculated to assess agreement. Soft tissue was also analyzed to evalu-

ate its contribution to *in vivo* signals. Separately, a compact KXRF system pairing a Cd-109 source with a cryogen-free CdZnTe detector was tested with bone phantoms and human cadaver tibias to characterize detection limits and linearity. **Results:** Benchtop XRF showed excellent agreement with ICP-MS ($R^2 = 0.92-0.95$; $ICC \geq 0.959$) and a detection limit of 0.6 ppm. Portable XRF exhibited moderate correlation ($R^2 = 0.62-0.67$) and systematic underestimation but maintained strong agreement between *in vivo* and *ex vivo* measurements ($R^2 = 0.85$). Soft tissue contained ~0.75 ppm lead, below the pXRF detection limit, indicating that *in vivo* signals primarily reflect bone lead. The Cd-109 KXRF system demonstrated linear response up to 100 ppm ($R^2 = 0.987$) and achieved detection limits of 2-3 ppm under optimized conditions. **Conclusions:** These complementary efforts validate XRF as a non-destructive method for assessing bone lead in wildlife and demonstrate the potential of next-generation Cd-109 KXRF instrumentation to measure both cortical and trabecular bone biomarkers. Together, they advance tools for cumulative lead exposure assessment in ecological and public health contexts.

PS 3771 Development and Validation of an HPLC Assay to Measure Anti-Oxidant GSH and Lipid Peroxidation Metabolite MDA to Understand Oxidative Stresses in a Hepatocellular Model

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Background and Purpose: Cadmium (Cd) is a highly toxic environmental and industrial pollutant associated with metabolic syndrome (MetS), obesity, type II diabetes, and metabolic dysfunction associated fatty liver disease (MAFLD). Chronic liver disease rates are the highest in the nation in New Mexico, mainly in Hispanic and Native American communities (36.4/100,000 in 2022). Cd exposure drives cellular oxidative stress via mitochondrial dysfunction to generate reactive oxygen species (ROS). Glutathione (GSH) is the key cellular antioxidant that neutralizes damaging free radicals (ROS) and maintains homeostatic balance and cellular health. Tissues contain 1-10 mM GSH in the reduced form. Reduced GSH levels are a biomarker of oxidative stress, indicating poor cellular health. Lipid peroxidation (LPO) is a cell-damaging process triggered by ROS reacting with polyunsaturated fatty acid residues of phospholipids in cells. Malondialdehyde (MDA) is a highly reactive and toxic byproduct of LPO. Thus, accurate quantification of cellular GSH and MDA levels is helpful to evaluate oxidative stress in cells. In the current study, we established and validated methods to quantitatively measure GSH and MDA using high-performance liquid chromatography (HPLC). **Methods:** Measuring GSH levels in biological samples is difficult due to the rapid autooxidation of GSH, leading to an underestimation of GSH levels. The sulfhydryl groups on the cysteine residues of GSH can be blocked by a thiol alkylating agent such as N-ethylmaleimide (NEM), preventing artificial oxidation. The resulting GS-NEM conjugate is detectable by high-pressure liquid chromatography (HPLC) with a diode array detector (DAD). Measuring MDA levels in biological samples requires a derivatization step, for which 2-thiobarbituric acid (TBA) is commonly used. The resulting MDA-TBA₂ conjugate can be measured fluorometrically by HPLC with fluorescence detection (FLD) or with a DAD. The HPLC method is rapid and reliable. **Results:** We established a linear range of detection from 25 μ M to 1 mM for GSH-NEM. The established MDA-TBA₂ linear range was from 55 to 273 nM. We measured the GSH and MDA levels in two epithelial liver cancer cell lines, HepG2 and HUH7, cultured under normoglycemic (5.5mM) and hyperglycemic (15 mM) conditions representing non-diabetic and diabetic states. MDA levels were increasing through Cd exposure and hyperglycemia across both cell lines. GSH levels were increasing under the Cd exposure conditions in both hyperglycemia and normoglycemia conditions, indicating a compensatory mechanism used by the cells to protect themselves against oxidative stress. **Conclusions:** Our HPLC GSH and MDA quantification assays are a valuable tool to understand the impact of chronic, low-dose exposures of Cd (CLEC) on cellular oxidative stress of hepatocellular cells. Our assays are valuable adjuncts to understanding the long-term impacts of heavy metal exposures on hepatic function.

PS 3772 Evaluating the Fibrogenic Effects of Tungsten Particulate Exposure in 3T3 and HFL1 Fibroblasts.

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Background and Purpose: Tungsten (W) is a heavy metal increasingly recognized as an emerging environmental toxicant. Inhalation of W particulates is a key route of human exposure, especially in occupational settings where air concentrations in hard metal manufacturing factories have been reported as high as 6 mg/m³. Inhalation exposure to particulates has been associated with an increased risk of pulmonary and cardiovascular diseases. However, the systemic implications of W-induced lung injury remain unexplored. Recent epidemiological studies have identified a correlation between W exposure and elevated risk of cardiovascular diseases and stroke, but the mechanistic pathways linking pulmonary exposure to cardiac dysfunction have yet to be elucidated. Prior work from our lab has demonstrated that acute inhalation of W particulates induces IL-1 β driven pulmonary inflammation and early signs of cardiac injury, with repeated exposures leading to increased cardiac expression of pro-inflammatory and pro-fibrotic markers. These findings suggest that W exposure

could be contributing to cardiovascular disease through IL-1 β -mediated inflammation and cardiac remodeling pathways. **Methods:** This study explores how W particulate exposure affects the secretion of pro-inflammatory and pro-fibrotic mediators in THP-1 human immune cells, and how these signals affect fibroblast activation and gene expression *in vitro*. Two complementary THP-1 macrophage models were used to characterize W-induced secretion profiles. THP-1 cells were first differentiated with M0 macrophages with 5 μ L of 10 μ M PMA, then exposed for 48h under four conditions: Control, W, LPS, and LPS+W (W concentration - 200ng/mL). In a parallel experiment, THP-1 cells were polarized to an M1 state with LPS and subsequently exposed to W particulates to examine W's effect in a primed inflammatory context. Fibroblast responses were examined in 3T3-L1 mouse fibroblasts and HFL1 (human lung fibroblasts) cell lines to capture tissue-specific effects of W-induced inflammation. The 3T3-L1 and HFL1 fibroblasts were treated for 72 hours with supernatant collected from THP-1 cells exposed to 200 ng/mL W particulates. RNA was isolated, and gene expression was analyzed using qPCR to assess pro-inflammatory (TNF- α , IL-1 β , IL-6) and pro-fibrotic (TGF- β 2, α -SMA, Col1a1, Col3a1, MMP-2, & MMP-9) gene markers. **Results:** Preliminary results indicate that exposure to supernatant from W-exposed THP-1 cells increased gene expression of MMP-9 and TGF- β 2 in 3T3-L1 fibroblasts. Co-treatment with LPS (LPS+W) did not further enhance this response. W exposure did not lead to expression of inflammatory makers TNF- α , IL-1 β in the fibroblasts. Cytokine and remodeling mediators will also be quantified in THP-1 supernatant by ELISA to define condition-specific secretion patterns driving fibrotic changes. These results will guide downstream fibroblast exposures and support optimization of indirect vs direct particulate exposure systems. **Conclusions:** These data will provide insight into how W-mediated pulmonary inflammation could contribute to the development of cardiac fibrosis and define gene expression patterns in fibroblasts that could serve as indicators of early fibrotic remodeling following exposure. Funding R21OH012552

PS 3773 Disruption of Early Human Red Blood Cell Development by Arsenite and Its Primary Metabolite Monomethylarsonous Acid

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Background and Purpose: Chronic exposure to heavy metals is linked to adverse health outcomes, such as anemia. Arsenite (the trivalent inorganic form of arsenic, AsIII), specifically, causes hematotoxicity in both *in vivo* and *in vitro* models. However, little is known about the relative hematotoxicity of the AsIII methylated metabolites. Preliminary *in vitro* studies indicate that the major trivalent AsIII metabolite, monomethylarsonous acid (MMAIII), is more toxic than its parent inorganic arsenical. Therefore, this study investigated the differential hematotoxicity of AsIII and MMAIII in primary human bone marrow CD34+ hematopoietic progenitor cells (HPCs) from male and female donors with varied backgrounds. **Methods:** To evaluate effects on growth and differentiation mediated by AsIII and MMAIII, CD34+ cells obtained commercially from STEMCELL Technologies were cultured in two parallel systems. The first system utilized HemaTox Erythroid™ medium which is selective for erythroid development, and the second system comprised of H4330 MethoCult™ medium which supports both erythroid and myeloid progenitors. HPCs cultured in both conditions were exposed to low concentrations of AsIII or MMAIII. **Results:** At 3- and 7-day timepoints, viability and growth of early RBCs was assessed using acridine orange/propidium iodide staining. Erythroid differentiation at these same timepoints was assessed using flow cytometry by comparing CD71+ and CD235a+ marker expression. AsIII and MMAIII reduced the growth and differentiation of early human RBCs in a dose-dependent manner at the 7-day timepoint. Flow cytometry revealed impaired erythroid differentiation following MMAIII treatment, with early RBCs failing to mature into CD235a+ cells. Impacts on hematopoietic differentiation were further evaluated via colony formation in H4330 MethoCult™ medium after 14 days. Colony-forming assays demonstrated significant reductions in erythroid colony forming units (BFU-E and CFU-E) following exposure AsIII and MMAIII in a dose-dependent manner. **Conclusions:** In summary, these findings indicated that early stages of human RBC development are highly susceptible to hematotoxicity from AsIII and MMAIII, with MMAIII eliciting greater toxicity than its parent arsenical. Future work aims to understand how arsenic biotransformation plays a role in impaired erythropoiesis.

PS 3774 The RAD50 Zinc Hook Motif as a Vulnerable Target in Heavy Metal-Induced Genotoxicity

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Background and Purpose: DNA double-strand breaks (DSBs) are linked to development of breast, ovarian, prostate and other cancers. The MRN complex is one of the first responders to DNA double-strand breaks (DSBs), while its disruption impairs DSB repair. The MRN complex consists of the proteins MRE11, RAD50 and NBN. To form functional MRN complex, two RAD50 molecules must bind one zinc (Zn) ion via their

respective Zn hooks forming a tetrahedral Zn-S complex (CXXC-CXXC). This Zn binding allows RAD50 to bind damaged DNA and enables DNA repair. Ataxia telangiectasia mutated (ATM) is activated by MRN complex. Cadmium (Cd), a non-mutagenic metal, causes suppression of ATM activation in breast epithelial cells. Exposure to another Zn displacing metal, inorganic trivalent arsenic (iAs), also causes suppression of ATM activation in skin keratinocytes. Here, we evaluate whether iAs and Cd directly bind to the RAD50 Zn hook motif and displace Zn, providing a mechanism for the observed inhibition of ATM activation caused by each metal. **Hypothesis:** iAs and Cd will bind to the RAD50 Zn hook motif and displace Zn. **Methods:** Synthetic P. furiosus RAD50 Zn hook peptide and UV-Vis spectrophotometry was used to characterize metal-peptide interactions. The peptide (1 Eq, 100 μ M) was incubated with increasing concentrations of Zn (0 - 5 Eq; 0 - 500 μ M), Cd (0 - 5 Eq; 0 - 500 μ M), or iAs (0 - 10 Eq; 0 - 1000 μ M) for 30 min at room temperature followed by monitoring absorbance change to assess binding of Zn (220 nm), Cd (240 nm) and iAs (270 nm). For Zn displacement, the peptide (1 Eq, 100 μ M) was incubated with Zn (1 Eq, 100 μ M) for 30 min followed by incubation with increasing Cd/iAs concentrations (0 - 10 Eq; 0 - 1000 μ M) for another 30 min, followed by assessing absorbance changes. Binding/displacement constants were calculated by curve fitting (Michaelis-Menten/allosteric sigmoidal) as appropriate. **Results:** Allosteric sigmoidal model provided the best fit for all metal binding kinetics. Cd (K_{half} = 0.21 Eq) and Zn (K_{half} = 0.20 Eq) had similar binding affinity for the RAD50 Zn hook motif, ~10-fold higher than that of iAs (K_{half} = 2.16 Eq). Cd displaced Zn from the RAD50 Zn hook motif (Dissociation K_{half} = 0.33 Eq, 33 μ M). **Conclusions:** Metal binding to the RAD50 Zn hook motif likely promotes carcinogenesis by inhibition of DSB repair pathways that normally prevent genomic instability. The marked difference in binding affinity of Cd and iAs for the RAD50 Zn hook suggests that Cd and iAs may differentially compromise cellular DNA-repair capacity. **Grant support:** American Cancer Society PF-23-1153675-01-DMC and NIH-NIEHS grants R01ES027778, P30ES030283, and 1K99ES036286.

PS 3775 Arsenic disrupts autophagosome-lysosome fusion in a zinc dependent manner

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Background and Purpose: Chronic environmental arsenic exposure causes skin and lung cancers, but the molecular mechanisms are poorly understood. We identified that chronic trivalent inorganic arsenite (iAs) exposure at population relevant 100 nM concentration activates autophagy while suppressing downstream protein degradation. We do not understand how environmental iAs exposure uncouples autophagy activation from autophagic protein degradation. Zinc (Zn) regulates the critical autophagosome-lysosome fusion (ALF) step of autophagy that connects autophagy initiation and protein degradation. iAs disrupts zinc dependent processes. **Hypothesis:** We hypothesized that iAs suppresses autophagy by compromising ALF in a zinc dependent manner. **Methods:** We exposed three human skin (HEKn, Ker-CT and HaCaT) and lung (HSAEpC, HBEC3-KT and BEAS-2B) cell lines to iAs (0/100 nM; up to 48 h) with or without Zn or manganese supplementation (0/1 μ M; up to 48 h). HaCaT and BEAS-2B cell lines were exposed chronically to iAs (0/100 nM; 8 weeks) with concomitant Zn supplementation (0/1 μ M). We quantified ER stress (XBP1 splicing) employing Reverse Transcriptase-Polymerase Chain Reaction), autophagy initiation using immunoblots and completion of autophagy using immunoblots and immunofluorescence. ALF was measured employing the GFP-LC3-RFP-LC3 Δ G fluorescent autophagic flux in a 96-well plate format. Data was analyzed using unpaired, two-tailed t-test with Welch correction or two-Way ANOVA with Tukey's post-hoc test as appropriate, $p < 0.05$ was considered significant for all tests. **Results:** Environmental iAs exposure specifically targeted the ALF step of autophagy to suppress autophagic protein degradation across multiple skin and lung cell line models. iAs suppressed ALF in a Zn dependent and possibly zinc specific manner. Physiological Zn supplementation (1 μ M) prevented and rescued against iAs-induced suppression of ALF and autophagic protein degradation in the short and long-term. **Conclusions:** Our work provides a framework to understand the precise molecular mechanisms by which chronic environmental iAs exposure disrupts global protein degradation, inducing proteotoxicity contributing to multi-organ carcinogenesis. **Funding:** This research was supported in part by grants T35ES014559 and P30ES030283 from the National Institute of Environmental Health Sciences and by a grant DBG-23-1143457-01-CDP from the American Cancer Society

PS 3776 Associations Between Environmental and Biological Metal Concentrations and Self-Reported General Physical and Mental Health Status

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Background and Purpose: Rural populations in the United States face disproportionate environmental health risks due to proximity to industrial sources, limited infrastructure, and reduced access to healthcare. Our prior analyses in Hartford City, Indiana, identified elevated soil and indoor-dust lead (Pb) and copper (Cu) concentrations near a local metal-recycling facility, with levels significantly decreasing by

distance based on regression and GIS analyses, indicating localized contamination sources. The purpose of this analysis is to examine whether metal exposures measured in environmental and biological media are associated with self-reported physical and mental health among adult residents of this rural community. **Methods:** A community-engaged, cross-sectional study enrolled 80 adults. Soil, indoor dust, and toenail samples were analyzed for metals using X-ray fluorescence and spot urine samples were analyzed for metals by inductively coupled plasma mass spectrometry. Additional data were collected via an online survey. Health outcomes were self-reported physical and mental health in the past 30 days (poor vs good). Multivariable logistic-regression models compared tertiles of metal concentrations with health outcomes, adjusting for age, education, BMI, and urine creatinine. **Results:** Mean age was 59.4 years (standard deviation: 14.2); 65% were female. Poor physical health was significantly associated with higher soil arsenic (tertile 3 vs. 1 adjusted odds ratio (aOR): 6.9; 95% confidence interval (CI): 1.35, 35.4), copper (tertile 3 vs. 1 aOR: 7.3; 95% CI: 1.35, 35.4), and iron (tertile 3 vs. 1 aOR: 12.3; 95% CI: 2.29, 65.8). The association of poor mental health with soil iron (tertile 3 vs. 1) was also statistically significant (aOR: 22.6; 95% CI: 1.07, 4.77). Other comparisons were not statistically significant. **Conclusions:** Findings suggest that localized soil metal contamination may be associated with poorer self-reported physical and mental health among residents living near the metal-recycling facility despite limited evidence suggesting that residents living near the facility demonstrate a culture of denial and are more reluctant to participate in the study than residents living further away. However, these results should be interpreted cautiously given the small sample size. Nevertheless, this community-engaged study offers valuable insight into an under-studied rural population. Larger, longitudinal investigations integrating objective exposure metrics and biomarker validation are warranted to clarify causal relationships and inform equitable, community-driven environmental health interventions.

PS 3777 Investigation of dynamic changes in pain sensitivity induced by methylmercury in mice

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Background and Purpose: Methylmercury is a neurotoxic substance that is ubiquitous in the environment and causes severe neurological disorders in many mammals, including humans. Previous studies on methylmercury toxicity mostly reported the decline of sensory functions (mainly motor functions, vision, hearing, and somatosensory systems) and revealed responding pathological disorders in peripheral nerves, dorsal root ganglia, and the cerebral cortex. In recent years, pain disorders caused by methylmercury exposure have been suggested to encompass a diverse spectrum, including not only decreases in pain sensitivity (hypoalgesia) but also neuropathic pain symptoms such as hyperalgesia and allodynia. Thus, although methylmercury exposure can reportedly cause hyperalgesia and hypoalgesia, no study has examined the relationship between these pain disorders and methylmercury toxicity under the same conditions. In addition, no animal model reproduces the biphasic changes of hyperalgesia and hypoalgesia under the same exposure conditions. Therefore, in this study, we created a long-term exposure model in mice and examined dynamic changes in pain sensitivity over time under the same exposure conditions. **Methods:** C57BL/6 male mice exposed to methylmercury chloride (final concentration 30 ppm) in drinking water *ad libitum* were temporally evaluated for changes in pain sensitivity using the von Frey test and Hargreaves test. Furthermore, to clarify the neuronal basis of these dynamic changes, we performed morphological and functional analyses using neuronal activity and number of spinal dorsal horn neurons as indicators. **Results:** The results show that hyperalgesia was induced from 3 to 7 weeks after methylmercury exposure, followed by hypoalgesia from 13 to 15 weeks after exposure. Furthermore, immunohistochemical staining of neuronal activity in spinal cord dorsal horn neurons reveals a dramatic increase in c-Fos-positive neurons (activated neurons) after 4 weeks of methylmercury exposure and a decrease in the number of activated neurons after 14 weeks of exposure. In addition, the amount of mercury accumulated in the spinal cord dorsal horn increased depending on the duration of exposure. These results suggest that gradual dysfunction of spinal dorsal horn neurons, dependent on the amount of methylmercury accumulated, is involved in the observed biphasic change in pain sensitivity. **Conclusions:** This study is the first to examine dynamic changes in pain sensitivity due to methylmercury exposure in a mouse model and provides important information for investigating the molecular mechanisms involved in methylmercury-induced sensory impairment.

PS 3778 Does Hexavalent Chromium Exposure Impact The Gut Microbiome of Guinea Pigs?

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Background and Purpose: Hexavalent chromium [Cr(VI)] is a known environmental contaminant with widespread exposure through inhalation of polluted air and ingestion of contaminated drinking water. Cr(VI) was recently identified as the number one

chemical hazard in U.S. public drinking water in 2024. The current maximum contaminant levels (MCLs) for total Cr [Cr(VI) and Cr(III)] in drinking water from the World Health Organization (0.05 mg/L) and the U.S. Environmental Protection Agency (0.1 mg/L) were set based on contact dermatitis, and thus these MCLs warrant more investigation. Further, the highest level of Cr in U.S. drinking water was reported at 5.4 mg/L in 2014. Cr(III) is considered non-toxic, while Cr(VI) is toxic. While Cr(VI) exposure in drinking water has been investigated as a potential factor for tumor incidence in the gastrointestinal (GI) tract of rodents, the impacts on the gut microbiome are understudied and unknown. This is a critical knowledge gap, given that GI-related pathologies in humans have increased. **Methods:** Female and male 10.5-11.5 month-old Hartley guinea pigs were exposed to sodium chromate [0 or 5 mg/L Cr(VI)] in drinking water for 90 days. Following completion of the study, the cecal contents were collected, and the microbiome DNA was extracted and sequenced using primers for the V4-V5 region of the 16S rRNA by the Hubbard Center for Genome Studies at the University of New Hampshire. Raw sequences were then analyzed using Quantitative Insights into Microbial Ecology (QIIME2) and assigned to taxonomic classification based on the SILVA138 database. **Results:** There were no statistical differences between in alpha- and beta- diversity between the control and the Cr(VI) exposure (5 mg/L). *Caproiciproducens*, *Eubacterium ventriosum*, *Lachnospiraceae* (NK4A136), NK4A214 group, and *Parabacteroides*, all rare taxa, were significantly decreased ($p < 0.05$) in the Cr(VI) group. Conversely, another rare taxon, *Eubacterium runiantium*, was significantly increased ($p < 0.05$) in the Cr(VI) group. No observable differences were seen between biological sexes. **Conclusions:** While Cr(VI) in drinking water for 90 days did not alter the overall alpha- and beta-diversity in the cecum of guinea pigs, it did significantly alter reduce key rare taxa. These observed taxa that were decreased are associated with different GI tract disorders, including irritable bowel syndrome (IBS). These data are the first to suggest a link between Cr(VI) drinking water exposure and negative gut microbiota changes and indicate that Cr(VI) exposure in drinking water may pose a threat as a precursor or initial "hit" to IBS or other gut-related disorders. Future work is aimed at chronic exposures of Cr(VI) in drinking water and investigations into other microbiota regions in guinea pigs. This work was supported by NIEHS grant R35ES032876 (JPWSr).

PS 3779 As3MT Activity Alters MASLD Phenotypes Following Arsenic Exposure in Mice

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Background and Purpose: Chronic exposure to inorganic arsenic (iAs) is associated with several metabolic diseases in humans, including atherosclerosis, diabetes mellitus, and metabolic dysfunction-associated steatotic liver disease (MASLD). In the liver, mouse studies have shown that iAs exposure can induce phenotypes consistent with MASLD, such as steatosis, inflammation, and fibrosis. The enzyme arsenic-3-methyltransferase (As3MT) plays a central role in iAs metabolism, yet its contribution to hepatotoxic outcomes upon arsenic exposure remains unclear. While methylation facilitates arsenic clearance, it can also generate reactive intermediates that may exacerbate liver injury. Understanding how differences in As3MT activity influence arsenic-induced hepatotoxicity is key to clarifying disease mechanisms and identifying at-risk polymorphisms. We hypothesize that reduced As3MT activity increases susceptibility to arsenic-induced hepatotoxicity, thus exacerbating MASLD phenotypes. **Methods:** To address this, C57BL/6 mice with varying capacities for arsenic methylation were used: wild-type (WT) mice, which metabolize iAs efficiently; As3MT knockout (KO) mice, which lack methylation capacity; and humanized As3MT (huAs3MT) mice, which exhibit an intermediate rate of metabolism. Male and female mice (6-8 weeks old) were exposed ad libitum to 200 ppb iAs in drinking water for 5 weeks. Following exposure, liver and serum samples were collected for histological and biochemical analyses. Steatosis was assessed using oil red O staining of frozen liver sections to quantify lipid accumulation. Immune cell infiltration was evaluated via immunostaining for CD45 (total leukocytes) and CD68 (macrophages). Collagen deposition, indicative of fibrosis, was visualized using picrosirius red staining. **Results:** Initial analyses revealed that a 5-week exposure to 200 ppb iAs resulted in approximately a 5x increase in lipid accumulation in WT mice. Female WT mice exhibited nearly 10x higher staining intensity compared to males for both control and arsenic exposed conditions. No corresponding changes were observed in CD45 or CD68 immunostaining or in collagen deposition, suggesting an absence of overt inflammation or fibrosis at this exposure duration. The same outcomes are being assessed in KO and huAs3MT mice to determine how differences in arsenic methylation capacity influence these hepatic endpoints. Initial observations suggest a change in CD45 and CD68 immunostaining in the livers KO mice. **Conclusions:** These initial observations suggest that As3MT activity contributes to the development of MASLD phenotypes following inorganic arsenic exposure, with evidence of sex-dependent variation among WT mice. Ongoing work is examining how arsenic metabolism affects liver vasculature and immune organization, including potential changes in endothelial cell fenestration and the spatial distribution of immune cells in these different mouse models. Together, these efforts will help clarify how As3MT activity modulates the balance between arsenic detoxification and hepatotoxicity.

PS 3780 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)-Elicited Disruption of Trace Metal Homeostasis in Wild-Type and Hepatocyte-Specific Aryl Hydrocarbon Receptor (AHR) Null Mice

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Background and Purpose: Trace metals are vital for enzymatic function in processes such as electron transfer, DNA replication, and antioxidant defense. Constant aryl hydrocarbon receptor (AHR) activation by persistent organic pollutants, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), results in hepatic transcriptome dysregulation. Changes in transporter and metalloprotein expression lead to trace metal imbalance and oxidative stress, potentially exacerbating liver damage. We aim to determine the effect of TCDD-induced AHR activation on trace metals in hepatocytes and the resulting systemic alterations in trace metal homeostasis in wild-type and hepatocyte-specific AHR null mice. **Methods:** This was accomplished by intraperitoneal injection of corn oil vehicle or 75 mg/kg tamoxifen to conditionally knock out AHR in hepatocytes of male *Ahr^{d1v375A}* Alb-CreERT2 mice prior to oral gavage with sesame oil vehicle or 3, 10, or 30 µg/kg TCDD every four days for 28 days. Inductively coupled plasma mass spectrometry (ICP-MS) and hepatic RNA-Seq data were integrated to identify changes in trace metal levels in liver extracts and serum, and correlated with the differential expression of trace metal transporters and chaperones in the liver. **Results:** Out of 15 metals analyzed in the liver, 8 changed in wildtype (WT) and 9 changed in hepatocyte-specific AHR knockout (hep-AHR-KO) animals. In serum, 2 of the 15 changed in WT and hep-AHR-KO samples. Specifically, TCDD decreased hepatic copper levels while simultaneously increasing serum copper levels from hep-AHR-KO mice. The changes correlated with less repression in the copper exporter *Atp7b* in the hep-AHR-KO liver samples compared to WT. Hepatic zinc levels were decreased by TCDD in both WT and hep-AHR-KO samples, however AHR ablation in hepatocytes attenuated the change when compared to WT. There was no correlation with genes associated with zinc homeostasis. **Conclusions:** Hepatocyte-specific AHR ablation altered the expression of metal homeostasis genes, which affected hepatic and serum trace metal levels. These results suggest AHR activation by TCDD disrupts hepatic trace metal homeostasis which may subsequently impact the cellular redox balance, metalloenzymes, and metal-dependent forms of regulated cell death.

PS 3781 THE IMPACT OF APOLIPOPROTEIN E IN THE TOXICITY OF HEAVY METAL MIXTURES

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Background and Purpose: The apolipoprotein E (APOE) gene polymorphism has been linked to various human diseases, including Alzheimer's disease (AD), obesity and diabetes, as well as coronary and peripheral vascular diseases. Research has shown that the presence of the APOE polymorphism in isolation is insufficient for triggering pathological decline. However, the presence of other risk factors, such as metal ions, is necessary to interact with apolipoprotein E and elevate the probability of developing these diseases. This systematic review aimed to elucidate this hypothesis. **Methods:** We conducted a systematic review of international databases such as PubMed and Web of Science to examine the impact of heavy metals and their mixtures on apolipoprotein E (ApoE) isoforms, to establish the vulnerability of ApoE carriers and to identify the mechanisms underlying ApoE-mediated neurotoxicity in the presence of heavy metals. We searched the literature using the keywords 'apolipoprotein E', 'ApoE', 'heavy metals', 'lead', 'mercury', 'cadmium', 'arsenic', 'chromium', 'manganese', 'neurodevelopmental toxicity', 'cognitive decline', 'neurological decline', 'memory loss' and 'IQ scores'. **Results:** The identified epidemiological studies revealed a link between exposure to low-to-moderate levels of lead, mercury and cadmium, both individually and in combination, during pregnancy and breastfeeding, and reduced cognitive and fine motor scores, as well as decreased IQ scores. The effects were more pronounced in ApoE4 carriers. The identified studies on humanized ApoE3-KI and ApoE4-KI mice and rats showed an additive toxic effect between cadmium, lead, manganese, and mercury, in conjunction with the APOE4 gene, leading to memory and locomotor impairment, as well as cognitive impairment. **Conclusions:** As daily exposure to metals in real life rarely involves just one element, it is necessary to clarify the possible antagonistic or synergistic effects of heavy metal mixtures in the presence of apolipoprotein E. The results of this study inform on the development of personalized prevention and treatment strategies for neurodevelopmental and neurodegenerative disorders in humans and future research strategies.

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Background and Purpose: Arsenic (As) and uranium (U) are toxic metal(loid)s and co-occurring drinking water contaminants in rural communities in North America. Although chronic exposure to low concentrations of As and U is common, most *in vivo* toxicological studies of As and U rely on acute exposure to large concentrations of these elements. Therefore, there is a need to assess the health risk of As and U exposure in human-relevant exposure conditions. In our previous work, life-long exposures to low (100–200 µg/L) concentrations of As and U in drinking water were not sufficient to worsen atherosclerosis or related cardiometabolic phenotypes. However, it is possible that either 1) there were cardiometabolic effects at these concentrations, but that such effects were only at the molecular, subclinical level, or 2) that low concentration As and U exposure did not affect cardiometabolic health, but disturbed a different disease pathway we did not directly measure. Therefore, we conducted a multi-omic analysis of liver tissue to broadly assess subclinical hepatic responses to As and U in a hypothesis generating manner. **Methods:** Livers were collected from atherogenic (apoE^{-/-}) mice exposed to 112 µg/L As, 200 µg/L natural U, both, or neither (control) from conception until collection at 14 weeks of age. We extracted mRNA and DNA for bulk transcriptomic and DNA methylation analysis with total RNA-seq and methyl-seq, respectively. The metabolome and lipidome were quantified with combined trapped ion mobility spectrometry and time of flight mass spectrometry after dual-phase extraction of the aqueous and organic fractions of homogenized tissue. Each -ome was first analyzed individually by identifying significantly differentially expressed genes, differentially methylated regions, and differentially abundant metabolites and lipids in exposed groups relative to control. Since all four analyses were performed on adjacent tissue from the same mouse, we additionally conducted an integrated multi-omic analysis with DIABLO to identify molecular pathways across all -omes that differed between exposure groups. **Results:** The molecular effects of As, U, or combined As and U exposure in liver tissue were mild across all -omic layers. Exposure groups largely failed to separate in multi-omic analyses, suggesting that molecular signatures were similar regardless of As or U exposure. In single-ome analyses, gene set enrichment analysis of differentially expressed genes suggested that exposure to U upregulated oxidative stress and amino acid metabolism pathways, while As exposure down regulated fatty acid metabolism. Pathway analysis of the metabolomics data with Mummichog demonstrated significant up regulation of folate synthesis in As-exposed mice and upregulation of galactose and glycerolipid metabolism in U-exposed and combined As/U exposed mice, respectively. **Conclusions:** Overall, 112 µg/L As, 200 µg/L U, or both 112 µg/L As and 200 µg/L U only mildly affected the molecular profile of liver tissue from exposed mice. Of the four -omes we assessed, the metabolome was the most sensitive to As/U exposure. Thus, it is possible that human-relevant doses of As and U disturb hepatic metabolism, but not sufficiently to induce overt metabolic disease.

PS 3783 Bayesian Network with Bootstrapping Analysis of the Effect of Exposure to Welding Fume Metal Mixtures on Cognitive Abilities, Motor Functions and Mood Symptoms

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Background and Purpose: Mild steel welding generates fumes which are comprised mainly of iron, manganese, copper, zinc and aluminum metals. Previous studies have shown decreased Cognitive and Motor functions, and increased Mood symptoms (CMM variables) of welders associated with occupational exposure to individual metals, in particular manganese, from welding fumes. Only a little work has been done on the association between CMM variables and metal mixtures. The aim of this study is to expand on studies investigating the exposure to individual metals, to identify how particular CMM variables are associated with the cumulative lifetime exposure of welders to metal mixtures at different welder exposure bi-stratifications. **Methods:** A cohort of 47 full-time career welders and a non-exposed, matched control group of 42 shift workers were recruited from a local truck trailer manufacturer. Nineteen CMM variables are studied: a battery of neuropsychological tests was administered to the welders to assess performance on 10 cognitive domains and 5 motor domains, and four classes of mood symptoms were assessed using the Brief Symptom Inventory mood survey. The five lifetime welding fume metals breathed in by a welder are approximated using a cumulative exposure model developed by Ward et al (2018), making use of personal air samples from each welder as well as their work history to estimate a lifetime cumulative exposure index (CEI), given in units of mg/m³ x yrs. Various learned Bayesian Network analyses of the effect of different welding fume metal mixtures and welder exposure bi-stratifications on cognitive abilities, motor functions and mood symptoms are conducted. The bi-stratifications procedure involved multiple percentile dichotomizations of the data to allow testing multiple welder exposure levels. The bi-stratifications procedure involved multiple percentile dichotomizations of the data to allow testing multiple welder exposure levels. Bootstrapping the Bayesian Network allows bias corrected and accelerated tests of significance of whether there is a significant association between CMM vari-

ables and metal mixtures at different welder exposure bi-stratifications. **Results:** For verbal fluency, processing speed, implicit learning and fine motor dexterity nondominant hand CMM variables, welders perform worst when exposed to either Mn or Zn and sometimes Al at most welder exposure bi-stratifications. The data gives no clear consensus as to which individual metal exposure(s) most negatively affects welders' cognitive flexibility. Verbal fluency is most negatively affected by exposure to the MnZn mixture pair at all welder exposure bi-stratifications. Cognitive flexibility is most negatively affected by any pair of metal exposures which includes Al, such as FeAl, CuAl, ZnAl or MnAl. Although no clear consensus, many metal exposure pairs including Mn negatively influence processing speed. Although no clear consensus, many metal exposure pairs including Mn or Zn negatively influence implicit learning. Motor dexterity (nondominant) is most negatively affected by any pair of metal exposures which includes Mn, such as MnZn, MnCu, and MnFe. Controlling for exposure level, welders' performance was worse (significantly or not) when exposed to mixtures with 3 or more metals than with 2 or fewer number of metals for most CMM variables. Furthermore, controlling for metal mixture, welders perform worse (significantly or not) when subjected to higher 35% or 30% welder exposure bi-stratifications for some CMM variables. **Conclusions:** This study shows how particular CMM variables (cognitive, motor and mood test scores) are associated with the cumulative lifetime exposure of welders to metal mixtures at different welder exposure bi-stratifications. Significant negative associations between exposures to welding fume metal mixtures, particularly those related to manganese and welder's processing speed, implicit learning and fine motor dexterity have been revealed at different welder exposure bi-stratifications. Welders were found to perform significantly worse on CMM tests for increasing numbers of metals in metal mixtures and when subjected to high metal exposures.

PS 3784 Cadmium toxicity by the disruption of glucose transportation system in human proximal tubular cells

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Background and Purpose: Cadmium (Cd) is an environmental contaminant that causes renal toxicity. However, the transcriptional pathways underlying proximal tubular injury remain incompletely defined. In this study, we focused on the transcription factor myocyte enhancer factor 2A (MEF2A), the activity of which is among those reduced by Cd treatment in human proximal tubular (HK-2) cells. Moreover, we investigated whether Cd disrupts the MEF2A-GLUT4 axis and cellular energy production. **Methods:** HK-2 cells were exposed to Cd and assessed for MEF2A transcriptional activity, MEF2A nuclear protein levels, GLUT4 (SLC2A4) expression, intracellular glucose and ATP level, and cytotoxicity. Knockdown of target genes was conducted by the siRNA transfection against MEF2A and SLC2A4. Renal SLC2A4 mRNA was quantified in mice chronically exposed to Cd (6 or 12 months). **Results:** Cd markedly inhibited the activity of transcription factor MEF2A in HK-2 human proximal tubule cells, which generated significant cytotoxicity in the cells. One of the glucose transporters, GLUT4, was downregulated not only by Cd treatment but also by MEF2A knockdown. Knockdown of SLC2A4, encoding GLUT4, eliminated both cell viability and Cd toxicity. Cd treatment or SLC2A4 deficiency reduced the cellular concentration of glucose. ATP levels were significantly reduced not only in the cells treated with Cd for 6 h but also in the SLC2A4-knockdown cells. The levels of SLC2A4 mRNA in the kidney of mice exposed to Cd for 6 or 12 months were significantly lower than those in the control group. **Conclusions:** Cd exerts cytotoxicity in proximal tubular cells by disrupting MEF2A activity and downregulating GLUT4, leading to impaired glucose availability and ATP depletion. These findings identify the glucose transporter pathway as a key determinant of Cd renal toxicity and provide a mechanistic link between transcriptional dysregulation and energy failure in the proximal tubule.

PS 3785 Spatial characterization of immune cell infiltration in the aortic sinus plaque using a multiplex PhenoCycler assay following arsenic-induced atherosclerosis in mice.

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Background and Purpose: Arsenic is known to exacerbate atherosclerosis, characterized by the development of plaques in large arteries. As plaques progress, they narrow vessels and become unstable resulting in heart attack or stroke. Atherosclerotic plaques contain a variety of cells, including vascular smooth muscle cells, endothelial cells, and numerous immune cells like macrophages, T cells, B cells, mast cells, and dendritic cells. Arsenic also promotes hypertrophy and fibrosis in cardiac tissue linked to dysregulated immune signaling pathways that cause damage to the heart. Our latest single cell multiome studies of CD45⁺, plaque-resident immune cells suggested that arsenic changes macrophage communication between cell types within the plaque. However, these studies did not provide spatial context or take into consideration other cell types. Multiplex imaging techniques, such as PhenoCycler, are powerful tools for spatially characterizing cell-cell interactions. Here, we created an 18 marker PhenoCycler panel to visualize the various

cells in murine atherosclerotic plaques and surrounding cardiac tissue following exposure to arsenic +/- high fat diet. We hypothesize that the change in the diet and/or the arsenic exposure will trigger an immune cell infiltration into the atherosclerosis plaque and modify communication between cells. **Methods:** Male and female ApoE^{-/-} mice were exposed to either tap water (Ctl) or 200 ppb arsenic (As) in drinking water for 13 weeks while being fed a low-metal purified diet (PD) or a high fat diet (HFD). Formalin-fixed paraffin-embedded (FFPE) sections of heart aortic sinus with atherosclerotic plaque were assembled in a tissue microarray (TMA) and stained using the PhenoCycler technology from Akoya. The staining panel consisted of 18 markers: a cardiac cell marker (Myh6), a hematopoietic cell marker (CD45), myeloid cell markers (CD11b, F4/80, CD206, and CD86), cell adhesion markers/ligands (ITGB1, CD44, ITGA5, and Spp1), a T-cell marker (CD3e), a smooth muscle cell marker (aSMA), endothelial cell markers (CD31 and CD146) and a segmentation marker (Actin). Myh6 was exceptionally detected using Streptavidin-biotin. The fluorescent nuclear dyes 4',6'-diamidino-2-phenylindole (DAPI) and DRAQ5 were included to aid nuclear segmentation. Data were analyzed using QuPath and CytoMAP. **Results:** We successfully created an analysis pipeline for characterizing atherosclerotic plaque and surrounding heart area optimized for our specific context. We observe an increase in the abundance of immune cells in the plaque and the surrounding heart tissue dependent on the treatment used (low purified vs high fat diet, with and without arsenic). Using this spatial profiling, we see a change in the cell-cell interaction between Monocytes/Macrophages and CD206⁺ Macrophages in female compared to male mice. Also, the presence of the Monocytes/Macrophages correlate with T cells and neutrophils. Next, we can compare the correlations between a cell phenotype (e.g. macrophage) and the expression of a marker (e.g. SPP1, CD44). With this, we address one of the findings from our previous multiome studies regarding the prediction that arsenic would change the preferred binding partner of SPP1 from CD44 to integrin receptors (ITGA5, ITGB1). **Conclusions:** We have successfully developed a PhenoCycler panel to spatially visualize and provide insights on cell locations, cell-cell interactions and cell heterogeneity in the arsenic-induced atherosclerotic plaque and surrounding heart tissue in our ApoE^{-/-} mice. This method could help determine what drives plaque instability and progression or what makes "vulnerable" plaques more prone to rupture and deserves to be further explored using our full dataset.

PS 3786 Toxic effects of the environmental low-dose cadmium and obesogenic microbiome metabolite delta-valerobetaine on muscle health

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Background and Purpose: Sarcopenia, the age-related loss of muscle mass and function, drives frailty, morbidity, and loss of independence, yet its environmental determinants remain poorly defined. We recently found that the intestinal microbiome produces a metabolite, delta-valerobetaine (VB), that causes obesity in young mice and is associated with aging and lower lean mass in humans. Cadmium (Cd), a heavy metal with a 10-to-30-year biological half-life, accumulates with age through intake from food, water, air pollution, and tobacco smoke. The objective of this study is to examine the effects of low-dose Cd and VB, alone and combined, on muscle health in aged mice. **Methods:** Twenty-month-old male C57BL/6J mice received standard mouse food (LabDiet 5053) *ad libitum* and drinking water containing CdCl₂ (3.3 mg/L), VB (1.62 mM), Cd+VB, or vehicle control (Cont) *ad libitum* for 12 weeks (n=10 per group). Protocols were approved by the Emory IACUC. Body weight and water intake were recorded weekly. After exposure, skeletal muscle Cd and VB levels were quantified by inductively coupled plasma-mass spectrometry and high-resolution metabolomics, respectively. Muscle damage and function were assessed by serum creatine kinase, gastrocnemius muscle histopathology by H&E with central nucleated fiber quantification, macrophage immunostaining, and beam traversal testing. **Results:** Muscle Cd and VB concentrations increased in their respective exposure groups versus Cont. Cd levels were higher with combined exposure than Cd alone (Cd+VB: 3.0 ± 0.4 ng/mg vs Cd: 2.3 ± 0.3 ng/mg; P< 0.05), indicating that VB potentiates Cd accumulation in aged muscle. Cd+VB elevated serum creatine kinase compared with Cont (270 vs 75 U/L; P<0.01) and prolonged beam traversal time (12 s vs 8 s; P< 0.05). Gastrocnemius muscle histology showed more central nucleated fibers with Cd+VB than Cont (20% vs 10%; P< 0.05), and macrophage infiltration increased >2-fold (P< 0.01). **Conclusions:** Chronic low-dose Cd and VB delivered in drinking water accumulate in aged skeletal muscle, with VB enhancing Cd burden and increasing toxicity. Combined exposure is associated with muscle damage, inflammation, and impaired physical performance, supporting environmental Cd and microbiome-derived VB as modifiable contributors to muscle aging and degeneration. In principle, methods to decrease Cd and/or VB could have a considerable population impact by protecting against sarcopenia and frailty in aging populations.

PS 3787 A Survey of DNA Damage and Metal Exposure in American Alligators (*Alligator mississippiensis*) in Florida

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Background and Purpose: One Environmental Health is a research strategy that studies toxicants while integrating human, wildlife, and ecosystem health to establish a more comprehensive understanding of health. We used this approach to study American alligators (*Alligator mississippiensis*), which are apex sentinel organisms and are regularly exposed to environmental toxicants. Because of their long lifespan, use of both terrestrial and aquatic habitats, broad diet, and ability to bioaccumulate metals, alligators serve as effective indicators of heavy metal pollution, which poses risks to both aquatic ecosystems and human health. Metals are widespread environmental pollutants that pose serious ecological risks, and surface water contamination remains a major concern in Florida. In particular, beryllium, chromium, nickel, arsenic, and cadmium are of critical concern because they are classified as known human carcinogens by the International Agency for Research on Cancer. Chronic exposure of alligators to these pollutants can induce DNA damage, potentially compromising their genetic and epigenetic integrity and, in turn, impairing reproductive success and population viability. In this study, we applied the One Environmental Health perspective to examine DNA damage and blood metal levels in alligators from three locations in Florida. **Methods:** We sampled alligators at three sites in Florida: Merritt Island National Wildlife Refuge (Merritt Island), Lake Apopka, and Lake Woodruff. After hand-capturing alligators, blood samples were collected from the occipital sinus and then put on ice. After sample collection, each alligator was examined for any external injuries or behavioral abnormalities (e.g., lethargy) while recording head length, snout-vent length, total length, sex, and age. Lymphocytes were isolated, and the alkaline comet assay was used for measuring DNA damage. Metal analysis was performed using inductively coupled plasma mass spectrometry (ICP-MS). Statistics were performed using GraphPad Prism 9.4.0. Data were expressed as the mean ± SEM. Statistical differences were assessed using a two-tailed Student's unpaired t-test or unpaired t-test with Welch's correction for normal distribution, Mann-Whitney test for non-normal distribution. When comparing two variables, a two-way ANOVA was used for sex and age. **Results:** We found alligators in Merritt Island National Wildlife Refuge had significantly higher DNA damage levels compared to those in Lake Apopka and Lake Woodruff. This trend was consistent across both sexes, with no observed sex differences. Similarly, DNA damage levels were significantly higher in both adult and juvenile alligators from Merritt Island compared to the other locations. Notably, juvenile alligators exhibited higher DNA damage than adults, with animals from Merritt Island, exhibiting particularly elevated levels. Except for cadmium, no significant differences were observed in the blood concentrations of chromium, arsenic, and nickel among alligators from these three locations. Both male and female alligators from Merritt Island had lower cadmium levels than those from the other two lakes, while female alligators from Lake Apopka exhibited higher nickel concentrations. Juvenile alligators showed higher blood levels of chromium and cadmium, whereas adults had higher nickel concentrations. **Conclusions:** Our findings highlight location- and age-dependent differences in DNA damage among alligator populations, with Merritt Island emerging as a hotspot of genotoxic stress. Metal levels alone did not fully explain this pattern, elevated DNA damage (especially in juveniles) signals potential environmental health risks that merit closer scrutiny through expanded contaminant profiling and mechanistic studies. This work was supported by the National Institute of Environmental Health Sciences [R35ES032876 and R01ES016893 to JPW; and T32ES011564 to JHT, RMW, and JPW].

PS 3788 Cell death mechanism of novel iron-containing terpyridine metal complexes for breast cancer cells

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Background and Purpose: Metal complexes have long been utilized as anticancer agents, such as cisplatin which is a platinum-based drug well-known and widely used for treating various solid tumors. A major challenge in anticancer drug development is achieving high selectivity to maximize cancer cell death while minimizing toxicity to normal cells. Terpyridine (tpy) metal complexes have been extensively investigated for decades due to their broad applications in functional materials, supramolecular chemistry, catalysis, and biomedical research, including anticancer, antibacterial, and antifungal studies. Previous work from Cheng's laboratory demonstrated that iron-based tpy complexes exhibit selective cytotoxicity toward either MCF-7 breast cancer cells or MDA-MB-468 triple-negative breast cancer cells. The present study further investigates the detailed cell death signaling mechanisms of the Fe-tpy (Fe9) complex. **Methods:** Protein level changes in MCF-7 and MDA-MB-468 cells treated with Fe9 for 72 hours at its IC50 concentration were assessed using the Full Moon Biosystems Explorer Antibody Array, which contains 656 highly specific and well-characterized antibodies targeting multiple signaling pathways. Fold changes obtained from the array were analyzed using QIAGEN Ingenuity Pathway Analysis

(IPA). Western blotting was performed to validate the expression of apoptosis-related proteins, including Bad, Bax, Bak, p53, Bcl-2, and Bcl-XL. **Results:** The array data showed that treatment of MCF-7 with Fe9 increased the expression of Bax and Bad (fold changes: +1.079 to +1.979), while decreasing Bak, Bcl-2, Bcl-XL, and p53 (fold changes: -1.002 to -1.195). In MDA-MB-468, Fe9 increased Bax and Bak (fold changes: +1.026 to +1.179) but decreased Bad, Bcl-2, Bcl-XL, and p53 (fold changes: -1.001 to -1.270). Western blot analysis validated the IPA results, showing that Fe9 reduced Bcl-2 expression by 44% in MCF-7 and 31% in MDA-MB-468, and decreased Bak and Bad in MCF-7 by 39% and 43%, respectively. However, other validated proteins did not consistently reflect the IPA findings. **Conclusions:** In conclusion, treatment with Fe9 modulates key apoptotic regulators in both MCF-7 and MDA-MB-468 breast cancer cells, suggesting its potential to promote cell death via the intrinsic mitochondrial pathway. The observed downregulation of Bcl-2 in both cell lines, along with decreases in Bak and Bad in MCF-7 cells, supports a pro-apoptotic effect, although discrepancies between array and protein data indicate possible post-translational regulation or pathway-specific modulation. Given the concurrent reduction of p53 expression, these findings suggest that Fe9 may induce apoptosis through a p53-independent mechanism. Future studies should further investigate the mechanisms underlying Fe9-induced cell death, including the potential involvement of oxidative stress and ferroptosis by assessing markers such as GPX4, as well as exploring time- and dose-dependent effects to better understand its therapeutic potential in different breast cancer subtypes.

PS 3789 Spatial transcriptomic profiling of glial and neuronal cells during mouse brain aging

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Background and Purpose: Age is a critical variable that influences both baseline brain function and susceptibility to toxic exposures. Also, aging induces widespread molecular and cellular alterations in the brain, leading to functional decline and increased susceptibility to neurodegenerative disorders. **Methods:** To elucidate the spatial and cell-type-specific transcriptional changes underlying brain aging, we performed spatial transcriptomic profiling of midbrain regions from one-month-old and one-year-old mice. Spatial transcriptomics is a technique that measures gene expression within an intact tissue to demonstrate where different cells and their transcriptional activities are located. Using a high-resolution spatial transcriptomics platform with a neuroinflammation-focused gene panel, we mapped gene expression changes across neuronal and non-neuronal cell populations. **Results:** We observed a reduction in glutamatergic excitatory neurons in the thalamus and cerebral cortex of aged brain, suggesting disrupted thalamocortical communication. Overexpression of inhibitory interneurons in the caudate putamen, coupled with decreased parvalbumin-expressing interneurons in the hypothalamus, indicated a shift toward excessive inhibition and potential autonomic dysfunction. An increased abundance of reactive microglia, immune-interacting epithelial cells, and vascular-associated astrocytes across aged brain regions as compared to young reflected an active neuro-immune crosstalk. In the corpus callosum and caudate putamen, a significant reduction in oligodendrocyte precursor cells, a sign of aging, was accompanied by an increase in myelinating oligodendrocytes, an indication of ongoing pathological condition. Collectively, our analyses reveal distinct transcriptional reprogramming in astrocytes, microglia, oligodendrocytes, and endothelial cells, marked by upregulation of inflammatory and stress-response pathways and downregulation of synaptic and metabolic genes in neurons in aged brain as compared to young. **Conclusions:** Together, these findings suggest that aging recapitulates features of chronic neuroinflammation, characterized by imbalanced excitatory-inhibitory signaling in cortical and thalamic regions and region-specific reactive gliosis in the corpus callosum and caudate putamen.

PS 3790 Astrocytic STING signaling mediates wildfire smoke-induced neurotoxicity in primary murine cells

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Background and Purpose: Global increases in wildfire events necessitate assessment of extrapulmonary health effects associated with smoke exposure. Mounting literature highlights the potential of this toxicant to induce neurologic consequences, occurring through unknown mechanisms. Chronic wildfire smoke exposure has been shown significant to disrupt parenchymal gene expression similarly to signatures of neurodegeneration. There is further a growing body of work demonstrating glial activation precedes overt loss of neurons in toxicant-based models of these disorders. The cGAS-STING signaling pathway, which coordinates interferon responses to cytosolic nucleic acids, has recently been implicated in the pathogenesis of age-related degeneration, despite little focus on astrocytes. Together, these data motivated our present assessment of STING-dependent activation in astrocyte-enriched primary mixed glial cells as a mechanistic contributor to smoke-induced neurotoxicity. **Methods:** Simulated wildfire smoke was generated using a laboratory furnace to combust Douglas fir needles at a smoldering temperature. Particulate matter was

collected on filters and extracted via methanol solvent exchange. To ensure the relevance of our smoke extract in a neurotoxic context, we applied two biophysical techniques to characterize particle size, transmission electron microscopy (TEM) and dynamic light scattering (DLS). Wildtype (WT) astrocyte-enriched mixed glial cultures were established from murine neonates and subsequently dosed with smoke extract at half-log concentrations spanning 1-1000 ng/mL. These cells were assessed for cytotoxicity using a permeability-based cell viability assay. To further probe the effect of sub-lethal smoke exposure on astrocytic STING activation, we applied high content immunofluorescence microscopy and deep learning-based image analysis. Additionally, we performed RNAseq on smoke-exposed cells compared to vehicle controls to establish patterns of transcriptomic disruption induced by this toxicant. We next employed a mouse strain with genetic STING ablation (STINGgt) to assess the extent of STING involvement in response to smoke. We compared cytokine and chemokine secretion patterns in WT and STINGgt culture supernatant via the Luminex multiplex immunoassay platform. Lastly, to assess the relevance of astrocytic reactivity to neurodegeneration, we exposed primary cortical and hippocampal neurons to glial conditioned media (GCM) from smoke-treated WT and STINGgt glial cultures. **Results:** TEM analysis resulted in an ultrafine particle size of 60.89 nm ($\pm$ 21.02) and fine particle size of 541.4 nm ($\pm$ 280.4) which was corroborated by DLS with ~90% of the sample particle size at 81.70 nm ($\pm$ 13.02) and ~10% of the particle size at 466.8 nm ($\pm$ 123.1), well within the accepted range of particles capable of direct, olfactory transport to the central nervous system. We next established the LD50 [smoke] in glia to be 747.5 ng/mL at 12 hours of exposure, preceding the doubling time of these cells. We further applied sub-lethal smoke to characterize astrocytic STING activation, reporting significant increase in the number of astrocytic perinuclear pSTING+ foci and nuclear pIRF3+ area via trained machine learning network analysis. Transcriptomic analysis revealed changes in gene expression (603 DEGs, 670 ⁻ DEGs) that mapped to pathways of immune cell recruitment and communication as well as central protein-protein interaction hubs of Tnf and Il1b. We then examined toxicologic endpoints in STINGgt cultures, and report an inability to calculate the LD50 [smoke] for the same dose range due to a strong protective effect of genetic STING ablation. Further, we observed significantly less nuclear pIRF3 signal and TNF- α , IL-6, IFN- γ , and CxCl1 release in the GCM of STINGgt cells. Lastly, WT smoke GCM induced substantially greater neurotoxicity (~50%) when compared to vehicle GCM or STINGgt smoke GCM (~25%) treatments. **Conclusions:** These data suggest that WFS exposure elicits acute glial cytotoxicity and glial-induced neurotoxicity which appear to be mediated through STING signal transduction. This work motivates subsequent assessment of the role that wildfire smoke exposure plays in STING-induced glial reactivity and neuroimmune crosstalk *in vivo* to further parse out relevance of these phenomena to the phenotypes of neurodegeneration.

PS 3791 Protective Effects of the Rho-Kinase Inhibitor Fasudil Against Wildfire Smoke-Induced Neuroinflammation at the BBB

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Background and Purpose: Wildfires across the United States have steadily increased over the past 40 years. Smoke from wildfires can cause cardiovascular and respiratory diseases, with new studies highlighting potential neurological outcomes¹. The purpose of the following studies are to delineate the dynamics of peripheral immune populations in response to woodsmoke, the mechanism of infiltration into the brain, and the treatment options available. This study is the first to deliver a comprehensive timeline of neuroinflammation following wildfire smoke inhalation, collecting tissue from animals 1, 14, and 28 days after exposure. Air pollution has previously shown to impact the blood-brain barrier which serves an essential role in mitigating neuroinflammation. Therefore, we introduced the Rho-kinase inhibitor fasudil to strengthen this barrier and demonstrate an endothelial-dependent mechanism of peripheral immune infiltration. **Methods:** Fasudil was administered via osmotic mini pumps which provide a consistent dose over 21 days. Mice are exposed to either filtered air or a woodsmoke exposure (0.5mg/m³ every other day for 14 days, 4hr/day). Timeline studies will follow these mice over the course of exposure, and extend past 1, 14, and 28 days after exposure. Left hemispheres will be collected for high dimensional flow cytometry analysis using the Aurora Cytex. Markers for peripheral immune infiltration included T cells (CD3, CD4, CD8), adhesion molecules (VCAM, ICAM) resident immune cells (CD11b, TMEM119, ACSA-2) and other inflammation-dependent markers. **Results:** Results demonstrate the presence of CD4 T cells in response to woodsmoke inhalation and the decrease of this population over time. VCAM and ICAM expression also showed similar trends, peaking 1 day after our 14 day exposure paradigm. Treatment with fasudil demonstrates the depletion of VCAM expression which indicates the endothelial-dependent nature of woodsmoke-induced neuroinflammation. Results were conferred with immunofluorescent imaging of the hippocampus with analysis of astrocytes, microglia, and presence of albumin. Albumin levels were elevated with woodsmoke exposure, indicating a leaky blood-brain barrier. Activated astrocytes, quantified via GFAP, and microglia, quantified via Iba-1, were also elevated after exposures. Fasudil lowered levels of albumin in the brain as well as levels of activated astrocytes and microglia. **Conclusions:** Overall, we demonstrate the ability of woodsmoke to cause indirect neuroinflammation through the recruitment of peripheral immune populations. We also observed a

blood-brain barrier dependent mechanism of neuroinflammation through albumin leakage and glial activation. Further research detailing the mitigation of toxicity could lead to public health-based recommendations following air pollution events.

PS 3792 Wildfire Smoke-Induced Neurotoxicity and the Role of Ovarian Hormone Signaling

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Background and Purpose: Wood smoke (WS) from wildfires is a growing public health concern due to the increase in extreme weather events and drier climates. Neurological sequelae following wood smoke (WS) exposure an expanding area of study. Wood smoke from wildfires is known to contribute to a range of adverse health outcomes, and growing evidence points to significant neurological and neuroinflammatory effects. Current research has begun to explore how WS impacts the aging brain, yet an important gap remains: little is known about how endocrinological phenotypes that commonly co-occur with aging may influence toxicological susceptibility. **Methods:** In these studies, we exposed mice (n=10/ group) to an acute WS protocol (~0.5mg/m³), compared to filtered air (FA, controls). We compared: 1) male and female mice 2) Sham and OVX mice and 3) mice subjected to accelerated ovarian failure using the 4-vinylcyclohexene diepoxide (VCD) mouse model and 4) WT and GPER^{-/-} mice. **Results:** Emerging data from our lab has determined that wood smoke exposure statistically significantly increases pro-inflammatory cytokines in the brain such as IL-6, CXCL-1 and TGF- β (p<0.05), in male mice, but not ovary-intact females following acute WS exposure, based on a 2x2 factorial ANOVA. On the other hand, ovariectomized females ((OVX), ovarian hormone deplete mice) demonstrated a statistically significant (p<0.05) increase in CCL-2 and CXCL-1 in the brain, compared to Sham females. In a mouse model of accelerated ovarian failure, we found significant (p<0.05) single-cell transcriptomic alterations in astrocytes, oligodendrocytes, cholinergic and glutamatergic neurons following acute WS exposure. Preliminary data from our laboratory suggests that ovarian hormones, estradiol and progesterone, and g-coupled protein estrogen receptor (GPER) may mediate neurovascular sequelae, based on laser speckle imaging following acute WS exposure. **Conclusions:** The sum of these data suggested a robust mechanistic role of ovarian hormones, that naturally decline with age in females, via GPER on neuroinflammatory and neurovascular responses following acute WS exposures.

PS 3793 Disrupted GABAergic Signaling and Microglial Activation Underlie Behavioral Disinhibition in a Preclinical Model of Gulf War Illness

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Background and Purpose: Gulf War Illness (GWI) is a chronic multisymptomatic disorder affecting veterans of the 1991 Gulf War, characterized by neuroinflammation, cognitive impairment, and altered affective and social behaviors. Exposure to the acetylcholinesterase inhibitor, pyridostigmine bromide (PB) and the insecticide permethrin (PER) has been implicated as a key environmental trigger underlying long-term central nervous system alterations. Prior studies, including work from our laboratory, have reported a loss of blood-brain barrier (BBB) integrity in preclinical models of GWI, facilitating the entry of danger-associated molecular patterns (DAMPs) such as high-mobility group box 1 (HMGB1) into the brain and initiating a neuroinflammatory cascade. However, the contribution of peripheral immune cells, particularly infiltrating T lymphocytes in driving microglial activation and subsequent neuronal dysfunction remains poorly understood. Further, the impact of these exposures on social behavior and mechanisms for inhibitory control also remains poorly defined. To address these gaps, the present study investigates how combined PB and PER exposure influences T cell-microglia interactions, inhibitory control, and social behavior in a preclinical mouse model of GWI. **Methods:** Adult C57BL/6J mice were administered PB (2 mg/kg body weight) and PER (200 mg/kg body weight) orally, every other day for a month along with restraint stress for 10 minutes, while control mice received vehicle (0.6% DMSO in PBS). Mice were then maintained Post 7 months of exposure, behavioral assays were performed including the Elevated Plus Maze (EPM) to assess anxiety/disinhibition, the Rotarod test for motor coordination, and the Three-Chamber Social Interaction test to evaluate sociability and social novelty preference. The animals were euthanized and blood, vital organs such as small intestine, liver, kidneys and brain. Molecular techniques such as western blotting, immunohistochemistry, immunofluorescence and ELISA were accessed to evaluate markers associated with GABAergic synaptic (GAD65/67) and microglial activation markers (IBA1, CD68). **Results:** Behavioral assessments revealed significant alterations in anxiety-related and social behaviors in GWI chemical exposed mice compared to controls. In the Elevated Plus Maze (EPM), GWI-exposed mice spent a greater proportion of time in the open arms, indicative of behavioral disinhibition. This behavioral pattern suggests a role of possible impaired inhibitory control and increased risk-taking or hyperactivity behavior in GWI mice rather than true anxiolytic effects. In the Rotarod test, GWI-exposed mice demonstrated a prolonged latency to fall relative to control animals. While this might indicate improved motor coordination, in the context of their disinhibited phenotype, the increased latency likely reflects altered motor responsiveness. In the three-chamber test, exposed mice showed differential social

preference, spending comparable time in social and non-social chambers, indicative of altered sociability than controls. Preliminary molecular analyses revealed altered expression of GABA-synthesizing enzymes GAD65 and GAD67, indicating compromised inhibitory neurotransmission. This was accompanied by increased microglial activation, as evidenced by elevated IBA1 and CD68 immunoreactivity within the prefrontal cortex and hippocampus regions critical for behavioral regulation and social processing. Collectively, these findings suggest that chronic PB and PER exposure induces persistent neuroinflammation and alteration of GABAergic inhibitory tone, contributing to the observed behavioral disinhibition and social deficits in GWI mice. **Conclusions:** Combined PB and PER exposure induces persistent behavioral disinhibition and social deficits in mice, accompanied by altered GABAergic signaling and microglial activation in brain regions regulating social behavior. These findings support the hypothesis that neuroinflammation-driven loss of inhibitory tone contributes to social and cognitive dysfunction in GWI, providing mechanistic insight into the underlying long-term neurological sequelae of Gulf War toxicant exposure.

PS 3794 Sex-independent dopaminergic and region-specific amino acid neurotransmitter dysregulation across brain regions in two Gulf War Illness mouse models

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Background and Purpose: During the 1990-1991 Persian Gulf War, soldiers were exposed to toxicants such as: pyridostigmine bromide (PB), permethrin (PM), N,N-Diethyl-meta-toluidine (DEET), and sarin. After, 25-30% of the 700,000 soldiers, of which 49,000 were women, developed Gulf War Illness (GWI), a chronic, multi-symptom illness, with many exhibiting memory, mood, and cognitive deficits potentially linked to neurochemical dysregulation. GWI exposure exhibits dopaminergic and serotonergic dysregulation in the ventral hippocampus (VHIP), striatum (STR) and prefrontal cortex (PFC). However, these findings remain limited, primarily acute, with sex differences in cognitive and mood deficits being unexplored at a neurochemical level. To address this and account for female veterans, sex-specific neurotransmitter changes were investigated using high-performance liquid chromatography with electrochemical detection (HPLC-ECD) and western blot of limbic and striatal regions in two preclinical GWI models. **Methods:** Eight-week-old male and female C57BL/6J mice were exposed to Gulf War chemicals; The first model (PB/PM) exposed mice to 0.7mg/kg PB and 200mg/kg PM or vehicle DMSO, via daily intraperitoneal (IP) injections for 10 days. The second model (PB/DEET/CORT/DFP) mice were exposed to daily subcutaneous injections of 2 and 30 mg/kg of PB and DEET respectively, or vehicle saline for 14 days. On days 8-14, mice were also administered corticosterone (CORT) via drinking water (200 mg/L in 1.5% EtOH/H₂O) *ad libitum*; control mice were given 1.5% EtOH vehicle water during this period. On day 15 mice underwent a single IP injection of 3.75 mg/kg of the sarin surrogate: diisopropyl fluorophosphate (DFP) or vehicle saline. After 9 months post-exposure, mice were euthanized, and brains were collected and analyzed for neurochemical changes in five regions: the PFC, STR, dorsal hippocampus (DHIP), VHIP, and lateral septum (LS). Tissue samples were analyzed using HPLC-ECD to quantify monoamine and amino acid neurotransmitter analytes: 3-methoxytyramine (3-MT), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), dopamine (DA), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), norepinephrine (NE), glutamine (GLN), L-glutamate (GLU), and gamma-aminobutyric acid (GABA). Neurotransmitter homeostatic and synaptic function-related proteins were analyzed via western blot probing for tyrosine hydroxylase, dopamine transporter, synaptophysin, vesicular monoamine transporter 2 and PSD-95 in the PFC, STR, and VHIP normalized to β -actin. **Results:** Global effects, model-specific, and sex-specific neurochemical effects of GWI were observed. Modulation of DA and/or its metabolites was evident in both sexes and models in all regions of interest, excluding the DHIP. Hippocampal GABA was decreased in males, while PFC 5-HT increased independent of GWI model. Male PB/PM-specific inhibitory/excitatory (I/E) neurotransmitter perturbations were observed, specifically, decreased GABA in the STR and increased GLU in the PFC. PB/PM also decreased 5-HT and/or its metabolite was prevalent in DHIP, VHIP, and STR. PB/DEET/CORT/DFP GLN in limbic structures was significantly reduced independent of sex. PB/DEET/CORT/DFP-specific DHIP DA dysregulation was sexually dimorphic increasing and decreasing in females and males, respectively. **Conclusions:** Overall, GWI-induced neurochemical dysregulation was more prevalent in male mice. Brain neurotransmitter and protein concentrations 9 months following PB/PM exposure reflect a neuroenvironment consistent with tonic shifts in activity in the PFC, STR and VHIP, while PB/DEET/CORT/DFP induced dopaminergic depletion in the STR and PFC. Male hippocampal GABA depletion impacts I/E balance and may drive memory and mood perturbations observed in preclinical models. The combination of region-specific neurotransmitter and protein dysregulation mirrors symptoms of memory recall deficits, depression, anxiety, and post-exertional malaise still reported by veterans with GWI. This underscores heterogeneity in symptom severity following the Gulf War with respect to soldiers' sex and severity of toxicant exposure; both must be considered in the development of efficacious therapeutics.

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Background and Purpose: Polychlorinated biphenyls (PCBs) are persistent environmental toxicants linked to developmental neurotoxicity. Inhalation exposure to lower-chlorinated congeners, such as PCB52, is increasingly recognized as a current route of human exposure. Critical gaps remain regarding how PCB52 perturbs glial bioenergetics and redox signaling. This study investigates whether PCB52 and its hydroxylated (4-OH-PCB52) and sulfated (4-OH-PCB52-S) metabolites induce mitochondria-centered toxicity in astrocytes and microglia, and whether astrocyte differentiation enhances susceptibility. **Methods:** C6 astrogloma cells were differentiated with dibutyl- α -cAMP/theophylline. Differentiation was validated by increased GFAP immunofluorescence. Viability (MTT, CellTiter-Blue) was measured in glucose and, to force oxidative phosphorylation (OXPHOS), galactose media to derive IC50 values. BV-2 microglia were assayed in parallel. Cellular ATP was quantified under glucose vs galactose conditions. Oxidative/nitrosative stress was imaged in differentiated C6 and primary rat astrocytes by CellROX (general ROS), MitoSOX (mitochondrial superoxide), and DAF-FM (NO/RNS; DEA-NONate positive control). BV-2 TNF- α output was benchmarked to LPS/IFN- γ . **Results:** Differentiation increased astrocyte susceptibility, with a potency order of 4-OH-PCB52 > 4-OH-PCB52-S > PCB52. In C6 cells, IC50 values (μ M) shifted from 11.7 to 12.1 (PCB52), 3.1 to 2.1 (4-OH-PCB52), and 8.9 to 4.7 (4-OH-PCB52-S) in undifferentiated vs differentiated cells, indicating a differentiation-linked vulnerability most pronounced for the hydroxylated metabolite. Mitochondrial dependence was evident in the glucose-to-galactose (Glc->Gal) paradigm and mirrored by ATP. For differentiated C6 in galactose, IC50 shifted to 10.1 μ M (PCB52), 0.9 μ M (4-OH-PCB52), and 2.1 μ M (4-OH-PCB52-S). Correspondingly, cellular ATP declined sharply in galactose, with a collapse near ~2 μ M 4-OH-PCB52 in both differentiated C6 and BV-2 cells, aligning temporally with loss of viability and pointing to OXPHOS liability rather than nonspecific cytotoxicity. BV-2 microglia followed the same potency order as differentiated C6 cells but were modestly more tolerant (IC50, μ M: PCB52 10.3, 4-OH-PCB52 3.0, 4-OH-PCB52-S 8.7). Despite the viability drop with 4-OH-PCB52, TNF- α release remained low relative to LPS/IFN- γ , suggesting that early mitochondrial stress is not tightly coupled to acute pro-inflammatory responses. Imaging corroborated a mitochondria-centric mechanism. In differentiated C6, CellROX revealed widespread ROS with PCB52 and 4-OH-PCB52-S, while MitoSOX showed the strongest mitochondrial superoxide signal with 4-OH-PCB52 and moderate increases with PCB52 and its sulfate metabolite; menadione and antimycin behaved as expected positive controls. Primary rat astrocytes recapitulated these patterns, showing ROS/mito-ROS elevations with 4-OH-PCB52 (~4.7 μ M) and 4-OH-PCB52-S (~7 μ M). DAF-FM at 2 h detected punctate-to-perinuclear NO/RNS under 4-OH-PCB52 at IC50 and sub-IC50, exceeding vehicle and temporally coinciding with the early ATP drop; DEA-NONate produced the anticipated diffuse positive signal. **Conclusions:** Human-relevant PCB52 metabolites function as glial-directed mitochondrial stressors, with a clear astrocyte bias. 4-OH-PCB52 is the more toxic metabolite of PCB52, producing OXPHOS-dependent ATP loss, mitochondrial superoxide, and elevations in NO/RNS. BV-2 microglia are affected at slightly higher IC50 and exhibit limited immediate TNF- α production. Ongoing work will quantify electron-transport-chain liabilities in Glc->Gal conditions and implement HPLC-ECD dopamine uptake/turnover to functionally connect glial bioenergetic failure to neuron-relevant outcomes. This mitochondria-first framework underscores differentiated astrocytes plus Glc->Gal and DAF-FM/MitoSOX imaging as sensitive detectors of PCB52-metabolite toxicity.

PS 3796 Effect of silver nanoparticle related cytokine storm on neurons in insulin resistance state

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Background and Purpose: A link has been established between Alzheimer's disease (AD) and type 2 diabetes, and these patients are at a higher risk of developing AD. The prevalence of insulin resistance and type 2 diabetes is increasing at alarming rates worldwide. However, it was exhibited that silver nanoparticles (AgNPs) attenuate diabetic complications and insulin resistance in experimental diabetes. The clinical association between type 2 diabetes and AD leads to the hypothesis that periphery-derived pro-inflammatory molecules could influence pathogenesis in the central nervous system (CNS). Resident macrophages undergo a phenotypic shift to the classically activated proinflammatory type (M1) in high glucose and insulin containing medium of uncontrolled type 2 diabetes. Cytokines at high levels in AD-induced microvessels are not found in non-AD cerebral microcirculation and this was thought to contribute to the pathogenesis of neuronal injury and death in AD. AgNPs are known to penetrate to the brain and cause neuronal death. AgNP cytotoxicity is mainly dependent on the release of Ag⁺ ions. However, it is unknown

whether peripheral macrophages contribute to the detoxification of AgNP in the luminal compartment of the blood-brain barrier (BBB) and reduce the neuronal damage in the brain. The aim of this study was to explore the AgNP related alterations in the cytokine release and respective neuronal damage in insulin resistance state. **Methods:** Triple co-culture BBB model was established by seeding U937 macrophages and HBEC-5i endothelial cells into the luminal compartment and SH-SY5Y cells into the abluminal compartment. The cell culture model was treated with 250 mg/dL high glucose, 40 uU/mL insulin and 100 ng/mL AgNP either alone or in combination for 48 h. Thirty-three cytokines were measured by using multiplex assay in the supernatants, after a 48-h incubation period in a triple coculture. The cytokine levels obtained in medium containing with or without high glucose, insulin and AgNP were compared. The effects of AgNP exposure on cell viability and oxidative stress in coculture and single cell culture of SH-SY5Y neurons were compared with cytokine levels in insulin resistance state. **Results:** With the addition of AgNP to the medium, 12 cytokines were reduced in the luminal compartment, while the IL1Ra/IL1 β ratio decreased by 8%. In the abluminal compartment, MIP1 β , IL18, IP10, MCP1, IL15, IL1 β , and GM-CSF remained elevated, while the other five cytokines decreased. A 21% decrease was observed in the IL1Ra/IL1 β ratio. **Conclusions:** U937 cells, which represent the peripheral macrophages, weakened AgNP related neurotoxicity by coating the surface of nanoparticles with silver sulfide by probably increasing the cystathionine- γ -lyase (CSE)-mediated H₂S. Thus, all cytokine levels decreased in luminal compartment subsequent to AgNP exposure. All cytokines transported through the BBB, which was disrupted due to hyperglycemia, and reduced the inflammation index (IL1Ra/IL1 β) in the abluminal compartment by 21%. Coincidentally, oxidative stress decreased by 75%, while neuronal survival increased 3.7-fold. (This study was partially supported by TUBITAK, projects 214S112 and 112S437)

PS 3797 Acute neurotoxic effects of plastic antioxidant degradation products in rat cortical neurons

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Background and Purpose: Owing to their affordability and versatility, plastics have become integral to modern life. Plastics are extensively used in modern day products such as electronics and clothes. To enhance the durability and longevity of plastics additives are added, including antioxidants. Without additives, plastics would degrade more rapidly under prolonged exposure to stress factors, such as heat, UV-light, and mechanical wear. Antioxidants in plastics prevent oxidation by sacrificing themselves to neutralize free radicals, resulting in the formation of Antioxidant By-Products (ABPs). Given the widespread applications of plastics, humans are exposed to plastic degradation products including ABPs. There is currently a data gap in understanding the potential health impacts, especially neurotoxic effects of ABPs. Hence, the aim of this study was to investigate the acute neurotoxic effects of ABPs on rat cortical neurons. **Methods:** Effects on cell viability experiments were determined using an Alamar blue assay following exposure of rat cortical cells for 21 days, 48 hrs and 30 minutes to ABP 1-10 at concentrations of 0.01, 0.1, 1, 10 and 100 μ M. Additional experiments were performed using 48-well microelectrode array (MEA) plates to record changes in spontaneous neuronal activity evoked by acute exposure to ABPs. Cells were seeded at a density of 1×10^5 cells/well. Baseline neuronal activity was recorded on day 11 for 30 minutes using the Maestro Pro™ system. Following baseline recording, cells were exposed to ABPs at concentrations between 0.01 - 100 μ M, with an additional 30 minute recording. Data acquisition was controlled by Axion's Integrated Studio (AxIS version 2.6). Raw data was pre-processed to generate spike files. Spike detection was performed with AxIS's spike detector, and the results were further analyzed using the Neural Metrics Tool (v3.1.7, Axion BioSystems) and custom Excel macros. To investigate a potential mechanism of action, acetylcholinesterase (AChE) enzyme was measured. The enzyme solution was combined in a 48-well plate with or without exposure to ABP and incubated for 30 minutes, followed by addition of DTNB solution. Next, 6 mM acetylthiocholine was added to each well, and absorbance was measured at 436 nm for 10 minutes. ABPs 1, 2, 3, 4, 6, 7, 9, and 10 were purchased commercially, while ABPs 5 and 8 were synthesized and provided by LyondellBasell, as they are not commercially available. **Results:** ABPs 1, 2, 3, 5, 7, 8, 9, and 10 did not induce cytotoxic effects after 21 days of exposure, although significant reductions in cell viability were observed at 100 μ M for ABPs 4 and 6. Following a shorter 48-hour exposure, no cytotoxic effects were noted for ABP 6, while ABP 4 exhibited a significant decrease in cell viability at 100 μ M. Finally, no cytotoxic effects were observed for ABP 4 at any tested concentration after a 30-minute exposure. With respect to changes in neuronal activity, a significant reduction in the numbers of recorded spikes was observed at 100 μ M for ABP 1, 2, 3, 8 and 9. For ABP 4, a reduction in the number of spikes was observed at both 10 and 100 μ M, which was associated with a reduced number of bursts and network bursts. In contrast, ABP 5 and 7 increased the number of spikes at 10 and 100 μ M, while ABP 6 and 10 did not appear to affect neuronal activity. The changes in the number of spikes were often associated with comparable changes in the number of bursts and network bursts. AChE experiments showed an inhibition of AChE only by ABP5 (IC₅₀: 59 μ M), while the remaining ABPs did not affect AChE activity. **Conclusions:** Acute exposure to ABPs at concentrations between 10 and 100 μ M resulted in either reduced (ABPs 1, 2, 3, 4, 8, and 9) or increased (ABPs 5 and 7) spontaneous neuronal activity. The numbers of spikes, bursts, and network bursts were the most significantly affected parameters. ABP 5 was the only compound that inhibited acetylcho-

linesterase (AChE) activity. These novel neurotoxicological findings suggest that exposure to ABPs at high concentrations may adversely affect neuronal function and potentially pose a risk to human brain health.

PS 3798 A Human iPSC-Based Platform for Functional Toxicity Screening of CNS-Targeted RNA Therapeutics

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Background and Purpose: Human induced pluripotent stem cell (iPSC)-derived neural co-cultures represent an advanced *in vitro* model that recapitulates key structural and functional features of the human central nervous system (CNS). Such models enable the study of neuron-glia interactions and provide a more physiologically relevant context compared to traditional cell lines or animal-derived systems. Given the growing therapeutic interest in antisense oligonucleotides (ASOs) and the ongoing concerns regarding their potential CNS toxicity, iPSC-derived neural co-cultures offer a valuable platform to evaluate ASO safety, elucidate mechanisms of neurotoxicity, and support the development of safer oligonucleotide-based therapeutics. **Methods:** Building on evidence that ASO-induced neurotoxicity correlates with suppressed calcium transient activity and altered electrophysiology *in vivo*, we sought to develop complementary *in vitro* functional assays to better characterize ASO effects on neuronal activity. To this end, we established high-throughput calcium imaging and multi-electrode array (MEA) platforms containing human iPSC-derived neurons and astrocytes. The calcium imaging assay enables quantitative evaluation of spontaneous calcium transients, providing insights into network excitability and functional integrity, while the MEA assay allows for real-time monitoring of electrical activity across neuronal networks. Together, these physiologically relevant and scalable systems facilitate comprehensive assessment of ASO-induced functional perturbations and support early identification of potential neurotoxic liabilities. **Results:** Under vehicle conditions, key metrics—peak rate (0.15-0.25 Hz), amplitude ($\Delta F/F \sim 1.5\%$), and oscillation score (80-100%)—demonstrated <15% inter-well coefficient of variation, supporting assay reproducibility. Screening of 70 preclinical candidates, we classified ASOs into three categories: (1) highly toxic ASOs causing >60% suppression of calcium oscillation scores with no recovery—correlating with seizures and convulsions *in vivo*; (2) ASOs with partial recovery (20-30%) over 15 minutes—associated with milder behavioural phenotypes; and (3) non-toxic ASOs maintaining normal calcium activity even at 100 μM . Across candidates, *in vitro* calcium scores demonstrated ~80% correlation with rodent CNS toxicity. Furthermore, multi-electrode array (MEA) recordings were employed to quantify ASO-mediated alterations in neuronal network functionality by assessing spike amplitude, network burst frequency, and network burst interval. In alignment with calcium imaging results, a subset of ASOs elicited a pronounced decrease in spike amplitude concomitant with an increase in burst frequency, indicative of enhanced neuronal firing and network hyperexcitability. Conversely, other ASOs induced a pronounced “toxic” electrophysiological signature, characterized by substantial attenuation of spike amplitude and a >50% reduction in network burst frequency, consistent with disrupted synaptic integration and impaired action potential propagation within the neuronal network. Additionally, we used the platform to guide sequence modifications that reduced toxicity. One optimized ASO showed an improved calcium oscillation score of approximately 40% without loss of efficacy, confirming its utility for early-stage screening and structure-activity relationship optimization. **Conclusions:** This iPSC-based platform enables scalable, human-relevant, functional screening of ASO neurotoxicity, supporting the development of safer CNS-targeted RNA therapeutics.

PS 3799 Cyclopiazonic acid downregulates oxidative stress-related genes in SH-SY5Y human neuroblastoma cells

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Background and Purpose: Cyclopiazonic acid (CPA), a neurotoxin produced by fungi from the *Penicillium* and *Aspergillus* genera, triggers oxidative stress and neuronal injury, processes closely associated with the development of neurodegenerative diseases. This study investigates the oxidative stress induced by CPA in SH-SY5Y human neuroblastoma cells, focusing on the transcriptional regulation of key antioxidant and redox-related genes. **Methods:** SH-SY5Y cells were exposed to increasing concentrations of CPA (400, 500 and 600 nM) for 24 h. Total RNA was extracted using the RNeasy Prep™ RNA Cell Miniprep System, quantified with a Nanodrop2000 spectrophotometer and reverse-transcribed into cDNA using the TaqMan™ MicroRNA Reverse Transcription Kit. Quantitative real-time PCR (qPCR) was performed with SYBR Green detection in a StepOne Plus Real-Time PCR System to analyze the expression of *nrf2*, *nos2*, *ho1*, *cat*, *keap1*, *nqo1*, *gpx1* and *gsr*. Primer specificity and efficiency were confirmed by melting curve and standard curve analyses. **Results:** CPA treatment resulted in a concentration-dependent downregulation of oxidative stress-related genes. At 600 nM CPA, *nos2*, *nqo1*, *ho1*, *gsr* and *nrf2* mRNA levels decreased by 93%, 83%, 79%, 78% and 68%. *Gpx1* and *cat* showed consistent repression across all concentrations. *Keap1* expression remained unchanged at 400 nM but decreased by 45% and 66% at 500 nM and 600 nM, respectively. **Conclusions:** In conclusion, CPA markedly suppresses the expression of genes involved in the

antioxidant defense system of SH-SY5Y cells, disrupting redox homeostasis and promoting oxidative imbalance. These findings suggest that CPA-induced disruption of redox homeostasis could contribute to neuronal vulnerability observed in neurodegenerative disorders. **Acknowledgments:** This research was funded by MICIU/AEI /10.13039/501100011033/ FEDER, UE through the project PID2024-1605270B-I00. C. Martínez Alonso thanks the F.P.U. Grant (FPU21/04950) provided by the Ministry of Education. **Keywords:** cyclopiazonic acid, oxidative stress, SH-SY5Y cells, qPCR.

PS 3800 Evaluation of pesticide- and mycotoxin-induced cytotoxicity in differentiated SH-SY5Y spheroids using a reproducible 3D microchip model

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Background and Purpose: Three-dimensional (3D) cell cultures have advanced considerably in recent years, offering greater physiological relevance to *in vivo* systems than traditional two-dimensional (2D) models. These spheroidal cultures improve extracellular matrix organization, nutrient and oxygen gradients, and inter-cellular communication, providing more accurate tools for toxicological assessment. Current efforts focus on enhancing their sensitivity and reproducibility to better mimic *in vivo* conditions. The SH-SY5Y neuroblastoma cell line remains a key model in neurotoxicology due to its ability to differentiate into cholinergic or dopaminergic phenotypes, enabling the evaluation of neuronal damage induced by environmental contaminants such as pesticides and mycotoxins found in food matrices. **Methods:** Based on our previously established model of differentiated SH-SY5Y spheroids, we will generate 3D neuronal spheroids using Honeycomb Cell Spheroid Chips (394 microwells per chip) to ensure high reproducibility. After 22 days of differentiation under previously optimized conditions, spheroids will be exposed to the pesticides Amitraz and Chlorpyrifos, and the T2 mycotoxin. Oxidative damage will be assessed by measuring intracellular and mitochondrial reactive oxygen species (ROS) with CellROX™ and MitoSOX™, together with mitochondrial membrane potential ($\Delta\psi\text{m}$) evaluation using Rhodamine-123. Additionally, apoptosis and necrosis will be quantified by flow cytometry, and changes in neuronal enzymes such as acetylcholine transferase (ChAT) will be analyzed to identify potential alterations in neuronal functionality. **Results:** The generated cholinergic SH-SY5Y spheroids showed high reproducibility and stability, maintaining a uniform morphology and consistent size distribution with a sphericity index (SI) above 0.9 across replicates. Preliminary viability assays revealed reproducible dose-response curves, with IC_{50} values within the range previously reported for other human cell lines in 3D models, confirming the reliability of the established system. When evaluated individually, each contaminant induced a dose-dependent increase in reactive oxygen species (ROS). Similarly, late apoptosis rates increased in a dose-dependent manner after 24h after exposure. Furthermore, changes in the expression of acetylcholine transferase, key enzyme essential for acetylcholine synthesis and marker of the cholinergic neuronal phenotype demonstrated possible alterations in neuronal integrity function. **Conclusions:** Differentiated 3D SH-SY5Y models more accurately mimic *in vivo* neuronal organization and function, establishing a sensitive and reproducible system for evaluating the cytotoxic potential of foodborne contaminants. The implementation of these microchip-based spheroidal cultures enhances model scalability and reproducibility, strengthening their applicability as advanced tools for regulatory and mechanistic toxicology. **Acknowledgments:** This research was funded by MICIU/AEI /10.13039/501100011033 / FEDER, UE through the project PID2024-1605270B-I00. F. Franco-Campos thanks to the scholarship “Santiago Grisolia (CIGRIS/2021/073)”.

PS 3801 Deoxynivalenol induces apoptosis through mitochondrial dysfunction in a 3D model of SH-SY5Y neural spheroids

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Background and Purpose: Deoxynivalenol (DON) is a type B trichothecene produced by *Fusarium* species, commonly found in cereals and derived products. It is one of the most prevalent mycotoxins worldwide, and exposure to it can cause damage to multiple organs, including the gastrointestinal tract, liver, kidneys, brain, and immune system. Most studies on DON toxicity have been conducted using two-dimensional (2D) cell cultures, which do not adequately reproduce the cellular microenvironment. For this reason, three-dimensional (3D) models have emerged, as they better reflect cellular architecture and cell-cell interactions, making them more suitable for studying the cytotoxicity of mycotoxins. This study aimed to evaluate the effects of DON on mitochondrial function, reactive oxygen species (ROS) generation, and apoptosis activation in SH-SY5Y spheroids. **Methods:** SH-SY5Y spheroids were exposed to different concentrations of DON (2-16 μM) for 24 hours. Apoptosis and necrosis were assessed by flow cytometry using Annexin V-FITC/PI staining. The expression of apoptosis-related proteins (BAX, Bcl2 and caspase-3) was analyzed by Western blot, using β -actin as a loading control. The mitochondrial membrane potential ($\Delta\psi\text{m}$) was determined by rhodamine 123 (Rh123) fluorescence, and ROS generation was measured by flow cytometry using the CellROX reagent. **Results:** Flow cytometry analysis showed a significant increase in early apoptosis at 16 μM DON. Western blot results revealed an overexpression of the pro-apoptotic protein BAX, along with the

appearance of cleaved active fragments of caspase-3, confirming the activation of apoptotic pathways. Moreover, a loss of $\Delta\Psi_m$ was observed between 2 and 16 μ M. Finally, no increase in ROS was observed at any of the DON concentrations evaluated. **Conclusions:** It has been demonstrated that ROS do not contribute to the mitochondrial dysfunction produced by DON in SH-SY5Y spheroids. However, a direct effect on the mitochondrial membrane was observed, resulting in a loss of $\Delta\Psi_m$ and, consequently, the activation of apoptotic pathways. These findings highlight the relevance of 3D spheroid models as physiologically representative systems for studying mycotoxin toxicity. **Acknowledgments:** This research was funded by MICIU/AEI/10.13039/501100011033/FEDER, UE through the project PID2024-160527OB-I00. Martina Sánchez acknowledges the Generalitat Valenciana for the Santiago Grisolia Fellowship (CIGRIS/2023/071). **Keywords:** apoptosis, mitochondrial dysfunction, 3D neural spheroids, neurotoxicity

PS 3802 Effects of brevetoxin exposure on zebrafish novel object recognition capabilities

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Background and Purpose: Brevetoxins are known to cause harmful effects on vertebrates, including humans. These effects manifest themselves most commonly as upper respiratory symptoms such as nasal congestion, throat irritation, cough, and eye irritation. However, brevetoxin's neurological and cognitive effects on humans have not been studied extensively. A standard measurement of a vertebrate's cognitive activity is the novel object recognition test (NORT). Zebrafish (*Danio rerio*) exhibit anxiolytic behavior when presented with a novel object, indicating the recognition of something new and potentially threatening in its environment. Reduction of this behavior when exposed to brevetoxin indicates alterations to fish cognitive functions, which could have further implications for humans living in areas such as the Gulf Coast of Florida, which experience large brevetoxin releasing algal blooms. **Methods:** A novel object recognition test (NORT) was used to explore the effects that brevetoxin (PbTx-2) may have on cognitive ability since these tests serve as a proxy for measuring memory capabilities. We exposed male and female adult zebrafish (Female-15, Male-16) to 10, 12.5 and 15 nM PbTx-2 solutions and then allowed them to explore a testing tank with a familiar and novel objects on opposite sides of the tank. Results were recorded using EthovisionXT animal tracking and recording software. Mean time within a zone of proximity to each object, and latency to approach each object was recorded, and t-tests compared fish object preference before and after exposure to brevetoxin. Data on animal velocity, acceleration, and total distance moved were also collected. **Results:** There were trends in time around novel objects compared to familiar objects and the latency in which the fish approached the objects, but there were no statistical differences between control and intoxicated groups. Brevetoxin-exposed fish spend more time in proximity to both novel and familiar objects than nonexposed control fish. The data also revealed tangential trends of PbTx-2 on fish locomotion in that the intoxicated fish showed increased swimming velocity and total distance moved yet decreased acceleration compared to control fish. **Conclusions:** Overall, while none of the differences were statistically significant, trends indicate that brevetoxin exposure made the fish less anxious about the objects as they spent more time near novel and familiar objects after exposure to the toxin. We did let the fish wait 5 minutes between trials in a holding tank and previous literature suggests that is the limit of their object discrimination memory. New trials with a shorter inter-trial wait time will be performed in the future.

PS 3803 Zebrafish behavioral profiling as a tool for neuroactivity and neurotoxicity prediction

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Background and Purpose: The successful assessment of an agrichemical requires extensive toxicological testing in mammals, including evaluation of neurotoxicity, which often occurs late in the product development pipeline and consists of higher tiered animal studies. Concern related to safety and late-stage project failure remains a challenge for discovery programs, hence it is pertinent to implement high-throughput predictive models for neurotoxicity. Additionally, discovery programs support the application of the 3Rs (Replacement, Reduction, and Refinement), by exploring *in vitro* new approach methodologies (NAMs) for predictive testing. Zebrafish assays leverage a complete physiological system and enable medium to high-throughput screening to investigate neuroactivity of compounds. To profile behavioral phenotypes of 7 days post fertilization (dpf) larvae, a unique battery of stimuli has been designed that exposes the animals to light or vibrational stimuli at various intervals on a custom-engineered system. This method has proven effective in identifying new small molecules in pharmaceutical classes such as antipsychotics, sedatives, and anesthetics. In prior collaboration, we demonstrated that the technique of behavioral profiling is sensitive to modes of actions of agrichemicals. Here we evaluate transferability of a multi-stimulus zebrafish behavioral assay to a commercial system and determine whether 5 dpf reproduces 7 dpf neuroactive phenotypes. **Methods:** To replicate the method of the custom engineered system (Sauron), a similar

sequence of light flashes, strobes, solenoid taps, and vibrations were programmed on a commercially available zebrafish behavioral system (Multi-camera array microscope (MCAM)). To assess initial transferability across systems and larval ages, 7 dpf and 5 dpf larvae were tested on the MCAM and Sauron to compare baseline response across systems. To test the ability of the MCAM system to respond appropriately to neuroactive compounds, 5 dpf zebrafish were exposed to six compounds known from previous experimental data on the custom engineered method to induce strong behavioral phenotypes. These included Optovin (TRPA1 ion channel modulator), Etomidate (GABA_AR agonist), Bromocriptine (dopamine receptor agonist), Lidocaine (voltage-gated sodium channel blocker), Pentylenetetrazol (PTZ) (GABA_AR antagonist), and Haloperidol (dopamine and 5-HT receptor antagonist). Larvae were allowed to acclimate with chemical on board for one hour and were then exposed to the series of stimuli while being recorded. Motion index was calculated from the recorded video, and behavioral traces were compared to vehicle control. **Results:** Behavioral traces of 7 dpf larvae were similar across MCAM and Sauron systems; however, due to technical limitations, behavioral phenotypes are not directly comparable across systems. Traces between developmental timepoints (5 dpf vs. 7 dpf) appeared similar within and across systems. Variability between individual runs was much higher in 5 dpf than 7 dpf larvae when assessed via DTW distance matrix analysis. The neuroactive compounds produced the expected behavioral phenotypes and were distinguishable from vehicle. For example, zebrafish exposed to PTZ exhibited increased behavior (approximately +2 SD in comparison to vehicle) specifically in the violet light stimuli portion of the battery, consistent with the phenotype as observed in the custom engineered system. Optovin exposure produced the expected enhanced acoustic startle response. Etomidate exposure produced the expected behavioral response of sedation with enhanced response to solenoid stimuli. **Conclusions:** In conclusion, we have demonstrated the ability to capture strong and expected neuroactive behavioral phenotypes using a method developed for 5 dpf zebrafish on a commercially available system. Further testing is needed to investigate the potential use and appropriate scope of this system for neurotoxicity prediction. Overall, developing this method in a way that is accessible to other laboratories as a NAM may increase in the acceptance of and interest in zebrafish behavioral phenotyping as a tool for neuroactivity and potentially neurotoxicity screening.

PS 3804 Stimulatory Effects of Alcohol in As3MT Knockout Mice

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Background and Purpose: Few preclinical studies have examined the relationship between arsenic (As) and alcohol, despite evidence of interaction between the two. Recent work shows that alcohol exposure increases the absorption and excretion of As, highlighting the need to investigate their combined effects. The *As3mt* gene encodes the arsenic (+3 oxidation state) methyltransferase (As3MT) enzyme, which catalyzes the metabolism of inorganic As. Human genome-wide association studies have implicated As3MT in anxiety phenotypes and identified an interaction between alcohol consumption and pulse pressure. **Methods:** Based on this evidence, we hypothesized that loss of As3MT would alter anxiety-like behavior and the locomotor response to ethanol (EtOH). As3MT knockout (KO) mice (males and females, $n = 6$ per sex) were compared with wild-type (WT) C57BL/6J controls ($n = 5$ per sex). On days 1 and 2, mice received habituation sessions with saline injections (10 mL/kg, i.p.) followed by testing in an open field for 30 minutes. On days 3 and 10, they received 1 or 2 g/kg EtOH (20% v/v, i.p.), and locomotor activity was recorded for 30 minutes, with analyses focused on total and early (0-10 minutes) activity. **Results:** Significant sex differences emerged on days 1 and 3 ($p < .05$), while a main effect of genetic model (i.e., WT vs As3MT KO) was observed on day 3 ($p < .05$) for total activity. When assessing percent change in early activity on EtOH treatment days, there was a significant main effect of genetic model at each EtOH dose ($p < .05$), with KO mice displayed a greater change in activity at both doses ($p < .05$) compared to WT mice. Moreover, KO mice spend significantly less time in the center of the open field on days 1, 2, and 10 compared to WT mice ($p < .05$), indicating greater anxiety-like behavior. **Conclusions:** These findings suggest that As3MT may contribute to EtOH- and anxiety-related behavioral phenotypes. Ongoing studies will examine and assess how As3MT modulates alcohol responses in both arsenic-naïve and arsenic-exposed mice.

PS 3805 Cytochrome C Oxidase activity across the brain following acute hydrogen sulfide toxicity in mice

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Background and Purpose: Hydrogen sulfide (H₂S) is a highly toxic gas that targets tissues with high oxygen demand, such as the brain and heart. Acute H₂S toxicity is widely attributed to its inhibition of cytochrome c oxidase (Complex IV; COX), an essential component of the mitochondrial electron transport chain (ETC) responsible for producing more than 98% of cellular ATP. In the brain, COX activity is not uniformly distributed, and the extent of COX inhibition across brain regions following acute H₂S poisoning is poorly characterized. In our laboratory, we have observed that the neurotoxic effects of acute H₂S exposure—including neurotransmitter alterations and neuropathology—are also region-specific. Given the central role of COX in H₂S-induced neurotoxicity, we designed a pilot study to test the hypothesis that

acute H₂S exposure does not uniformly inhibit COX activity across the brain. This knowledge will be useful developing brain COX activity as a biomarker of H₂S poisoning. **Methods:** All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC). Male C57BL/6J mice (6-7 weeks old) were purchased from Jackson Laboratories (Sacramento, CA, USA). Three experimental approaches were used. In the first approach, mice were randomly assigned to two groups: a room-air control group and an H₂S-exposed group. Control mice were exposed to room air for 50 minutes in a whole-body exposure chamber. H₂S-exposed mice were exposed to 1000 ppm H₂S for 50 minutes, and cohorts of surviving mice ($n = 4/\text{time point/group}$) were euthanized at 5 minutes, 2 hours, 24 hours, or 72 hours post-exposure. Brains were micro dissected, and different regions were analyzed for COX activity. In the second approach, cohorts of twenty 6-7-week-old male C57BL/6J mice were divided into four groups. Control mice ($n = 5$) were exposed to room air for 60 minutes. H₂S-exposed mice were exposed to 1000 ppm H₂S for 15 minutes ($n = 4$), 30 minutes ($n = 4$), or 60 minutes ($n = 4$). Upon euthanasia, brains were rapidly removed and micro dissected for COX activity measurements. In the third approach, mice were exposed daily to either room air or H₂S (1000 ppm for 40 mins on day 0, and for 15 mins once daily) for up to 6 days, and cohorts were euthanized on days 1, 3, and 7. Brains were removed, micro dissected on ice, and stored at -80°C until analysis. COX activity was measured using a commercial Complex IV Rodent Enzyme Microplate Assay Kit (Abcam, Waltham, MA, USA) following the manufacturer's guidelines. Normality was assessed using the Shapiro-Wilk test. COX activity was analyzed using one-way ANOVA with Dunnett's post hoc multiple-comparison test. Statistical analyses were performed in RStudio (Version 2025.05.0+496). A $p\text{-value} \leq 0.05$ was considered statistically significant. **Results:** Across all experimental approaches, acute H₂S exposure did not uniformly inhibit COX activity across the mouse brain. The cortex was the most sensitive region, followed by the olfactory bulb and brainstem. Following a single acute 60-minute exposure to 1000 ppm H₂S, COX activity in the cortex was reduced to 8% of control levels, compared to 30% in the olfactory bulb, 62% in the brainstem, and 66% in the cerebellum. **Conclusions:** There is limited information mapping COX activity across brain regions following acute H₂S poisoning. Our preliminary data suggest that acute H₂S exposure does not uniformly inhibit COX activity across brain nuclei and regions. Additional studies are needed to correlate regional COX activity with neuropathology and neurochemical changes to clarify the role of this enzyme as a biomarker of H₂S-induced neurotoxicity.

PS 3806 Correlation of Brain MRI Alterations with Histopathologic Changes in a Cynomolgus Monkey with Cytokine Release Syndrome

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Background and Purpose: Cytokine release syndrome (CRS) is a systemic inflammatory condition caused by a various factors, including infection and drug administration and can sometimes cause CNS symptoms in severe cases. However, there was few information about animal model with severe lethal symptoms induced by chemotherapy. We experienced CRS with CNS symptoms in a cynomolgus monkey treated with paclitaxel. To investigate CRS-related changes and their distribution in a whole brain, we conducted an *ex vivo* MRI with three-dimensional volume reconstruction evaluation, and histopathologic examination of the brain to compared the respective results and evaluate their correlations. **Methods:** A 3-years-old male monkey once treated with paclitaxel at 6 mg/kg showed symptom suggesting infusion reactions from end of dosing with prolonged CNS clinical sign and was sacrificed moribund on the day after administration. Serum samples were collected sequentially from 5 min to 24 hr after dosing and was submitted to blood cytokine level measurement (Milliplex MAP kit NHP Cytokine Magnetic Bead Panel). After necropsy, the brain was fixed with 10% neutral buffered formalin and *ex vivo* MRI was performed on the fixed brain. A Bruker 7 Tesla MRI system (Biospec 70/40) was used, with an 86 mm inner diameter birdcage coil (T12053V3) for transmission and reception. Microscopic examination by Hematoxylin-Eosin (HE) staining, Immunohistochemistry (IHC) staining for fibrinogen was performed on the brain following *ex vivo* MRI. **Results:** In the cytokine measurement, serum IL-10, IL-6, and MCP-1 increased, while IL-8 decreased from 30 min up to 24 hours after dosing, compared to levels in background control pool. In the *ex vivo* MRI, the T2-weighted images revealed hypointense signals around the vasculature in the brainstem region. In the histopathology, there was localized dilatation of arteries and veins in the brainstem region where hypointense signals in MRI, suggesting abnormalities in the circulation. There was focal and very slight neuronal necrosis in the superficial layers of the cerebral cortex adjacent to the cerebral sulci. In the pia mater, reactive infiltration of inflammatory cells was also observed. In the anti-fibrinogen IHC staining, there were perivascular fibrinogen-positive areas with indistinct boundaries around blood vessels in the medulla oblongata. Although no clear changes were seen in these regions in HE stained sections and MRI images, the IHC findings suggested brain edema due to leakage of plasma components out of the vasculature. **Conclusions:** In the monkey presenting CRS with CNS symptoms, MRI using T2-weighted sequences revealed vascular lesions in the midbrain. Histopathologic examination with HE staining showed localized dilatation of arteries and veins in the brainstem region where MRI abnormalities had been detected. In addition to the areas with MRI abnormality, focal and very slight neuronal necrosis, mild inflammatory cell infiltration, and perivascular fibrinogen-positive areas were also observed. In conclusion, combination of MRI and histopathologic examination

could detect highly localized vasculature changes. It was considered that the brain edema caused by CRS contributed to CNS symptoms in this animal, suggested by MRI abnormalities, neuronal necrosis and fibrinogen-positive perivascular area.

PS 3807 Development of Novel Aminopyridines for Safe and Effective Treatment of Peripheral Neurological Disorders

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Background and Purpose: Aminopyridine-based voltage-gated potassium channel (VGKC) blockers, such as 3,4-diaminopyridine (3,4-DAP) and 4-aminopyridine, enhance neurotransmitter release by prolonging presynaptic action potentials. The FDA-approved compound 3,4-DAP provides symptomatic benefit in disorders characterized by impaired presynaptic release, including Lambert-Eaton myasthenic syndrome, myasthenia gravis, and congenital myasthenic syndromes. We have demonstrated that 3,4-DAP acts as a fast-acting reversal agent for botulinum neurotoxin (BoNT)-induced paralysis, a condition marked by reduced acetylcholine (ACh) release due to SNARE protein cleavage. However, widespread clinical use of 3,4-DAP is limited by its narrow therapeutic index, driven by dose-limiting Central nervous system (CNS) toxicity, and by five-fold interindividual variability in plasma exposure caused by N-acetyltransferase 2 (NAT2) polymorphisms. To overcome these limitations, we developed a new class of aminopyridine analogs designed to retain VGKC-blocking activity while reducing CNS penetration and eliminating NAT2-mediated metabolism. **Methods:** Using a structure-based pharmacophore model incorporating a protonatable ring nitrogen and a hydrogen bond donor at the 4-position, we synthesized analogs with targeted substitutions to optimize activity, permeability, and metabolic stability. Lead compounds were screened in a BoNT/A mouse model using unrestrained whole-body plethysmography; several candidates restored respiratory function to normal physiological levels at well-tolerated doses. Pharmacokinetic studies assessed NAT2-mediated acetylation of lead analogs using human liver S9 fractions (*in vitro*) and mouse plasma and urine (*in vivo*) following single-dose infusion. Acetylated metabolites were quantified using highly sensitive LC-MS analysis. *Ex vivo* nerve-muscle preparations and ion channel recordings confirmed potent VGKC inhibition associated with motor neuron repolarization and presynaptic ACh release. **Results:** Lead aminopyridine analogs demonstrated robust efficacy in reversing BoNT/A-induced respiratory impairment in mice, restoring normal function at safe doses. Pharmacokinetic analysis revealed resistance to NAT2-mediated acetylation both *in vitro* (human liver S9 fractions) and *in vivo* (mouse plasma and urine). Electrophysiological studies confirmed strong VGKC blockade and enhanced presynaptic neurotransmitter release. **Conclusions:** Our findings support the advancement of NAT2-resistant aminopyridine analogs as fast-acting therapeutics for BoNT intoxication and other peripheral neuromuscular disorders. This work validates an aminopyridine drug discovery platform with potential applications in additional indications, including pain, muscular dystrophy, and seizure disorders.

PS 3808 A Comprehensive In Vitro Six-Cell Human NVU Model for Coupled Evaluation of BBB Function, Immune Response, CNS exposure, and Neurotoxicity

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Background and Purpose: Neurotoxicants and CNS-active therapeutics encounter the highly restrictive blood-brain barrier (BBB), yet most *in vitro* models lack sufficient cellular complexity to predict human-relevant permeability, immune activation, and neurotoxicity. To address this, we developed a physiologically aligned, six-cell human neurovascular unit (NVU) model in a two-compartment format that reconstructs the spatial organization of the BBB and brain parenchyma. On the apical (blood-facing) membrane, human astrocytes, pericytes, and HBEC-5i endothelial cells are cultured in direct contact to establish a restrictive BBB layer, where co-seeding microglia are integrated with astrocytes to impart immune responsiveness at the barrier interface. The basal (brain-facing) compartment contains human neurons, oligodendrocytes, and microglia to recapitulate parenchymal structure and enable measurement of downstream neurotoxic effects. **Methods:** Human primary astrocytes, pericytes, and HBEC-5i endothelial cells were sequentially seeded onto poly-L-lysine-coated 0.4 μm Transwell® inserts to generate a direct-contact BBB layer. HMC3 microglia were co-seeded with astrocytes to establish immune surveillance at the barrier interface. The basal chamber was populated with SH-SY5Y or primary human neurons, MO3.13 oligodendrocytes, and HMC3 microglia to form a parenchymal compartment. Barrier integrity was quantified using Transendothelial Electrical Resistance (TEER) and apical-to-basal apparent permeability (Papp) of lucifer yellow and 4 kDa FITC-dextran. Structural and functional BBB phenotype was evaluated by immunocytochemistry for tight-junction and adhesion proteins (ZO-1, claudin-5, occludin, PECAM-1), cytoskeletal organization (F-actin), and efflux transporters (MRP2, P-gp). Neuroinflammatory activation was assessed using changes in Papp and microglial markers CD68 and IBA-1. Downstream neurotoxicity was evaluated using the neuronal injury marker Tau and neuronal integrity marker MAP2. **Results:** The direct

contact triculture model (endothelial cells, astrocytes, pericytes) established a functional baseline barrier. Addition of neurons generated a four-cell NVU and significantly increased structural BBB markers, including ZO-1, claudin-5, occludin, PECAM-1, F-actin, and efflux transporters (P-gp, MRP). Neuronal integration also improved barrier function, reducing FITC-dextran Papp from 5.83×10^{-6} to 5.34×10^{-6} cm/s. The NVU retained physiological immune sensitivity, with LPS (5 µg/mL) increasing Papp to 10.4×10^{-6} cm/s. Incorporation of microglia produced spatially dependent modulation of barrier integrity. Apical microglia markedly tightened the barrier (Papp $2.06\text{--}2.23 \times 10^{-6}$ cm/s), whereas basal-only microglia induced only modest changes, demonstrating that microglial position—not cell density—is the primary determinant of BBB regulation. Dual apical-plus-basal microglia further enhanced immune responsiveness and strengthened neuron-microglia signaling. An apical astrocyte to microglia ratio of 1:5 achieved both robust immune activation and physiologically relevant neuronal outcomes, including changes in Tau and MAP2 expression and associated alterations in axonal morphology. Completion of the six-cell NVU with oligodendrocytes stabilized neuronal networks and produced the most restrictive barrier observed lucifer yellow permeation rates (Papp 0.6×10^{-6} cm/s) almost 10 times lower compared with the triculture BBB model alone. NVU disruption was induced using potassium cyanide to assess neurotoxicity using the six-cell model. The incorporation of microglia and oligodendrocytes enhanced the barrier tightness and increased the stability of neuronal networks during metabolic stress. This increased resistance supports the use of the six-cell NVU model as a physiologically relevant system for neurotoxicity assessment. **Conclusions:** This optimized six-cell NVU model establishes a structurally and functionally complete human-relevant BBB system capable of capturing permeability, neurotoxicity, inflammation, and neuronal structural alterations within a single platform. Its improved barrier tightness, immune competence, and neuronal readouts position it as a powerful tool for mechanistic toxicology, neuroinflammation studies, and CNS drug screening.

PS 3809 Scalable and well-defined human CNS co-culture platforms suitable for modelling excitatory-inhibitory neuron imbalances in drug discovery and safety studies

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Background and Purpose: Cortical neuronal circuits are composed of two principal neuronal classes: glutamatergic excitatory neurons and GABAergic inhibitory neurons (IN). The inputs of IN provide cortical networks with the ability to balance spontaneous and evoked excitatory activities, preventing runaway excitation. Aberrant IN function and resultant excitatory-inhibitory (E/I) imbalances are implicated in various neurological disorders including autism, epilepsy, and schizophrenia. Furthermore, these imbalances can arise from astrocytic dysfunction, given their vital role in the turnover of glutamate released into the synaptic cleft. There is a demonstrable need for scalable methodologies to generate human *in vitro* models that reliably recapitulate E/I imbalances for high-content drug screening and toxicology assays. **Methods:** Leveraging our deterministic cell programming technology, opti-ox™ (optimised inducible overexpression), we have successfully generated the core cell types essential for establishing a platform to investigate E/I neuron imbalances: GABAergic neurons (ioGABAergic Neurons), glutamatergic neurons (ioGlutamatergic Neurons), and astrocytes (ioAstrocytes), derived from human induced pluripotent stem cells (iPSCs). **Results:** Comprehensive molecular characterisation via immunocytochemistry, RT-qPCR, and single-cell RNA-sequencing demonstrates that cultures consist of over 95% pure GABAergic neurons, which express the canonical markers GAD1, GAD2, VGAT, DLX1, and DLX2, and exhibit GABA positivity. Notably, somatostatin (SST) was the sole GABAergic subtype-specific marker detected, further accentuating the purity of the ioGABAergic Neurons. Moreover, three independently manufactured lots of ioGABAergic Neurons exhibited highly equivalent transcriptomic profiles, confirming the consistency and scalability inherent to the opti-ox technology. Functional validation through Microelectrode Array (MEA) assays demonstrated that ioGABAergic Neurons inhibit the excitatory activity of ioGlutamatergic Neurons in a ratio-dependent manner, and that the E/I balance can be further modulated by pharmacological agents targeting GABAergic signalling. In addition, we have developed protocols to culture ioGlutamatergic Neurons with glial cells, specifically oligodendrocyte-like cells (ioOLCs), microglia (ioMicroglia) and astrocytes (ioAstrocytes). These models afford valuable opportunities for investigating the mechanisms underlying immune activation and demyelination, thereby facilitating drug discovery in these therapeutic areas. Furthermore they are critical for drug safety studies as they enable the construction of more complex multi-cell models capable of recapitulating key cellular interactions in the human brain. **Conclusions:** The opti-ox driven co-culture platforms offer a means to model intricate CNS interactions accurately *in vitro*, thereby advancing our understanding of the principles underpinning E/I imbalances and directly supporting human-relevant neurotoxicity assays and drug discovery for debilitating neurological disorders.

PS 3810 Phenotypic Screening Platforms for Neurotoxicity and Neurorepair in CNS Drug Safety Assessment

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Background and Purpose: The high attrition rate in CNS drug development underscores the need for predictive, mechanistically relevant *in vitro* models that support both efficacy and safety profiling. We developed complementary phenotypic screening platforms using human iPSC-derived neurons to evaluate neurotoxicity and regenerative potential under disease-relevant conditions, aiming for alignment with recommendation frameworks like OECD for *in vitro* neurotoxicity testing. **Methods:** In one approach, we adapted the Cell Painting assay to profile morphological changes in ioGlutamatergic neurons either carrying pathogenic mutations or being exposed to neurodegenerative disease relevant triggers like βAmyloid. To complement neurotoxicity profiling, we developed neurite outgrowth and axonal regeneration assays using iPSC-derived dopaminergic and motor neurons (including neurodegenerative disease-relevant mutants). **Results:** Cell Painting assays have been widely adopted for morphological profiling to uncover mechanisms of action, disease phenotypes, and toxicity prediction. A modified staining protocol incorporating β-III tubulin enhanced visualization of neuronal architecture. Principal Component Analysis revealed distinct phenotypic separation between mutant and wild-type neurons, with dose-dependent shifts upon β-amyloid oligomer exposure. These shifts correlated with increased neurofilament light chain (NfL) release—a translational biomarker of neurodegeneration—highlighting the assay's sensitivity to neurotoxic insults. OECD guidelines alignment in this case is particularly related to neurite morphology and network integrity. Neurodegeneration was induced via chemically defined triggers, with high-content imaging and viability measurements as key readouts. Regeneration was assessed using axotomy in microfluidic devices, enabling real-time tracking of axonal repair. These assays align with the neurite outgrowth and network formation endpoints in the OECD DNT *in vitro* battery and offer additional insight into functional recovery, complementing current regulatory tools. **Conclusions:** Together, these platforms provide scalable, multiparametric tools to assess compound-induced neurotoxicity and neurorepair potential. Their alignment with OECD test guidelines and guidance documents supports integration of neurotoxicity risk evaluation in early-phase drug development.

PS 3811 Assessing the neurotoxic and cardiotoxic potential of botanical extracts using microelectrode array (MEA) recordings

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Background and Purpose: The global use of botanical extracts in medicinal, cosmetic, and dietary products is steadily increasing. However, these complex mixtures vary in composition due to differences in plant growth conditions, extraction methods, and processing, which can affect their biological activity and safety. Despite their natural origin, botanical extracts have been linked to adverse effects, including neurotoxicity and cardiotoxicity. Assessing the safety of these products is challenging because of their chemical diversity and multiple modes of action. Conventional animal-based toxicological testing is often unsuitable, as it is costly, time-consuming, and ethically debated. Consequently, there is growing interest in applying new approach methodologies (NAMs) to evaluate the toxicity of botanical mixtures. The present study aimed to evaluate the suitability of microelectrode array (MEA) recordings to assess the neurotoxic and cardiotoxic potential of 16 botanical extracts by deriving dose-response profiles and activity patterns in neuronal networks and human induced pluripotent stem cell-derived cardiomyocytes. **Methods:** For neuronal activity measurements, primary cultures of rat cortical neurons were grown on 48-well MEA plates. For assessing cardiac activity, human iPSC-derived cardiomyocytes were grown on 96-well MEA plates. Cells were cultured in 5% CO₂/95% air atmosphere at 37°C until use after 10–15 days in culture. MEA recordings were used to determine changes in spontaneous neuronal network activity and spontaneous cardiomyocyte beating, respectively, following acute (30 min) exposure to 16 botanical extracts. All botanical samples were tested at a dose of 50 µg/mL down to the no-observed effect level (NOEL). The botanical extracts were provided by the Botanical Safety Consortium (<https://botanicalsafetyconsortium.org/>; Mitchell et al., 2022). Details on the sourcing, chemical analysis, and botanical details can be found at NIEHS's CEBS website (<https://cebs-ext.niehs.nih.gov/cebs/paper/15717>). Botanicals were selected based on existing literature with respect to toxicity or safety, from human, animal, or mechanistic studies (Kanungo et al., 2024). **Results:** Our results demonstrate that exposure to extracts from ashwagandha, comfrey, and Asian ginseng had limited effects on neuronal network activity, whereas aristolochia, ephedra, green tea, milk thistle, trypterygium and usnea resulted in a dose-dependent, mild inhibition of neuronal activity, albeit with distinct neuronal activity phenotypes. Extracts of kava, kratom, and yohimbe potentially inhibited neuronal activity, with cessation of activity at 50 µg/mL. Despite differences in the maximum degree of inhibition, all these inhibitory extracts show NOELs of 1–5 µg/mL. Exposure to aconite, blue cohosh, goldenseal and oleander results in specific activity phenotypes characterized by hyperexcitation and/or intensification of (network) burst activity, with extracts from aconite, gold-

enseal and oleander being most potent with NOELs of 0.25 - 0.5 µg/mL. Our result from MEA recordings using human iPSC-derived cardiomyocytes show that extracts from ashwagandha, blue cohosh, comfrey and Asian ginseng have limited cardiotoxic potential, whereas aristolochia, green tea, milk thistle, and usnea have only a mild cardiotoxic potential. Extracts of aconite, ephedra, goldenseal, kava, kratom, oleander, tripterygium and yohimbe result in profound cardiotoxicity, evidenced by distinct effects on beat rate, spike amplitude and field potential duration, with extracts from goldenseal and oleander being most potent with a NOEL of 0.05 µg/mL. **Conclusions:** Diverse botanical extracts have a clear neuro- and cardioactive potential. The distinct activity phenotypes suggest that the botanical extracts have diverse modes of action, which are partly shared by the nervous system and the heart. Despite challenges related to *in vitro-in vivo* extrapolation, our combined data highlight the applicability of MEA recordings of neuronal and cardiac activity for hazard identification and potency ranking of botanicals.

PS 3812 Comparative Biodistribution of an Antisense Oligonucleotide following Intrathecal Administration via Three Injection Techniques in Rats

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Background and Purpose: Effective delivery of antisense oligonucleotides (ASOs) into the central nervous system (CNS) is essential for evaluating therapeutic potential in preclinical studies. Intrathecal delivery in animal models is the preferred route of administration due to its translational relevance for human route of administration for CNS-targeted oligonucleotide therapies. However, intrathecal delivery presents technical and anatomical challenges that can influence drug delivery efficiency, consistency, and biodistribution, particularly into the brain. **Methods:** Our laboratory has developed and refined procedures to allow for reliable intrathecal administration in rats. To compare delivery approaches, 14-week old Sprague Dawley rats received a single dose of ASO via one of three methods: (1) percutaneous direct injection, (2) direct injection via surgical approach, and (3) administration through an implanted catheter. Animals were monitored for 3 weeks post-dose, after which they were euthanized. Clinical signs, body weight, and clinical pathology parameters were evaluated. **Results:** CNS tissues, including brain and spinal cord regions, were collected and analyzed for ASO concentration using a hybridization electrochemiluminescence bioanalytical method. Robust ASO exposure was observed in both spinal cord and brain tissues. As expected, all three methods yielded high concentrations in the spinal due to proximity to the injection site. However, differences in distribution efficiency along the neuroaxis into the brain were observed. ASO concentrations in the brain - the primary target tissue - ranged between 0.47 to 7.58 ng/mg across the 3 methods. Notably, direct puncture methods resulted in higher brain concentrations compared to the catheter-based approach. Mean ASO concentrations in the caudal cortex were 4.91, 7.58, and 3.72 ng/mg for direct percutaneous puncture, direct puncture via surgery, and the catheter method, respectively. In the middle cortex, concentrations were 3.24, 4.21, and 2.12 ng/mg, and in the cerebellum, 4.97, 5.78, and 3.91 ng/mg respectively. Distribution consistency across animals was assessed using the coefficient of variation (CV). In the spinal cord (cervical, thoracic, and lumbar levels), the CV varied between 0.19 and 0.48 for the direct puncture methods (percutaneous and surgical approaches) while it was 0.41 to 0.75 for the catheter-based method. In the brain (caudal cortex, middle cortex, and cerebellum), the CV varied between 0.29 and 0.38 for the direct puncture methods compared to 0.59 to 0.61 for the catheter-based method. These findings suggest that both surgical and percutaneous intrathecal injection methods provide more reliable and uniform ASO distribution across CNS tissues compared to the catheter-based approach. Direct injection methods offered improved consistency and distribution into the brain. Additionally, transient clinical signs and body weight changes were observed in catheter implanted animals, likely related to the procedure itself. Clinical pathology changes indicative of inflammation were noted in groups receiving surgical and catheter-based dosing. Histopathological changes such as vacuolation and nerve fiber degeneration - consistent with class effects of intrathecally administered oligonucleotides (Berman Booty et al., 2023; Frazier, 2015) - were more prominent in catheter-implanted animals. **Conclusions:** These results are valuable for optimizing delivery strategies in nonclinical CNS-targeted oligonucleotide therapeutic development.

PS 3813 Antioxidant Effects of Partridgeberry Leaf Extract against Perfluorooctanesulfonic Acid (PFOS) and Methylmercury in *Caenorhabditis elegans*.

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Background and Purpose: Perfluorooctanesulfonic acid (PFOS) and methylmercury (MeHg) are environmental contaminants that co-occur in aquatic ecosystems. These compounds bioaccumulate as they move up trophic levels, posing significant risks to human and environmental health. PFOS is a synthetically fluorinated compound that was initially incorporated into products for its ability to resist both oil and water. Although it has been phased out of production due to adverse health effects, it persists in the environment, deemed a "forever chemical." Methylmercury

is a neurotoxic heavy metal that is produced when inorganic mercury enters aquatic environments and undergoes microbial methylation. It then dissolves in water and becomes concentrated in marine organisms. Despite their distinct chemical properties, both PFOS and MeHg exert their toxic effects in part by inducing oxidative stress. Oxidative stress arises from an imbalance between the production of reactive oxygen species (ROS) and the availability of antioxidants. Accumulation of oxidative stress has been implicated with the development of age-related neurodegenerative diseases. This project will explore the protective effects of naturally occurring antioxidants in partridgeberry (*Mitchella repens*) leaf extracts (PBE) against the combination of PFOS and MeHg toxicity in the nematode worm, *Caenorhabditis elegans*. Partridgeberry is a plant that is native to eastern North America and has an extensive history within the medicinal practices of Indigenous tribes. Historical records indicate Indigenous Americans treated pain associated with childbirth and rheumatism with tea created from their leaves. Our hypothesis is that if *C. elegans* are pre-treated with partridgeberry leaf extract before PFOS and methylmercury exposure, then there will be increased survival, increased mitochondrial function, and decreased oxidative stress. **Methods:** This hypothesis will be tested with dose-response survival curves, lifespan assays, quantification of total ROS and glutathione (markers of oxidative stress), and quantification of ATP (marker of mitochondrial function). Prior dose-response curves and oxidative stress experiments have determined that the 5% dose of PBE (LD20 value) has the most antioxidant benefit in *C. elegans*. Therefore, all experiments will be conducted on *C. elegans* in the following treatments with and without 5% PBE pretreatment: WT worms (control), MeHg (LD20 value), PFOS (LD20 value) and PFOS + MeHg (both LD20 values). WT worms will be synchronized before treatment to ensure that all worms are in the L1 life stage. Then, they will be pre-treated with 5% PBE for 30 minutes before treatment of either MeHg, PFOS, or the combination for 30 minutes. **Results:** Previous research in the Newell lab has determined PBE to have a significant polyphenolic content of 1432 ± 280 mg GAE/g. Additionally, PBE pretreatment followed by treatment of the known oxidant manganese chloride resulted in a two-fold decrease in the total ROS, increased glutathione levels, increased ATP production, and a 30% increase in lifespan. Through dose-response survival curves, we have confirmed an LD20 value of 1 µM for methylmercury and are in the process of confirming an LD20 value of 100 µM for PFOS. Our expected results are that PBE pretreatment followed by PFOS and/or methylmercury exposure will decrease total ROS, increase glutathione levels, increase ATP production, and increase *C. elegans* lifespan. **Conclusions:** This is the first study to assess PBE as an antioxidant in PFOS and MeHg-treated worms. The findings of this study could provide insight into how dietary factors could mitigate the toxic effects of PFOS and MeHg, with potential implications for guiding future public health recommendations.

PS 3814 Curating Developmental Neurotoxicity in the Toxicity Reference Database (ToxRefDB)

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Background and Purpose: The US Environmental Protection Agency (EPA) has regulatory authority to evaluate pesticides and industrial chemical substances in the United States, as statutorily required by the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and Toxic Substances Control Act (TSCA), respectively. Animal studies are often conducted as part of the chemical evaluation to assess potential hazard and for certain uses. To facilitate the reuse of data from legacy animal studies, the US EPA's Toxicity Reference Database (ToxRefDB) is a publicly available, structured data resource for *in vivo* toxicity information with over 6000 guideline or guideline-like human-relevant studies for over 1000 chemicals. ToxRefDB is largely comprised of curated animal study data from repeat dose studies conducted according to OECD and EPA Health Effects Series 870 guidelines, and many coming from reviews of pesticide registrant-submitted toxicity studies known as data evaluation records (DERs) from the U.S. EPA's Office of Pesticide Programs (OPP). Ongoing curation efforts since 2009 have expanded ToxRefDB to incorporate toxicity studies from additional sources, making ToxRefDB a premier resource for qualitative and quantitative animal toxicity information. Given ToxRefDB-applicable studies follow guideline specifications, any deviations from the guideline are noted, allowing missing (i.e., not tested) endpoints or negative (i.e., tested with no effect observed) effects to be inferred. A developmental neurotoxicity (DNT) study is a specialized toxicity study to screen for adverse effects of pre- and postnatal exposure on the development and function of the nervous system, focusing on the characterization of offspring survival, motor activity, auditory startle response, learning, memory, and brain pathology. The relative diversity of DNT study design variants presents a challenge in creating an adequate format to capture study data as well as apply a standardized vocabulary representing the unique outcomes contained across studies. Given the significant value in developing an aggregated resource of DNT studies that have been conducted, this work presents efforts to extract and collate DNT guideline and other DNT-relevant *in vivo* studies in the structured ToxRefDB format. **Methods:** An important component of ToxRefDB is its controlled vocabulary for studies and effects observed for enhanced data quality. Most terminology was standardized to better reflect the terminology used in the guidelines and study reporting. DNT studies are complex with the guideline requirement for observational and functional assessments collected at specified time points. Given the variations in assessment times and flexibility in tests that are permissible for consideration under the guideline, observations under the ToxRefDB controlled vocabulary often include required time periods associated with the neurological endpoint category.

With the controlled effect vocabulary established and appropriate studies identified, the ToxRefDB document extraction process utilized an application-based modular workflow for curating the heterogeneous and complex *in vivo* study designs. A multi-layer review process was implemented to ensure data integrity and minimize data entry error. **Results:** A major update in ToxRefDB v3.0 was to expand the controlled vocabulary and create a new guideline profile to curate DNT guideline-adherent and "non-guideline" DNT-relevant studies. ToxRefDB v3.0 (August 2025) includes 141 DNT and DNT-relevant studies on 96 chemicals. 9,640 total effects were extracted across 1,680 dose treatment groups. Types of curated effects are differentiated as treatment-related, statistically significantly different from the control group, or critical, deemed adverse by study reviewer. Within the subset of DNT and DNT-relevant studies, 3,194 treatment-related and 390 critical neurological effects were extracted. **Conclusions:** Capturing DNT study data in a relational database enables a systemic and interoperable review of chemical exposures at neurological endpoints. This data extraction effort produced an important resource to benchmark the performance of new approach methods, such as the DNT *in vitro* battery, to support predictive toxicology applications and reduce animal testing. *This abstract does not necessarily reflect U.S. EPA policy.*

PS 3815 High-Throughput Cytokine Detection Platform for Evaluation of Chemical Induced Microglial Activation

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Background and Purpose: Environmental chemical exposure is implicated in neurodegenerative disorders, primarily through neuroinflammation. This study aimed to identify and validate suitable *in vitro* microglial models for assessing cytokine responses to potential neurotoxins, with a specific focus on human induced pluripotent stem cell-derived microglia (hiMG). **Methods:** We evaluated the cytokine secretion profiles of four microglial cell types (hiMG, HMC3, IM-HM, and BV2) upon lipopolysaccharide (LPS) stimulation using cytokine arrays. Subsequently, we developed a homogeneous time-resolved fluorescence (HTRF) assay platform in a 1536-well plate format for high-throughput screening using hiMG cells. We then assessed the response activity of hiMG to various stimuli, including interferon gamma, catecholamine compounds, and nine environmental chemicals with documented cytokine-inducing potential. **Results:** Our findings showed that hiMG cells exhibited cytokine response patterns that most closely resembled *in vivo* conditions, displaying significant increases in interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) levels upon LPS stimulation, with TNF-α being uniquely expressed in hiMG. The HTRF assay demonstrated robust performance, with a 3.71-fold and 2.62-fold increase in the assay window for IL-6 and TNF-α secretion, respectively, after LPS treatment, yielding EC50 values of approximately 50 ng/mL and 90 ng/mL. All nine tested environmental chemicals stimulated IL-6 and TNF-α production, with three compounds (e.g., picoxystrobin) significantly stimulating both cytokines. **Conclusions:** This study successfully established a reliable high-throughput platform utilizing hiMG cells for detecting the inflammatory effects of environmental toxicants. This platform provides valuable insights into their neuroinflammatory potential and potential implications for neurodegenerative disorders.

PS 3816 Reducing animal use in rat fertility studies through use of historical control data and statistical methods

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Background and Purpose: This study evaluates the potential of replacing/reducing conventional control animals in rat fertility studies and improving statistical analysis by leveraging historical control data (HCD). **Methods:** This analysis was conducted using data sourced from a single test facility using consistent animal attributes and study procedures and consisted of 13 datasets from 12 rat fertility studies conducted between 2018 and 2023. For ICH guideline required fertility endpoints, we have evaluated full and hybrid Virtual Control Group (VCG) and Bayesian statistical approaches in comparison with standard statistics used for concurrent control groups (CCG). **Results:** Findings from this pilot study demonstrate that the sensitivity and specificity of most fertility endpoints were generally similar between CCG and full or hybrid VCGs. Bayesian analyses leveraging all available HCD offered similar or superior sensitivity and specificity to CCGs and VCGs (full or hybrid) for all fertility endpoints, with some exceptions. Retrospective implementation of these approaches on previously conducted fertility studies illustrated similar statistical performance across all approaches in addition to the benefit of reduced animal use. **Conclusions:** Overall, this effort illustrates the first steps to improve sensitivity and reduce animal use with VCG (full or hybrid) or Bayesian approaches for required fertility endpoints in the rat.

PS 3817 Ovarian Toxicity of Benzyl Paraben and Butyl Paraben

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Background and Purpose: Benzyl paraben (BzP) and butyl paraben (BuP) are chemicals that are used as preservatives and antimicrobial agents in cosmetics, pharmaceuticals, and foods. BzP and BuP have been detected in urine, placental tissue, and ovarian follicular fluid and are understudied with regards to their endocrine activity, raising concerns on their impact to the reproductive system. In this research we tested the hypothesis that BzP and BuP are toxic to the ovary and behave similarly to other structurally similar endocrine disrupting chemicals, such as phthalates and terephthalates, by altering hormone levels and impacting cell growth and gene expression. To investigate these concerns, we studied the impact of exposure to BzP and BuP on the mouse ovary with a particular interest in the development of follicles, the structures that contain developing oocytes and steroidogenesis, the process of steroid hormone synthesis in the ovary. **Methods:** We performed 96 hr *in vitro* studies in which follicles were dissected from ovaries of adult female CD-1 mice and cultured with vehicle control (DMSO) or 0.1-100 µg/mL of BzP or BuP. Follicle growth was measured every twenty-four hours. At the end of the culture period, media were collected to analyze steroid hormone concentrations and follicles were collected to analyze gene expression. We used enzyme linked immunosorbent assays (ELISA) to measure the hormones estradiol, testosterone, progesterone, and pregnenolone. We also used quantitative polymerase chain reaction (qPCR) to assess gene expression related to steroidogenesis, cell cycle, and apoptosis. **Results:** We found that 100 µg/mL BzP and BuP were highly cytotoxic to follicles. BzP at 1 µg/mL significantly increased levels of estradiol and BzP at 10 µg/mL also significantly increased levels of testosterone. BuP did not significantly alter hormone levels at or below 10 µg/mL. Fabbp4 and Cdkn1a were significantly upregulated from 10 and 1 µg/mL BzP, respectively. **Conclusions:** Our results suggest that BzP and BuP are cytotoxic to the ovary and disrupt hormone levels and signaling at doses below cytotoxicity, suggesting potential endocrine disruption. Further study should be done on the toxicity of these chemicals to ensure they are safe and sustainable for use.

PS 3818 The Effects of Short-term Exposure to Di-(2-ethylhexyl) phthalate (DEHP) and Diisononyl phthalate (DiNP) on Markers of Female Reproductive Aging

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Background and Purpose: Phthalates such as di-(2-ethylhexyl) phthalate (DEHP) and diisononyl phthalate (DiNP) are used as plasticizers in polyvinyl chloride plastics, flooring, food packaging, personal care products, and medical tubing. Phthalates are endocrine disruptors, which affect many hormonal functions. Humans are continuously exposed to phthalates via oral, dermal, and inhalation routes. Research suggests that environmental factors such as exposure to endocrine disruptors might accelerate the onset of female reproductive aging. Ovarian aging is characterized by oxidative stress, fibrosis, inflammation, inflammasome activation, and disrupted expression of telomere associated genes (TAGs). We previously found that long-term exposure to phthalates affects markers of reproductive aging. However, little was known about the long-term consequences of phthalate short-term exposure on markers of ovarian reproductive aging. Thus, the goal of this work was to test the hypothesis that short-term phthalate exposure has long-term effects on markers of ovarian aging. **Methods:** Young adult CD-1 female mice were orally dosed with vehicle, DEHP (20 µg/kg, 200 µg/kg, 20 mg/kg or 200 mg/kg), or DiNP (20 µg/kg, 100 µg/kg, 20 mg/kg, and 200 mg/kg) daily for 10 days. Mice were euthanized at 15 months post exposure and ovaries were collected for the assessment of markers of ovarian aging. **Results:** Exposure to DEHP at different concentrations (20 µg/kg, 20 mg/kg, and 200 mg/kg) altered the levels of 17 inflammation markers compared to control, with ICAM-1, IGFBP-6, and LDL affected at all DEHP concentrations. Additionally, DEHP exposure increased the expression levels of *Il-13*, but did not affect the expression of antioxidant enzymes or TAGs compared to control. Exposure to DiNP at different concentrations (20 µg/kg, 20 mg/kg, and 200 mg/kg) altered the levels of 16 inflammation associated molecules compared to control, with fetuin and LDL R affected at all tested DiNP concentrations. DiNP also increased the expression of *Il-13* and decreased the levels of the inflammasome associated molecule *Nlrp3* compared to control. Furthermore, DiNP exposure at the lowest concentration decreased the expression of the TAG *Terc*, whereas DiNP exposure at higher concentrations increased *Terc* expression levels compared to control. DiNP exposure did not affect the expression of antioxidant enzymes compared to control. **Conclusions:** In conclusion, short-term exposure to DEHP and DiNP have long-term effects on markers of ovarian aging. Specifically, DEHP and DiNP mainly target inflammation pathways and TAGs in the mouse ovary. R01 ES028661, R01 ES034112, T35 OD011145.

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Background and Purpose: Endocrine disrupting chemicals (EDCs) are compounds that interfere with hormone biosynthesis and may contribute to the development of chronic diseases such as cancer. The prostate, being a hormone-dependent organ, is particularly sensitive to the action of EDCs both during development and in maintaining homeostasis in adulthood. However, most studies evaluate the effects of EDCs in isolation, distancing themselves from actual patterns of human environmental exposure. Given this, this study characterized molecular changes in cell survival and death pathways and possible changes in collagen synthesis in the prostatic microenvironment of rats exposed, from intrauterine life to adulthood, to an environmentally relevant mixture of EDCs. **Methods:** Pregnant Sprague-Dawley rats were randomly divided into two experimental groups: Ctrl (vehicle: corn oil, by gavage) and EDC Mix (32.11 mg/kg/day of a mixture of 12 compounds — phthalates, pesticides, UV filters, bisphenol A, and butylparaben—diluted in corn oil, 2 mL/kg). The treatment was administered from gestational day 7 (GD7) to postnatal day 21 (PND21). After weaning (PND22), male offspring of the F1 generation continued to be exposed to the mixture until PND220, when they were euthanized (CEUA 8556/8559). The ventral prostate of the animals was collected, weighed, and stored at -80°C. Total RNA was extracted with TRIzol®, treated with DNase I, and reverse-transcribed into cDNA. The expression of the genes caspase 3 (*Casp3*), caspase 9 (*Casp9*), Bcl-2-associated X (*Bax*), B-cell leukemia/lymphoma 2 protein (*Bcl-2*), AKT Serine/Threonine Kinase 1 (*Akt*), phosphoinositide 3-kinase (*Pi3k*), mechanistic target of rapamycin (*Mtor*), collagen type I (*Col1a1*) and collagen type III (*Col3a1*), metalloproteinase 2 (*Mmp2*), metalloproteinase 9 (*Mmp9*), and metalloproteinase inhibitors 1 (*Timp1*) and 2 (*Timp2*) were quantified by RT-qPCR (endogenous reference gene: *Rpl30*) and analyzed by the comparative Delta Ct method. The data obtained were analyzed by parametric (Student's t-test) and nonparametric (Mann-Whitney, for *Pi3k*) statistical tests. **Results:** No statistically significant differences were observed in the expression of genes related to cell proliferation and apoptosis between the Ctrl and EDC Mix groups. Regarding collagen synthesis and deposition, no statistically significant differences were found between the control and EDC Mix groups, both in gene expression and MMP enzymatic activity. **Conclusions:** The findings suggest that prolonged exposure to the mixture of EDCs may not induce detectable changes in these molecular markers under the proposed conditions and analysis windows. The absence of effect may be related to post-transcriptional regulation, individual variability, adaptive response, and the temporality of gene expression. In addition, previous studies by the same research group identified a reduction in the proportion and total content of collagen fibers in the prostatic stroma under the same experimental model, as well as the presence of invasive adenocarcinoma, suggesting that other molecular mechanisms, such as epigenetic ones, may be involved in stromal remodeling and cell death and survival pathways not evidenced by the analyses performed in this study (Funding: FAPESP 2024/00912-4; 2018/24044-0).

PS 3820 **Phthalate Replacements Show Similar Effects as Phthalates on Gene Expression and Hormone Production in Mouse Ovarian Follicles**

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Background and Purpose: Phthalates are ortho-substituted phthalic acid esters that are used as plasticizers to make polyvinyl chloride plastics more flexible. Two of the most common phthalates are di(2-ethylhexyl) phthalate (DEHP) and benzyl-butyl phthalate (BzBP). After entering the body, DEHP and BzBP are broken down into monoester metabolites, mono(2-ethylhexyl) phthalate (MEHP) and monobenzyl phthalate (MBzP), respectively. Previous studies indicate that phthalate monoesters are endocrine disrupting chemicals, interfering with hormone production and signaling, which has spurred the use of structural isomers as replacements. However, there is a lack of research on if terephthalate alternatives show similar toxicity as the phthalates they replace. The purpose of this research is to compare the toxicity of phthalate monoesters to their para-substituted isomers to identify how ring substitution affects the toxicity of these chemicals. **Methods:** Ovarian follicles were collected from adult female CD-1 mice. The follicles were cultured for 96 hours in vehicle control or 100 µg/mL of MEHP, mono(2-ethylhexyl) terephthalate (MEHTP), MBzP, or monobenzyl terephthalate (MBZTP). After the culture period, the follicles were collected for analysis of gene expression related to hormone production, apoptosis, and cell growth and proliferation using quantitative polymerase chain reaction (qPCR). Genes were chosen because they were impacted by phthalate exposure in previous studies. Culture media were collected for measuring hormone levels of progesterone, estradiol, testosterone, and androstenedione using enzyme linked immunosorbent assays (ELISAs). **Results:** MEHP significantly impacted 16 of the

21 genes studied and MBZP affected 10 of the genes. In general, similar effects were seen between phthalate and terephthalate treatments, with some exceptions. In steroidogenesis genes, both MEHP and MEHTP downregulated Hsd17b1, along with MBZTP. MBzP and MBZTP also both upregulated gene expression for Star. In peroxisome proliferator-activated receptor (PPAR) controlled genes, MEHP and MEHTP both upregulated expression of Plin1. Additionally, both MBzP and MBZTP upregulated Fabp4. Terephthalates also showed unique toxicity for some genes. MEHTP was the only treatment to significantly upregulate gene expression of the PPAR gene Angptl4 and cell cycle genes Cdkn2b and Ki67. Similar effects were also observed for hormone levels. Both MEHP and MEHTP treatments decreased estradiol levels, and all treatment groups were found to significantly increase progesterone levels. **Conclusions:** Our findings suggest that though ring substitution may change the magnitude of phthalate effects, terephthalates show similar impacts on gene expression and hormone production. This is important because terephthalates are proposed as safer alternatives to phthalates in consumer products. Similar effects as phthalates means that terephthalates may also lead to endocrine disruption and health concerns, specifically for female reproductive health.

PS 3821 **Evaluation of Data Inclusion of Dams with No Viable Fetuses in Maternal Data Analysis in Rat Embryo-Fetal Development Studies**

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Background and Purpose: Dams with no viable fetuses (NVF, dams with all implantation resorptions or dead fetuses at necropsy) in rat embryo-fetal development (EFD) studies are not uncommon and the causes can be test article-related or incidental occurrences. Data interpretation of the EFD studies are commonly performed in two ways: excluding or including the NVF dams for maternal data analysis (e.g., body weight, food consumption). The debate of whether to include NVF dams in maternal data analysis has been focusing on the biological differences between dams with NVF and viable fetuses, however, comparison of data analysis results conducted by using study data with or without NVF dams has not been performed. **Methods:** This is a retrospective analysis of Rat EFD studies conducted at the Labcorp Greenfield location between 2018 to 2025. Rat EFD studies with NVF dams were selected to compare the data analysis results and the overall conclusion made with or without NVF individuals. Studies selected included NVF dams noted as incidental occurrences (e.g., in control, in treated, or in both control and treated groups), as well as treatment-related NVF dams. Maternal data were statistically analyzed using analysis of variance (ANOVA) with and without NVF dams for each study and results were compared. **Results:** Including or excluding NVF dams in maternal data analysis reached the same overall study conclusion on treatment-related effects. However, for studies with treatment-related NVF dams findings, including NVF dams in the maternal data analysis showed more impacted maternal data points (e.g., treatment-related reduced weight and/or food consumption) than excluding them. **Conclusions:** Because including NVF dams in the maternal data analysis provides the same conclusion as analysis conducted without NVF dams, but with more robust detection of treatment-related effects, including the NVF dams in the maternal data analysis for the EFD studies may enhance detection of treatment-related effects and is therefore suggested as a consideration.

PS 3822 **Investigating the Direct Effects of Phthalates on the Vaginal Microenvironment**

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Background and Purpose: The vaginal microenvironment is a critical component of female reproductive health, composed of epithelial, immune, and microbial elements that together maintain mucosal integrity, pH balance, and defense against infection. Under healthy conditions, *Lactobacillus*-dominated communities promote an acidic pH and mucosal homeostasis. In contrast, vaginal dysbiosis, defined by loss of *Lactobacillus* dominance and overgrowth of facultative anaerobes such as *Gardnerella vaginalis*, is associated with elevated risk of bacterial vaginosis (BV), sexually transmitted infections, preterm birth, and infertility. Recent epidemiologic evidence links exposure to endocrine-disrupting chemicals (EDCs), including phthalates found in feminine hygiene products, with higher incidence of BV and vaginal inflammation. Yet, the molecular mechanisms connecting phthalate exposure to epithelial dysfunction and microbial imbalance remain poorly understood. We hypothesize that phthalates disrupt the vaginal microenvironment through two mechanisms: (1) by activating inflammatory and stress-response pathways in vaginal epithelial cells, leading to weakened barrier integrity and altered cytokine signaling, and (2) by directly influencing the growth, metabolism, and acid-producing capacity of commensal and pathogenic bacteria. Together, these changes are predicted to shift the vaginal ecosystem toward a proinflammatory, less acidic, and dysbiotic state that may increase susceptibility to infection and reproductive complications. **Methods:** Human vaginal (VK2/E6E7) epithelial cell lines were cultured per ATCC guidelines and exposed to diethylhexyl (DEHP), dibutyl (DBP), diethyl (DEP), dimethyl (DMP), and diisobutyl (DiBP) phthalates, alone or as a mixture, at 0.001-1.0 µg/mL for 24 hours. Gene expression of proinflammatory and barrier-associated targets (IL-6, TNF-α, TGF-β1, occludin, and claudins 1-4) was assessed by quantitative RT-PCR,

and viability was confirmed via CCK-8 assay. Complementary bacterial assays were conducted with *Lactobacillus crispatus* (beneficial) and *Gardnerella vaginalis* (pathogenic), exposed to equivalent phthalate concentrations reflective of levels measured in feminine hygiene products. Bacterial growth and metabolic activity were quantified spectrophotometrically and through pH changes indicative of lactic acid production. **Results:** Phthalate exposure did not significantly alter epithelial viability but induced marked, cell-type-specific transcriptional responses. Vaginal epithelial cells (VK2) displayed significant upregulation of IL-6 and TNF- α , coupled with suppression of TGF- β 1 and tight junction transcripts (occludin, claudin-1), suggesting barrier weakening and proinflammatory activation. Cervical cell lines showed a blunted inflammatory profile, highlighting tissue-specific vulnerability. In bacterial assays, *L. crispatus* growth was significantly reduced following DEHP and DBP exposure, while *G. vaginalis* exhibited increased metabolic activity and pH elevation under the same conditions. These findings indicate that phthalates can directly impair beneficial bacteria while favoring opportunistic species, providing a plausible link between chemical exposure and dysbiotic microbial shifts. Together, epithelial and microbial results reveal convergent effects of phthalates on inflammation, barrier dysfunction, and microbial balance, three hallmarks of vaginal dysbiosis. Ongoing studies include quantifying cytokine protein expression, validating TEER and immunohistochemistry data (claudins, occludin), and testing mixed phthalate exposures for additive effects. **Conclusions:** These findings demonstrate that environmentally relevant phthalate exposures disrupt both epithelial and microbial components of the vaginal microenvironment. Direct exposure upregulates inflammatory mediators, reduces structural barrier gene expression, and selectively alters bacterial growth dynamics, collectively supporting a model where EDCs promote dysbiosis and impair reproductive mucosal health. This work establishes an *in vitro* framework for understanding the mechanisms linking phthalate exposure to vaginal inflammation and microbial imbalance, with implications for reducing EDC exposures in women's health products and mitigating reproductive health disparities.

PS 3823 Polycyclic Aromatic Hydrocarbons Disrupt Placental Gene Regulation in a Sex-Dependent Manner

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Background and Purpose: Our laboratory investigates mechanisms behind the increased risk of preterm birth (PTB) with environmental exposures. In this study, we utilized transcriptomics to determine how exposure to polycyclic aromatic hydrocarbons (PAHs) alters gene expression in primary cultured trophoblasts (ie placental cells) and what pathways are disrupted that may contribute to an increased risk of PTB. **Methods:** Primary trophoblasts were isolated and cultured from Cesarean delivered placentae within one hour of delivery (N=7, 4 female and 3 male). Cells were cultured for 48 hours after which cells were either treated with DMSO as a control, or 10uM of Benzo(a)pyrene for 24 hours. RNA was isolated and bulk RNA-seq was performed. Samples were pre-processed using the nf-core/magseq (v3.18.0) pipeline. Reads were aligned to the human reference genome (GRCh38, Ensembl release 111) using STAR. Differential expression analysis employed DESeq2 (v1.46.0) with apeglm log fold change shrinkage. Genes were considered differentially expressed at Benjamini-Hochberg adjusted p-value < 0.05 and |log2FC| > 0.6. Gene set enrichment analysis was performed using clusterProfiler (v4.14.4), MSigDB (v7.5.1) and ReactomePA (v1.50.0) against Gene Ontology Biological Processes, KEGG, Reactome, MSigDB Hallmark, and Disease Ontology databases. Statistical significance for pathway enrichment was determined using q-value < 0.05. All analyses were performed in R (v4.4.2) within Bioconductor (v3.20) docker container. **Results:** Significant sex-specific differences were observed in the transcriptomic response to PAHs in cultured primary human cytotrophoblasts. The female placental cytotrophoblasts exhibited a more robust transcriptomic response, characterized by a greater number of differentially regulated genes, with minimal overlap with the male cells. With BaP treatment, trophoblasts from female placentae had 205 unique genes that were differentially expressed that were not altered in trophoblasts from male placentae, compared with only 8 genes altered in the trophoblasts from male placentae. Biological pathways related to xenobiotic metabolism and detoxification were positively enriched, while pro-inflammatory pathways were negatively enriched in the female cytotrophoblasts. We also observed higher expression of the aryl hydrocarbon receptor (AHR) in female cytotrophoblasts compared with male (2.8-fold, p=0.047), suggesting a sex-specific regulatory role of this pathway. **Conclusions:** These findings highlight sex-specific mechanisms in placental responses to environmental toxicants and their potential contribution to disparities in PTB risk.

PS 3824 The Current Situation in Development and Reproductive Toxicity Evaluation of Pharmaceuticals using Non-Human Primates in Japan

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Background and Purpose: In recent years, increasing number of biopharmaceuticals have been developed. On the aspect of the binding affinity to target molecules, non-human primates (NHPs) are often used as relevant species for non-clinical safety evaluation. On the other hand, due to the ethical concern about animal testing, the use of NHP is being reconsidered. **Methods:** In this study, we investigated non-clinical studies using NHPs for development and reproductive toxicity (DART) evaluation of 72 products newly applied for marketing approval in Japan from 2014 to 2024. We mainly examined fertility assessment, study design of ePPND/EFD studies, relationships between the ePPND/EFD study results and mode-of-action (MoA). **Results:** Most of the products using NHPs for DART assessment were therapeutics antibodies. The endpoints for fertility evaluation were usually histopathological examination of the reproductive organs. While in some products, detailed assessment such as the measurement of hormones was conducted. As for ePPND/EFD studies, MoA-related endpoints were often confirmed and most of the findings observed in those studies were related to MoA or its secondary effects. **Conclusions:** The DART evaluation using NHPs are usually limited to the products with high species specificity and those products are rare to induce off-target toxicity. Therefore, the Weight of Evidence approach such as MoA based risk assessment for DART should be considered before animal testing in the balance of the existing data adequacy.

PS 3825 From Development to Ovarian Failure How the Tobacco Smoke Chemical Benzo[a]pyrene Disrupts Egg Quality in Mice

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Background and Purpose: Benzo[a]pyrene (BaP) is a reproductive hazard released as a by-product from the incomplete combustion of organic materials such as cigarettes, fossil fuels, wood, or grilled foods. BaP is highly detrimental to ovarian development. The ovarian reserve, or the finite pool of oocytes within the ovary, is established from primordial germ cells (PGCs) during development. Published work from the Luderer laboratory demonstrated prenatal exposure to BaP significantly reduced the number of germ cells, leading to premature ovarian failure, increased oxidative stress in surviving eggs, and reduced fertility in adulthood. The expression of meiosis-related genes in fetal PGCs, including *Tex12*, *Sycp1*, *Sycp2*, *Syce1*, *Stag3*, and *Dmc1* which transcribe proteins needed for the synaptonemal complex and DNA repair pathways that drive homologous chromosome crossing over, was also decreased. The meiotic spindle consists of microtubules that attach to chromosome kinetochores to ensure that each egg receives half of the chromosome set in which euploidy is reestablished upon fertilization. The meiotic window plays a pivotal role in preserving egg quality and genetic integrity. We hypothesized prenatal exposure to BaP would significantly reduce the quantity and quality of surviving oocytes by disrupting egg chromosome alignment, spindle quality, and ploidy. **Methods:** To test this hypothesis, we exposed pregnant F0 dams to 0, 0.033, or 2 mg/kg/day of BaP from 6.5-15.5 dpc which targets the mitotic proliferation window of F1 PGCs and the initiation of meiosis in oocytes. The 10-days of the 0.033 mg/kg/day BaP dose is equivalent to one-third of the cumulative mutagenic PAH dose to a highly, non-occupationally exposed woman throughout pregnancy after allometric species conversion. The 2 mg/kg/day dose is at the low end of doses of BaP used in published toxicological studies. The F1 generation were aged to post-natal day (PND) 35-45 when they were primed with 5IU of pregnant mare serum gonadotropin (PMSG) and superovulation was induced with 5IU of human chorionic gonadotropin hormone (hCG). Eggs were assessed for the following characteristics: a small perivitelline space (PVS), spherical shape, and the presence of a polar body. Eggs missing one or more of these characteristics were labeled as abnormal. Eggs were fixed and stained with antibodies against CREST, alpha-tubulin, and DAPI which labeled kinetochores, spindles, and chromosome structures within. Z-stack images of eggs were obtained, and spindle length, metaphase plate length, spindle volume, chromosome alignment, and ploidy were assessed using IMARIS software. **Results:** Corroborating previous results, the number of eggs collected from BaP-exposed mice did not differ from control (GEE, p>0.05). The prevalence of abnormal eggs (Kruskal Wallis, p=0.007), spindle length (GEE, p=0.005), and spindle volume (GEE, p=0.002) were significantly increased in BaP-exposed mice compared to control mice. Compared to control mice, percent aneuploidy was significantly higher in mice exposed to 0.033 mg/kg/day BaP (Kruskal Wallis, p=0.034). Chromosome alignment and metaphase plate width was not altered by BaP exposure (GEE, p>0.05). **Conclusions:** Prenatal BaP exposure compromised egg quality by increasing abnormal egg and spindle characteristics that are implicated in reduced developmental competence. This exposure also undermines the genetic integrity of eggs at a human relevant dose, which raises an important question of BaP effects on women's long-term reproductive, maternal, and prenatal health.

PS 3826 Chronic exposure to selenium through natural diets causes endocrine disruption and reproductive toxicity in zebrafish (*Danio rerio*): a trophic transfer study

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Background and Purpose: Aquatic ecosystems face increasing threats from contaminants linked to human activities, particularly the release of selenium from mining processes. While selenium is vital for essential physiological functions, its excessive accumulation poses significant toxicity risks. Although much is known about the developmental and neurotoxic implications of selenium in fish, its effects as an endocrine disruptor during realistic dietary exposure remain inadequately explored. **Methods:** In this pivotal study, we exposed the benthic oligochaete *Lumbriculus variegatus* to selenium, as selenomethionine at concentrations of 0, 50, and 200 µg/L over 14 days. The resulting tissue burdens were 0.65, 17, and 43 µg/g dry weight, respectively. Following this exposure, the worms were fed to adult zebrafish for 60 days, allowing us to assess a range of critical reproductive and endocrine parameters. **Results:** Our findings revealed no significant differences in body length or weight among the groups. However, marked increases in Gonadosomatic index were observed in female zebrafish exposed to Se, while Hepatosomatic index was elevated in both sexes at the highest exposure level. Notably, reproductive performance suffered, as indicated by decreased fecundity, smaller egg diameters, and diminished sperm motility and velocity. At the molecular level, dietary selenium disruption affected the hypothalamic pituitary gonad axis. Females exhibited a down-regulation of *gnrh*, *fshb*, *fshr*, *cyp19b*, *vtg*, and *era*, whereas males displayed mixed responses, including upregulation of *vtgr*, *cyp11a*, and *3βhsd*. Circulating estradiol (E2) levels decreased in females and the highest Se-treated males. Additionally, while 11KT levels remained stable in females, a decline was noted in males at the 17 µg/g Se-exposed group. Notably, vitellogenin (Vtg) levels decreased in females but increased in males. Histopathological assessments uncovered significant ovarian adhesion and oocyte degeneration in females, along with degeneration of Sertoli and Leydig cells in males. **Conclusions:** These results underscore that chronic dietary exposure to environmentally relevant selenium levels disrupts endocrine regulation, impairs gonadal development, and severely limits reproductive success in zebrafish. This research highlights the urgent need to address the ecological risks posed by selenium contamination in aquatic ecosystems.

PS 3827 Parental and Postnatal NDMA Exposure Alters Reproductive and Toxicological Profiles in F1 Rats

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Background and Purpose: Environmental and lifestyle habits are among the factors that contribute significantly to infertility worldwide. The Developmental Origins of Health and Disease (DOHaD) and Paternal Origins of Health and Disease (POHaD) hypotheses explore how parental health influences offspring development. Nitrosodimethylamine (NDMA) is a harmful environmental contaminant found in water, food and some drugs, with mutagenic, genotoxic and carcinogenic effects, promoting the formation of reactive oxygen species. Data on reproductive and developmental studies is limited, especially at low doses. The aim of this study was to assess the effects of parental (Maternal-M, Paternal-P, and MP-) and postnatal NDMA exposure on toxicological and reproductive outcomes in male and female F1 rats. **Methods:** The animals were exposed to control (distilled water, 1mL/kg/day) or to 7.2 ng/kg/day of NDMA. The F0 were divided into 4 groups: C- (control males X control females); M- (NDMA females X control males); P- (control females X NDMA males); and MP- (NDMA females X NDMA males), exposed from postnatal day (PND) 60 until mating, with continued exposure in females during gestation and lactation. Offspring were exposed from PND 22 until the end of sexual maturation, with 1 pup/sex/litter exposed to the vehicle (groups -) and the other to NDMA (groups +). The F1 offspring were evaluated for reproductive and toxicological parameters in adult life. **Results:** In male offspring sexual behavior, a reduction in the first mount and first intromission latency in the C+ and M+ groups was observed. No significant alterations were found in organ weight, hematological parameters, fertility test, sperm morphology/motility and Leydig cell count. In biochemistry analysis, elevated serum creatinine (P-), elevated urea (P-, C+, M+, P+ and MP+) and reduced total proteins (MP- and M+ groups) were found. In the staging of seminiferous tubules, there was a decrease in the percentage of stages I-VI (MP-, C+ and MP+) and an increase in stages VII-VIII (C+). Glutathione (GSH) and superoxide dismutase (SOD) were reduced in the testis (MP+), while the Malondialdehyde (MDA) showed high levels (MP-). In female offspring, no alterations were found in estrous cycle, sexual behavior, organ weight, hematological parameters or follicle count. However, there was a decrease of alkaline phosphatase (P- and P+) and an increase in gamma glutamyl transferase (M+). Furthermore, in the fertility test, females in the MP- group had a reduction of uterus + fetuses weight, number of implantations and live fetuses. SOD was reduced in the M-, P-, MP-, C+ and M+ groups, while catalase (CAT) and GSH were reduced in the P+ group. **Conclusions:** Low-dose NDMA exposure caused biochemical and oxidative stress alterations in offspring, with mild reproductive

effects, particularly after combined parental exposure. These results support the DOHaD/POHaD concepts and highlight the sensitivity of the reproductive system to even low environmental NDMA levels.

PS 3828 Comparative Toxicogenomics Database Analysis: Relevant Environmental Toxicant Mixtures and Preeclampsia

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Background and Purpose: Puerto Rico, a region characterized by a high concentration of Superfund sites and subsequent environmental chemical exposures, also exhibits a high rate of preeclampsia. Given the limited available information on how environmental toxicant mixtures impact preeclampsia, we sought to leverage publicly accessible data to analyze genes, molecular mechanisms and biological events linking relevant environmental toxicant mixtures with preeclampsia. **Methods:** Using the Comparative Toxicogenomics Database (CTD), a database of curated chemical-gene, chemical-phenotype, and chemical-disease associations, we prioritized a set of 70 chemicals found in maternal blood/urine samples as well 40 additional parent chemicals within the pregnant women enrolled in PROTECT (Puerto Rico Test site for Contamination Threats) study. We used CTD's statistical tools to test the curated chemical-disease associations and identified 8 chemicals, including heavy metals, phthalates and perfluoroalkyl substances with a direct marker or mechanistic relationship with preeclampsia. Through a comprehensive approach involving curated chemical-gene interaction analysis and CTD's set analyzer, we examined correlations of the 8 identified chemicals and their interactions with genes. Subsequently, we conducted pathway enrichment analysis on the resulting 29,009 chemical-gene interactions. **Results:** Among the statistically enriched pathways (p-value <0.01) for imputed genes, 16 pathways overlapped with the enriched pathways associated with the disease outcome of preeclampsia, including pathways such as: cytokine signaling in the immune system, extracellular matrix organization and biological oxidations. We utilized the integration of five types of curated interactions within the CTD to establish Chemical-Gene-Phenotype-Disease (CGPD)-tetramers (a 4-unit block of information connecting chemical-gene interactions with phenotypes and diseases). This strategy generated 843 CGPD-tetramers involving the 8 environmental toxicants associated with preeclampsia. The identification of 213 phenotypes and 21 genes shared within these CGPD-tetramers suggests crucial mechanistic roles for cellular proliferation, glucose homeostasis and inflammatory response. **Conclusions:** Our analysis demonstrates biological plausibility for environmentally relevant mixtures of toxicants to dysregulate key biological pathways, potentially contributing to preeclampsia/hypertensive disorders during pregnancy. These findings provide a rationale for future experiments in animal tissue or cell models testing diverse mixtures of toxicants.

PS 3829 Effect of *in Utero* Exposure to the Cannabis Compounds Tetrahydrocannabinol (THC) and Cannabidiol (CBD) on the Developing Fetal Gonads

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Background and Purpose: There is a growing rise in the use of cannabis and cannabis related products after its legalization in many states. As a result, cannabis use is widely accepted, but studies of its effects on *in utero* development are limited. Studies have shown that in adult male rats, cannabis sativa exposure decreased sperm counts, and in female mice low dose tetrahydrocannabinol (THC) exposure during adolescence decreased ovarian follicle reserve. Much of the focus has been around adolescent to adult exposure, but there is also a growing rise in the percentages of expectant mothers who using cannabis or cannabis related products during pregnancy. However, the impacts of exposure to cannabis during pregnancy on the developing fetal gonads is poorly understood. **Methods:** Pregnant female C57BL/6J mice were exposed by intraperitoneal injection to THC (5mg/kg), CBD (20mg/kg) or a vehicle dose (5% Tween 80 and 95% saline) from E6.5 to E16.5. Mice were sacrificed 24 hours after last injection. Fetal testes and ovaries (E17.5) were harvested, immediately fixed in 4% PFA for 3 hours and embedded in OCT. Embedded fetal gonads were cryosectioned at 7 micron thickness, and immunofluorescence was performed labeling germ cells using GCNA antibody and the cannabinoid receptor 1 (CB1R) using a CB1R antibody. **Results:** Sex dependent differences were found with fetal ovaries expressing no CB1R at E17.5, while fetal testes did express CB1R, confined only at the germ cells. For the testes, the percentage of germ cells positive for CB1R normalized to germ cell-specific marker (TRA-98) positive cells revealed no difference in the average percentage between treatment groups. **Conclusions:** Preliminary results reveal that there is a sex dependent difference of CB1R expression between male and female fetuses at E17.5. CB1R expression in the testes was confined to only the germ cells, emphasizing the role CB1R plays in testicular development at E17.5. Ongoing studies will look further into the expression of CB2R, germ cell apoptosis, and critical enzymes of the endocannabinoid system. These results will provide better information about fetal gonadal development and whether cannabis exposure *in utero* may affect the fetus' fertility in adulthood.

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Background and Purpose: Microplastics (MPs) and nanoplastics (MNPs) have become pervasive contaminants in the environment, detected in air, water, and soil worldwide. Emerging studies reveal that plastic particles can infiltrate multiple human organs, suggesting possible systemic health risks. However, evidence linking environmental microplastic exposure to reproductive outcomes remains limited. This study aimed to evaluate (1) the presence and composition of MPs in canine testes and human semen, (2) their potential associations with sperm quality parameters, and (3) the translational implications of environmental microplastic accumulation for male reproductive health. **Methods:** Freshly excised testes and epididymides were collected from 37 adult dogs (1-9 years old) undergoing routine neutering procedures at local veterinary clinics. Microplastic particulates were visualized using micro-Raman spectroscopy and quantified using pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) for 12 common plastic polymers, including polyethylene (PE), polypropylene (PP), polystyrene (PS), polymethyl methacrylate (PMMA), polyvinyl chloride (PVC), polyurethane (PU), polyethylene terephthalate (PET), nylon 6 (N6), and nylon 66 (N66). Testes were stratified into high- and low-MP burden groups, and sperm parameters—concentration, motility, viability, and morphology—were analyzed using computer-assisted sperm analysis (CASA). Lipid peroxidation was assessed by quantifying malondialdehyde (MDA) adducts in epididymal sperm. Parallel analyses were performed on semen samples collected from 16 men (aged 28-52 years) attending a fertility clinic. Samples were assessed for semen quality following WHO 6th Edition guidelines. Py-GC/MS quantified MNP polymers in whole semen and in fractionated samples (seminal plasma vs. sperm cell fraction). **Results:** In canine testes, Raman spectroscopy confirmed plastic particles (5-20 µm) identified as PS, PET, PFA, and LDPE. Total polymer concentrations ranged from 2.37 to 485.7 µg/g tissue, with PE, PVC, N66, SBR, and PMMA being predominant. Testes with higher MP loads (mean = 99.7 ± 14.2 µg/g) exhibited significantly elevated lipid peroxidation in sperm (MDA = 0.344 ± 0.04 µM/mg protein vs. 0.255 ± 0.04 µM/mg protein, $p < 0.05$) and altered motility kinetics, while sperm count and morphology remained unchanged. Elevated cadmium and cobalt levels were also associated with high-MP tissues, suggesting co-contamination effects. In human semen, MNPs were detected in all 16 samples, ranging from 10.79 to 334.30 µg/ml (mean = 116.69 ± 94.78 µg/ml). PE and PU were the most frequent polymers. Approximately 70% of MPs were located in the seminal plasma fraction, while sperm cell fractions contained minimal detectable levels. Although no statistically significant correlation was observed between total MNP concentration and standard semen indices, eight samples displayed at least one subnormal WHO parameter, indicating a potential subclinical impact. **Conclusions:** This combined canine-human study provides the first cross-species evidence of microplastic accumulation within mammalian male reproductive tissues. The findings demonstrate that microplastics are widespread in both dog testes and human semen, encompassing multiple polymer types and concentration ranges. Elevated microplastic burdens were associated with increased oxidative stress and altered sperm motility in dogs, while universal detection in human semen highlights the pervasiveness of exposure. Together, these results suggest that environmental microplastics may represent a previously unrecognized risk factor for male reproductive dysfunction. Future research should integrate larger human cohorts, mechanistic *in vitro* assays, and exposure modeling to determine causal relationships, evaluate co-exposure effects with heavy metals, and inform strategies for reproductive health protection.

PS 3831 Co-exposure to Polystyrene Nanoplastics and Phthalate Metabolites Mixture Disrupts Ovarian Cell Cycle and Oxidative Balance in Neonatal Ovaries *In Vitro*: A DOHaD Perspective

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Background and Purpose: The increasing production and pollution of plastics raise concerns due to their harmful effects on the environment and human health. In the environment, plastics degrade into microplastics (<5 mm) or nanoplastics (≤100 nm) that can reach humans and cause health effects. Plastic additives such as phthalates, classified as endocrine-disrupting chemicals (EDCs), are released into the environment and could attach to the hydrophobic surfaces of nanoplastics, leading to bioaccumulation and biomagnification. The ovary is essential for female reproduction, responsible for folliculogenesis, oogenesis, and hormone synthesis. Females are born with a finite number of primordial follicles, the follicular reserve, which determines fertility throughout life. Alterations in this reserve can lead to premature ovarian aging, characterized by a decline in fertility potential with age or premature ovarian failure. The Developmental Origins of Health and Disease (DOHaD) proposes that events occurring during early development determine susceptibility to diseases later in life. The establishment of the primordial follicle pool is closely related to the DOHaD concept, as it occurs during late gestation in humans and around post-

natal day (PND) 3 in mice. Establishment of the primordial follicle pool depends on tightly regulated cell cycle, oxidative stress, apoptosis, and hormonal signaling that ensures the survival of a reproductive reserve with optimal long-term fertility potential. Previous studies have demonstrated the effects of phthalates on reproductive health, highlighting the importance of investigating environmentally relevant doses and mixtures. Recent studies have also shown that nanoplastics can reach and accumulate in the ovary, inducing oxidative stress, apoptosis, and disrupting ovarian homeostasis. However, limited information is available regarding the effects of exposure to nanoplastics alone or in combination with phthalates on the neonatal ovary during follicular reserve establishment. **Methods:** Therefore, this study evaluated the effects of exposure to polystyrene nanoplastics (PS-NPs, nominal size 100 nm) alone or in combination with a phthalate metabolite mixture (MM) on neonatal mouse ovaries *in vitro*. Ovaries from CD-1 mice on PND4 were collected and cultured for 96 hours with vehicle control, PS-NPs (5 - 100 µg/mL), MM (0.01 - 10 µg/mL), or the combination of PS-NPs + MM at lowest and highest concentrations. Doses were based on follicular fluid levels and dose-response evaluation. The MM was based on the proportions of phthalate metabolites in the urine of pregnant women. After 96 hours, ovaries were collected (three pooled per replicate) and processed for RT-qPCR analysis ($n = 5-6$). **Results:** The exposure to PS-NPs or MM alone did not significantly affect the expression levels of the analyzed genes. However, co-exposure to PS-NPs and MM increased the expression of *Ccnd2* ($p = 0.001$) and *Cdk6* ($p = 0.038$) at the lowest combined dose compared with the control, indicating activation of cell cycle progression related to the G1/S phase transition and possibly enhanced activation of primordial follicle proliferation. Moreover, *Esr2* ($p = 0.03$) and *Amh* ($p = 0.03$) expression levels were also increased at the same combined dose, suggesting potential defects in follicular recruitment, as *Amh* is expressed by growing follicles and *Esr2* has been associated with the maintenance of the follicular reserve. At the highest combined doses, co-exposure to PS-NPs and MM decreased *Cdkn1c* ($p = 0.04$) and increased *Lamp1* ($p = 0.04$) expression compared with the control, suggesting facilitated cell cycle progression and alterations in lysosomal membrane integrity. Also, co-exposure to PS-NPs and MM decreased *Hmox1* ($p = 0.005$ and $p = 0.0009$) expression at both combined concentrations, indicating an impact on oxidative stress balance. **Conclusions:** These findings suggest that early-life co-exposure to PS-NPs and MM may disrupt ovarian development and compromise future reproductive health, which is consistent with the DOHaD concept.

PS 3832 Long term exposures to di(2-ethylhexyl) phthalate and diisononyl phthalate alter the Nrf2 signaling pathway in the mouse ovary

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Background and Purpose: Di(2-ethylhexyl) phthalate (DEHP) and diisononyl phthalate (DiNP) are plasticizers used in a wide range of consumer products including building materials, food and beverage containers, and children's toys. This is concerning because DEHP and DiNP can leach out of products and enter the human body. Furthermore, both short-term and long-term exposures DEHP and DiNP have been shown to exhibit endocrine disrupting abilities and lead to early reproductive aging in animal models. However, little is known about the mechanisms underlying phthalate-induced reproductive aging. Previous studies indicate that increased oxidative stress can accelerate the loss of ovarian follicles and lead to reproductive aging. Thus, this study tested the hypothesis that DEHP and DiNP increase oxidative stress in the mouse ovary. Specifically, this study focused on the effects of DEHP and DiNP on the Nrf2 signaling pathway because Nrf2 is a transcription factor that is responsible for regulating the expression of antioxidant proteins such as glutathione peroxidase, glutathione S-transferases, NAD(P)H quinone dehydrogenase 1, and peroxiredoxin-1. **Methods:** To test this hypothesis, female CD-1 mice aged 39-40 days were dosed through the chow with either vehicle control (corn oil), DEHP (0.15, 1.5, 1500 parts per million (ppm)), or DiNP (0.15, 1.5, 1500 ppm) for 1, 6, 9, 12, and 15 months. The ovaries from these mice were then collected immediately post-dosing and subjected to quantitative polymerase chain reaction analysis of the expression of factors in the Nrf2 signaling pathway. **Results:** At 1 month post exposure, DEHP and DiNP at 1500 ppm significantly decreased the expression of NFE2 like bZIP transcription factor 2 (*Nfe2l2*), the gene that encodes the protein NRF2, when compared to control. At 6 months post exposure, DEHP and DiNP at 1500 ppm significantly increased the expression of *Nfe2l2* when compared to control. At 9 months post exposure, DEHP at 0.15 ppm and 1.5 ppm and DiNP at 1.5 ppm and 1500 ppm significantly increased the expression of *Nfe2l2* when compared to control. At 15 months post exposure, DEHP at 0.15 ppm and 1.5 ppm and DiNP at 1.5 ppm and 1500 ppm significantly increased the expression of *Nfe2l2* when compared to control. **Conclusions:** Collectively, our data indicate that DEHP and DiNP exposure significantly affect the Nrf2 signaling pathway in the ovary. Given that the Nrf2 signaling pathway is crucial for regulating oxidative stress in the ovary, these data suggest that long term exposure to DEHP and DiNP can significantly affect oxidative stress pathways in the ovary.

PS 3833 **Febuxostat Has A Protective Effect On Maternal Nano-Titanium Dioxide Inhalation Induced Placental Glycogen Changes in Female Pups**

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Background and Purpose: Increased placental glycogen content during pregnancy is linked to gestational complications such as diabetes, preeclampsia, and intrauterine growth restriction in humans. Previously, we have shown that maternal inhalation exposure to nano titanium dioxide (nano-TiO₂) during gestation in rats impacts litter size, pup and placental mass, and increases labyrinth zone area and decreases junctional zone area. Additionally, we have investigated the role of xanthine oxidase (XO) in these mechanisms, and found that XO inhibition via febuxostat (FBX) has some protective effects against nano-TiO₂. Thus, we wanted to explore the possibility that glycogen storage is affected by FBX treatment during critical periods of placental development. We hypothesized that placental glycogen content would be elevated in the nano-TiO₂ exposed placentas compared to controls, and that FBX-treatment would restore glycogen levels to resemble control dams. **Methods:** Pregnant Sprague Dawley rats were randomly assigned to either sham-control or nano-TiO₂ groups. Dams were exposed (nano-TiO₂ concentration = 12 mg/m³; HEPA-filtered air 25 ml/min) for 6 hrs/d for 6 d between gestational day (GD) 10-19 before sacrifice on GD 20. Within those groups, rats were further assigned to early gestation FBX treatment (50 mg/L; GD 1-19), mid-gestation FBX treatment (50 mg/L; GD 13-19), or no FBX treatment. One male and one female placenta was taken from each dam fixed with paraformaldehyde, frozen, sectioned via cryostat at 10µm, and stained with Periodic acid-Schiff (PAS). ImageJ analysis was used to identify the following four parameters; average glycogen deposit area, average number of glycogen deposits, total glycogen area, and percent glycogen area by total tissue area. **Results:** Average glycogen deposit area was significantly increased in the female nano-TiO₂, mid-FBX exposed group compared to the female nano-TiO₂, early-FBX group (46144.11 ± 17406.69 AU vs. 11865.15 ± 1513.78 AU). Average number of glycogen deposits was also significantly increased in the female nano-TiO₂, no FBX treatment exposed placentas compared to female sham-control, no FBX treatment group (6.44 ± 3.42 vs. 1.78 ± 0.76). Finally, total glycogen area was significantly increased in the female nano-TiO₂, no FBX treatment group, compared to the female sham-control, no FBX treatment group (169372.67 ± 85206.07 AU vs. 58334.56 ± 14645.05 AU). **Conclusions:** These results highlight that nano-TiO₂ increases placental glycogen levels at a critical time point during the end of gestation, and FBX plays a modestly protective role, especially in female pups. The cellular mechanisms by which increased glycogen affects maternal and fetal health, specifically fetal growth, and how FBX modulates this response warrants further investigation.

PS 3834 **Alterations in early embryonic development by exposure to the pesticide temephos in male rats.**

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Background and Purpose: Temephos is an organophosphate pesticide widely used in Mexico and worldwide to control mosquito larvae that carrier diseases such as dengue, Zika, and chikungunya. The World Health Organization (WHO) classifies temephos in Category III (slightly toxic) and considers its use in drinking water safe. The Environmental Protection Agency (EPA) classifies temephos as moderately toxic. However, several studies have reported that temephos causes reproductive adverse effects in males, including decreased fertilization rate. This study aimed to evaluate the effect of temephos exposure in male rats on early embryonic development. **Methods:** Male Wistar rats were exposed to doses of 10 or 100 mg/kg/day/5d by gavage (WHO-NOAEL and LOAEL, respectively). Temephos was emulsified with saline solution (0.9% NaCl; vehicle). The euthanasia was performed 24 h post-treatment and spermatozoa were collected from the cauda epididymis by deferens. Female rats not exposed to temephos were used and superovulated by intraperitoneal injection of 30 IU of eCG hormone, followed by 50 IU of hCG hormone 50 h later. Euthanasia was performed 24 h after the hCG injection, the ovaries were retrieved, and the cumulus-oocyte complexes were collected. Fertilization was performed under *in vitro* conditions (in HTF medium supplemented with 4% BSA/37 °C/5% CO₂/5 h); for embryo development, the cells were transferred to KSOM medium + 4% BSA for 120 h under similar incubation conditions. **Results:** The dose of 10 mg/kg/d/5 d did not cause changes in the fertilization rate compared with the control group; however, it showed an increase in the number of cells arrested at the 8-cell stage. While exposure to 100 mg/kg/day for 5 days of temephos reduced the fertilization rate by 25%, showing a significant increase in undivided cells and a 74% decrease in morula stage cells. Embryos from the control group reached the blastocyst stage with a median value of 8.12%; however, embryos from the treated group (100 mg/kg/day) did not show blastocysts. **Conclusions:** These results suggest that exposure of male rats to doses corresponding to the NOAEL and LOAEL affect early embryonic development. Further studies are needed to elucidate the mechanism by which the larvicide temephos causes these developmental effects.

PS 3835 **Exposure to a Phthalate Mixture Decreases Ovulation Rates, Impairs Steroidogenesis, and Alters Angiogenesis In Vivo**

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Background and Purpose: Phthalates are ubiquitous, endocrine-disrupting chemicals that people are exposed to daily. They are rapidly metabolized to monoester metabolites, which are distributed throughout the body and have been shown to reach the ovary. Previous work in our laboratory and others have shown that exposure to phthalates impairs steroidogenesis, alters angiogenesis, and decreases ovulation rates in cultured mouse antral follicles *in vitro*. Because of this, we hypothesized that mice exposed to phthalates *in vivo* would have decreased ovulation rates via potential mechanisms involving disrupted steroidogenesis and angiogenesis. **Methods:** For these experiments, we utilized a mixture of six phthalate metabolites (MPTmix) that was derived using urinary phthalate levels in women. Mice were injected intraperitoneally (IP) with MPTmix (10 or 100µg/kg) or DMSO (vehicle control) beginning at postnatal day 22. IP injection of metabolites was prioritized to replicate the composition of phthalate metabolites that reach women's ovaries. On day 3, the mice were injected IP with 5IU pregnant mare serum gonadotropin to induce ovarian follicular development ± MPTmix. 48 hours later, animals were injected IP with 2.5IU human chorionic gonadotropin (hCG) to induce ovulation ± MPTmix. Animals were euthanized prior to hCG administration or at 18 hours post-hCG treatment. At collection, ovulation rates were assessed, serum were collected for hormone measurements, one ovary was flash frozen in liquid nitrogen for qPCR analysis, and the other ovary was fixed in 4% formaldehyde overnight before being paraffin embedded for histology. Tissue sections were mounted and stained with hematoxylin and eosin (H+E) for follicle/corpus lutea (CL) counting, and subjected to CD31 immunohistochemistry, a marker of endothelial cells for ovulatory vascular visualization. ImageJ was used to measure average CD31 integrated density, a measurement that assesses stain intensity and area. **Results:** Animals that received hCG had increased ovulation rates (mean=16.5) and fewer preovulatory follicles compared to DMSO 0hr controls (n=8-16; p<0.05); however, animals treated with hCG+100µg/kg had decreased ovulation rates (mean=8.33) relative to hCG controls (n=12; p<0.05), and 6/12 animals had <2 ovulations. Consequently, these animals had significantly fewer CLs per ovary section than hCG controls. Animals that received hCG+100µg/kg had lower but not significantly decreased ovulation rates (mean=10.17) and numbers of CLs, and 4/12 animals had <2 ovulations. Additionally, these animals had significantly more preovulatory follicles than those treated with hCG alone (n=8-12; p<0.05). Treatment with hCG increased serum levels of progesterone compared to DMSO 0hr (n=7-14; p<0.05), but progesterone levels were decreased in animals that failed to ovulate with hCG+100µg/kg compared to hCG (n=3-10; p<0.05). Additionally, animals that received hCG+100µg/kg had decreased serum estradiol levels and decreased expression of *Cyp11a1* and *Hsd3b1* relative to hCG (n=6-15; p<0.05). Immunohistochemistry staining revealed an increase in integrated density of CD31 staining with hCG treatment relative to DMSO; however, animals that received hCG+100µg/kg did not have this increase (n=7-11; p<0.05), suggesting impaired ovulatory angiogenesis and luteal formation. **Conclusions:** While hCG+10µg/kg did not have significantly decreased ovulation rates, the ovaries contained significantly more preovulatory follicles, and there were several animals that failed to ovulate. This suggests that this dose may still alter ovarian function, and more analysis is needed with this dose. Animals exposed to hCG+100µg/kg had decreased ovulation rates and fewer CLs. This may be due to vascular dysfunction, indicated by a decrease in integrated density of CD31 staining for blood vessels. Further, progesterone levels, estradiol levels, and expression of *Cyp11a1* and *Hsd3b1* were decreased in animals exposed to hCG+100µg/kg, suggesting dysregulation of ovarian steroidogenesis during ovulation. Supported by R01ES033767, P30ES026529.

PS 3836 **A weight-of-evidence review of the toxicological and epidemiological literature of the reproductive and developmental hazard of vinyl chloride monomer**

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Background and Purpose: The United States Environmental Protection Agency (EPA) designated vinyl chloride monomer (VC) as a High-Priority substance for risk evaluation under the Toxic Substances Control Act (TSCA) with reproductive/developmental outcomes as a health hazard of interest. The objective of this review was to independently assess the quality of available epidemiological and toxicological literature to determine the current weight-of-evidence for the relationship between VC exposure and reproductive/developmental outcomes. **Methods:** A review of English-language literature was conducted in PubMed and Scopus databases through October 7, 2024, and compared with reference lists from Agency for Toxic Substances and Disease Registry, Texas Commission on Environmental Quality, and EPA's Integrated Risk Information System assessments of VC. Inclusion criteria were met when the study included relevant population (e.g., animal, human), exposure (e.g., VC via oral, inhalation, or dermal route), comparator (e.g., sham or vehicle control, unexposed epidemiological controls), and outcome (e.g., reproductive/developmental). The quality of each included study was evaluated using the Draft Systematic Review Protocol Supporting TSCA Risk Evaluations for Chemical Substances. Studies were numeri-

cally scored from 1 to 4 (score of 1 indicating highest quality) on methodological and analytical characteristics, including study participation, exposure characterization, outcome assessment, potential confounding, and statistical analysis for epidemiological studies, as well as test substance, test design, exposure characterization, test organisms, outcome assessment, confounding control, and data presentation and analysis for toxicological studies. Individual metric scores were averaged to provide an overall ranking of *high* ($1 \leq X < 1.7$), *medium* ($1.7 \leq X < 2.3$), *low* ($2.3 \leq X < 3$), or *critically deficient* (score of 4 on any metric). **Results:** Ten toxicological and three epidemiological studies that assessed reproductive/developmental outcomes among VC-exposed subjects were reviewed. Of the animal studies, one was determined to be of high quality, four were of medium quality, and three of low quality. Two studies were critically deficient. High- and medium-quality studies demonstrated lack of statistically significant reproductive and developmental toxicity following VC exposure, although delayed ossification in offspring coincident with maternal toxicity was noted in mice. Though lower quality toxicological studies suggest potential for developmental toxicity in both mice and rats, effects were either not dose-dependent or occurred at doses that caused maternal toxicity evidenced by maternal mortality and reduced maternal body weight and food consumption. Of the human studies, two occupational case-control studies of women in PVC processing operations were of medium quality, and one ecological study was critically deficient due to likely exposure misclassification. In the medium-quality studies, odds of involvement in processing or heating of PVC were slightly elevated, though not statistically significant, among women who had spontaneous abortions compared to women who had successful births, as well as statistically significantly elevated among women with adverse birth outcomes compared to women with healthy births. Results of the reviewed epidemiological studies are limited by the small number of evaluated endpoints, small sample size, and lack of VC exposure characterization. **Conclusions:** Because of the limited nature of the available epidemiological studies, the epidemiological findings do not contribute significantly to a weight-of-evidence conclusion on reproductive or developmental toxicity studies for VC. However, the weight of evidence for reproductive/developmental toxicity from the available animal studies suggests that VC is neither a reproductive nor a developmental toxicant. Additional high-quality reproductive/developmental studies, particularly those assessing human VC exposure, would bolster confidence in the weight of evidence.

PS 3837 Environmental pollutants during pregnancy: phthalates and nanoplastics alter gut microbiota and intestinal morphology in pregnant rats

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Background and Purpose: Between 1950 and 2017, global plastic production reached approximately 9 billion tons, of which only 23.9% were properly disposed of. The remaining 76.1% accumulated in the environment, fragmenting into micro and nanoplastics (MNP), which constitutes a public health problem. Furthermore, a group of chemical compounds, phthalates, is continuously used as additives to confer flexibility and malleability to plastic products, such as hospital equipment, building materials, toys, food containers, etc. However, due to their chemical instability, they are easily released into the environment, causing toxic effects, and are classified as endocrine disruptors (EDs). Evidence indicates that both MNPs and phthalates can cross biological barriers and compromise the integrity of the gastrointestinal tract, altering the microbiota and impacting metabolic, immunological, and developmental functions. Considering that ingestion is the primary route of exposure to these substances, it becomes fundamental to understand their effects during critical developmental phases, such as gestation. This study aimed to evaluate the effects of gestational exposure to polystyrene nanoplastics (PS-NPs, nominal size 100 nm) alone or in combination with phthalate mixture (PM) on the composition of the microbiota and histology of colon tissue in pregnant rats. **Methods:** Pregnant rats (CEUA 1174020523) were distributed randomly into 4 groups: CTR: (control; vehicle); T1: 20 µg/kg/day phthalate mixture (PM); T2: 1 mg/kg/day of polystyrene nanoplastics (PS-NPs - 100nm); T3: 20 µg/kg/day PM + 1 mg/kg/day PS-NPs (100 nm). Doses were based on previous studies on environmentally relevant exposure. Treatment was administered orally from gestational day 5 (GD5) to GD20. At GD20, 6-8 pregnant rats from each group were anesthetized and euthanized for analysis. Feces were collected from the cecal region for analysis of the microbiome composition through sequencing. The colon region was collected for histological analysis, such as morphometry by hematoxylin and eosin (HE) staining, and goblet cell counting by periodic acid-Schiff (PAS) staining. The statistical test used was ANOVA, followed by Tukey, with $p < 0.05$ considered statistically significant. **Results:** The microbiome analysis showed an increase in class *Bacilli* ($p = 0.02$) and order *Lactobacillales* ($p = 0.04$) was observed in T2 and T3 vs. CTR, while class *Clostridia* ($p = 0.04$) and order *Clostridiales* ($p < 0.06$) decreased in T1 vs. CTR. Nanoplastics may trigger an inflammatory response favoring *Bacilli* growth while partially preserving *Clostridiales*, and may indicate impaired trophic functions and maintenance of the intestinal barrier, since this group is essential for the production of short-chain fatty acids. A lower Shannon index (diversity and equity of species) was observed in group T1 vs. CTR ($p = 0.04$), indicating lower richness and uniformity of the intestinal microbiota in this group. Morphometric analysis, through the quantification of colon areas by the *imageJ* program, indicated a reduced muscular layer in T3 ($p = 0.03$) vs. CTR, suggesting compromised intestinal motility. Using PAS staining, it was possible to count goblet cells in the colonic mucosa, and an increase in the number of

cells was observed in groups T1, T2, and T3 ($p = 0.01$) vs. CTR, and an increase in T3 ($p = 0.01$) vs. T1. This suggests a compensatory or adaptive response of the epithelium to protect the tissue, an initial defense process against short-term exposure by increasing mucin production. **Conclusions:** These findings suggest that the effects of phthalates on the gut microbiota may be modulated by the presence of nanoplastics, which may lead to alterations in gut integrity and metabolism, influencing critical metabolic functions in the host.

PS 3838 Single-Nucleus RNA-seq Reveals Cell-Type-Specific Transcriptional Alterations in the Ovary After Adolescent Per- and Polyfluoroalkyl Substances (PFAS) Mixture Exposure

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are highly persistent synthetic chemicals widely used in consumer products, leading to extensive environmental contamination. PFAS exposure has been associated with altered menstrual function, premature ovarian insufficiency, and disruptions in steroidogenesis in both humans and animal models. Although legacy PFAS such as perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) have been phased out, they remain detectable, and newer short-chain alternatives including undecafluoro-2-methyl-3-oxahexanoic acid (GenX/HFPO-DA) and perfluorobutanesulfonic acid (PFBS) are now common in environmental mixtures. Despite this, the effects of PFAS mixtures on ovarian biology are still not well characterized. Our prior work showed that adolescent PFAS mixture exposure impairs ovarian function in adulthood through reduced ovarian reserve, endocrine disruption, Hippo pathway alterations, and increased fibrosis, though the timing of these effects remained unclear. We therefore hypothesized that PFAS mixture exposure initiates ovarian changes during the exposure window rather than only later in adulthood. **Methods:** Female CD-1 mice (postnatal day 30) received daily oral dosing via pipette delivery to the cheek pouch with either vehicle (distilled water containing 1% DMSO; $n = 10$) or a PFAS mixture (0.1 mg/kg each of PFOA, PFOS, GenX, and PFBS; $n = 10$) for a 15-day exposure period ending at PND45. The selected dose was selected based on previously published LOAEL values for developmental toxicity in juvenile mouse models. Body weight was monitored weekly, and vaginal cytology was assessed for 10 days prior to tissue collection. At PND45, serum reproductive hormone concentrations were quantified by ELISA. Ovaries from a subset of animals ($n = 3$ per group) were processed for snRNA-seq to characterize cell-type-specific transcriptional responses to PFAS exposure. **Results:** Adolescent exposure to the PFAS mixture did not alter overall body mass or the weights of reproductive organs; however, liver weight was significantly elevated ($P < 0.05$). PFAS-treated females exhibited notable disruptions in estrous cyclicity, characterized by an increased proportion of time spent in estrus ($P < 0.05$) and a reduction in time spent in metestrus/diestrus ($P < 0.05$) compared with controls. These animals also experienced more frequent cycle transitions ($P < 0.05$). Despite these alterations in cyclicity, circulating concentrations of progesterone, estradiol, testosterone, and luteinizing hormone remained unchanged. snRNA-seq identified distinct ovarian cell clusters, including granulosa (mitotic, antral, atretic, and preantral), theca, luteal, stromal, epithelial, immune, and endothelial cells. Further, snRNA-seq revealed widespread transcriptional changes across ovarian cell populations after PFAS mixture exposure. The most pronounced alterations occurred in luteal and thecal cells, where genes involved in cholesterol/steroid biosynthesis and cell survival were robustly upregulated. Notably, Hippo pathway effector *Yap1* re-emerged among top differentially expressed genes, reinforcing its role in PFAS-mediated follicle activation and ovarian remodeling. **Conclusions:** The effects of environmental toxicants on female reproductive health depend heavily on the developmental window of exposure, as well as the dose and duration. Defining when PFAS-induced reproductive impairments first emerge is essential for evaluating long-term fertility consequences. Our findings of disrupted estrous cyclicity, coupled with cell-type-specific transcriptional shifts in ovarian steroidogenic pathways, point to early-onset reproductive disturbances. These results underscore the need to further dissect the mechanisms underlying these initial alterations.

PS 3839 Chronic Exposure of Mice to Propylparaben Disrupts Hormone Signaling and Promotes Fibrosis in the Uterus

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Background and Purpose: Parabens are a class of widely used synthetic preservatives. Their ability to prevent microbial growth and extend product shelf life leads to their widespread usage, particularly in cosmetics and personal care products. Propylparaben (PP), one of the most common parabens, is frequently detected in women due to higher product use. Studies have shown that women tend to have higher levels of paraben exposure compared to men, likely due to greater use of these paraben containing products. Parabens possess estrogen-mimicking properties and are considered endocrine disruptors, raising concerns about their long-term effects on reproductive health. However, the impact of chronic PP exposure

on uterine function remains poorly understood. **Methods:** Adult female CD-1 mice were orally dosed with corn oil (vehicle control) or PP (2, 20, or 200 µg/kg/day) for six months. Uteri were subjected to histology, immunohistochemistry (IHC), RNA expression analysis, Picrosirius Red staining for fibrosis, F4/80 staining for macrophage infiltration, and p-SHG for collagen organization. **Results:** Chronic propylparaben (PP) exposure led to myometrial thickening in the 2 µg/kg/day treatment group. Further analysis revealed that exposure to PP caused marked upregulation of estrogen receptor (Esr1/Erα) expression, particularly in the luminal epithelium and subepithelial stroma, as confirmed by IHC. In contrast, progesterone receptor (Pgr) expression was starkly reduced in the luminal epithelium, while stromal PGR expression remained largely unchanged. Consistent with these receptor changes, PP exposure downregulated multiple *Pgr*-regulated genes (e.g., Sox17, Ihh, Csf1, Cd44) and upregulated *Erα*-regulated targets (e.g., Ezh2, Hif2α, cFos, Tgfb1, Muc1). Additionally, inflammatory markers (e.g., Stat3, Il1b, Ccl2, Ccl12, Ccl17, Ccl21, Tnfα, Tnfr1, Nfkb) were elevated. Our studies revealed a significant increase in the population of macrophages in the uteri of mice exposed to PP, indicating heightened inflammation in response to this chemical. Fibrosis-related genes (Col1a1, Col3a1, Tgfb1, Tgfb1r) were significantly induced, and Picrosirius Red staining confirmed significantly enhanced collagen deposition in PP-exposed uteri. Second Harmonic Generation further delineated the character of collagen deposition in the endometrial layer. Our findings indicate that chronic PP exposure disrupts the estrogen-progesterone signaling balance by enhancing *Erα* activity while diminishing luminal epithelial PR expression. This hormonal imbalance is associated with transcriptional changes promoting inflammation, immune cell infiltration, and fibrotic remodeling of the uterus. **Conclusions:** Chronic exposure to PP induces uterine changes characterized by enhanced *Erα* signaling, loss of luminal PR, inflammation, and fibrosis. These alterations highlight potential risks for reproductive health in individuals with sustained paraben exposure. These findings may have significant implications for reproductive health, particularly for individuals with long-term exposure to parabens through the use of personal care products.

PS 3840 From *In Silico* Prediction to *In Vitro* Validation: Kaempferol Mitigates Perfluorodecanoic acid (PFDA)-Induced Granulosa Cell Toxicity

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Background and Purpose: Perfluorodecanoic acid (PFDA), a long-chain per- and polyfluoroalkyl substance (PFAS), has been implicated in ovotoxicity, but granulosa-cell mechanisms and tractable countermeasures remain uncertain. We implemented an *in silico* to *in vitro* workflow that first predicted PFDA injury nodes and kaempferol (KA) response pathways from network pharmacology and then experimentally tested those predictions in human granulosa (HGrC1) cells. **Methods:** Putative targets for PFDA and KA were integrated from public resources (e.g., CTD, ChEMBL, STITCH, PubChem) and intersected with ovotoxicity gene sets (GeneCards, OMIM). Protein-protein interaction (PPI) networks (STRING) were prioritized in Cytoscape using centrality metrics (MCC, MNC, degree, EPC, closeness). GO/KEGG enrichment (clusterProfiler; adjusted $p < 0.05$) predicted PFDA-evoked oxidative/inflammatory programs and KA-linked Nrf2-PPAR γ cytoprotection, which pre-specified readouts and dosing. HGrC1 cells were exposed to PFDA (20 µM) $\pm$ KA (3 or 10 µM) for 24-72 h. Outcomes included viability (MTT), intracellular reactive oxygen species (ROS; DCFDA), qPCR of antioxidant (CAT, GPX1, SOD2) and steroidogenic (PPARG, STAR, HSD3B1, HSD17B1) transcripts, and immunoblot of Nrf2-responsive proteins (HO-1, NQO1). One-way ANOVA with Dunnett's post-test compared treatments to vehicle controls. **Results:** Computational intersections yielded overlapping PFDA-ovotoxicity ($n=131$) and KA-ovotoxicity ($n=236$) genes. Networks/enrichment predicted activation of inflammatory (IL6, NFKB1, RELA) and oxidative-stress/xenobiotic pathways with PFDA and Nrf2-centered antioxidant and PPAR γ -linked cytoprotective programs with KA. Bench validation corroborated these predictions: PFDA (20 µM) decreased HGrC1 viability in a time-dependent manner, increased ROS, and downregulated PPARG and steroidogenic transcripts (STAR, HSD3B1, HSD17B1). Co-treatment with KA (3-10 µM) partially restored viability, reduced ROS, recovered antioxidant transcripts (CAT, GPX1, SOD2), and increased HO-1/NQO1 proteins consistent with Nrf2 activation, while rescuing PPARG and steroidogenic transcripts under PFDA stress. Thus, *in vitro* phenotypes aligned with *in silico* network predictions, nominating an Nrf2-PPAR γ protective axis. **Conclusions:** PFDA compromises granulosa-cell function through oxidative/inflammatory stress and disruption of steroidogenic/nuclear-receptor signaling. Guided by *in silico* predictions, KA mitigated these injuries by restoring antioxidant defenses and PPAR γ -linked transcription, experimentally validating a predicted Nrf2-PPAR γ axis. This prediction-driven network-to-bench approach identifies KA as a candidate modulator of PFAS-associated ovotoxic risk and yields mechanism-anchored entry points for *in vivo* studies.

PS 3841 Reproductive and Developmental Toxicity Assessment of Fragrance Ingredients: Considerations for Reprotoxic Classification in the Context of Maternal Toxicity

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Background and Purpose: Reproductive and developmental toxicity is a key aspect in toxicological risk assessment, particularly for fragrance ingredients with generally low human exposure. While real-world exposure is low, high-dose animal testing required for regulatory compliance may report adverse effects even for low-exposure substances. A central challenge is distinguishing true developmental toxicity from effects secondary to maternal toxicity. This review evaluates published reproductive and developmental toxicity data for two fragrance monoterpenoids, focusing on how maternal toxicity may influence developmental outcomes. Maternal and fetal no-observed-adverse-effect levels (NOAELs) were compared to identify patterns indicating secondary effects. The findings indicate that developmental outcomes are more likely driven by maternal toxicity than direct fetal effects, improving the scientific basis for classification under the European framework. **Methods:** Representative monoterpene fragrance compounds were analyzed using studies conducted according to OECD Test Guidelines 414 (Prenatal Developmental Toxicity Study), 421 (Reproductive/Developmental Toxicity Screening Test), 422 (Combined Repeated Dose with Reproduction/Developmental Screening), 443 (Extended One-Generation Reproductive Toxicity Study), 407 (Repeated Dose 28-Day Oral Toxicity Study), and 408 (Repeated Dose 90-Day Oral Toxicity Study). Studies selected included published, well-defined systemic, maternal, and developmental NOAELs, with reproductive and/or developmental toxicity indicators. Systemic, reproductive, and developmental parameters were compared, focusing on maternal endpoints and offspring outcomes. Maternal analysis included body and organ weights, food intake, biochemistry, histopathology, and thyroid hormones. Reproductive parameters included estrous cycle, mating performance, pregnancy rate, and implantation loss. Developmental parameters included fetal viability, pup weight, malformations, skeletal variations, among other parameters. **Results:** For the first monoterpene, dose-dependent maternal toxicity was observed, including reduced body weight, decreased food intake, and biochemical alterations, particularly at ≥ 600 mg/kg/day. Elevated bile acids indicated liver dysfunction, while creatinine and urea elevations suggested renal stress, contributing secondarily to developmental effects. Male reproductive toxicity at 750 mg/kg/day included testicular degeneration and absence of spermatozoa. Developmental effects, such as reduced fetal and placental weights, occurred only at high doses of 600 mg/kg/day in the presence of maternal systemic stress and undernutrition, with no evidence of direct teratogenicity. For the second monoterpene, maternal toxicity was pronounced at ≥ 720 mg/kg/day, including reduced gestational weight gain, decreased food intake, liver hypertrophy, altered biochemistry (elevated bile acids, cholesterol, creatinine kinase, and reduced potassium), and hormonal changes (reduced T4 at ≥ 800 mg/kg/day). Developmental effects, including reduced viability at starting at 300 mg/kg/day, lower pup weights at 1000 mg/kg/day, delayed sexual maturation, and increased post-implantation loss, appeared secondary to maternal toxicity. Paternal toxicity at ≥ 720 mg/kg/day included liver and kidney changes, abnormal sperm, and reduced spermatid counts, potentially affecting early embryonic development. At the dose of ≥ 800 mg/kg/day, a decrease in circulating T4 levels was also observed. These changes correlate with liver enzyme induction and are relevant because reductions in T4 may cause effects on pups. **Conclusions:** Adverse developmental outcomes for both fragrance monoterpenoids were primarily associated with high-dose maternal toxicity. Effects in offspring were observed only alongside maternal stress, making unclear the attribution to direct fetal toxicity. These findings emphasize maternal health as an early, sensitive indicator of reproductive risk. Integrating mechanistic understanding and human-relevant exposure data can refine hazard assessments, guide regulatory decisions, and improve safety characterization of fragrance ingredients.

PS 3842 Sex differences in gonadal toxicity of exposure to fine particulate matter (PM_{2.5})

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Background and Purpose: Open burn pits were widely used to dispose of waste in American military bases in Iraq and Afghanistan. As combustion sources, burn pits emit PM_{2.5}. Many studies investigating the effects of PM_{2.5} have focused on exposure to males. Since women make up increasing proportions of the armed forces, it is important that experimental studies include both males and females. Previous studies have reported that exposure to ambient PM_{2.5} decreased epididymal sperm counts in male mice; sperm counts are expected to recover due to the presence of germine stem cells. In contrast, the post-natal ovary lacks germine stem cells. We hypothesized that PM_{2.5} exposure permanently depletes the ovarian follicle reserve and has reversible effects on testicular sperm production. **Methods:** We exposed 2-months old, late adolescent, male and female wild type (WT) C57BL/6J mice and mice that express the wildtype human amyloid- β sequence (hA β -KI mice) to concentrated ambient PM_{2.5} (average concentration 169 ± 113 µg/m³) or filtered air (1.9 ± 1.2 µg/m³) for 5h/day, 4d/wk for 12 weeks. Mice were euthanized 24 h after the last exposure at age 5 mos or 9 months after the last exposure at age 14 mos. In a

separate experiment, 5 mos old adult female and male hA β -KI mice were exposed to concentrated ambient PM_{2.5} (average concentration 85.2 μ g/m³) or filtered air (9.1 μ g/m³) for 5h/day, 4d/wk for 12 weeks and were euthanized 24h after the last exposure at age 8 mos. In both experiments, females were euthanized on a day of the estrous cycle with vaginal cytology showing cornified epithelial cells, characteristic of estrus. Ovaries were cleared and processed for wholemount imaging with antibody against oocyte cytoplasmic-specific protein MSY2 and automated follicle counting based on oocyte diameter using Imlaris software. Immunostaining or immunofluorescence was performed on contralateral ovaries to identify effects of PM_{2.5} exposure on ovarian follicle cell proliferation (Ki67), apoptosis (activated caspase 3), and autophagy (LC3B). **Results:** There were no statistically significant effects of exposure on estrous cycle length in mice euthanized at age 5 mos, 8 mos, or 14 mos. Females of both strains exposed to PM_{2.5} and euthanized at age 5 mos had statistically significantly decreased total number of ovarian follicles/ovary (P<0.001, effect of exposure), primordial follicles (P=0.002) and antral follicles/ovary (P=0.004), while the decrease in primary follicles approached significance (P=0.089). Female hA β -KI mice exposed to PM_{2.5} as adults and euthanized at age 8 mos also had statistically significantly decreased primordial, antral, and total follicles/ovary (P=0.022, 0.020, and 0.046, respectively), as well as significantly increased percentages of primary follicles with Ki67-positive granulosa cells (P=0.019) and significantly increased percentages of LC3B-positive secondary and antral follicles (0.043 and 0.010, respectively). The percentage of ovarian secondary and antral follicles positive for activated caspase 3 approached significance (P= 0.076 and P=0.065, respectively). By contrast testicular sperm counts and seminiferous tubule pathology were not significantly altered by exposure to PM_{2.5} in either strain at age 5 months, in hA β -KI mice at age 8 mos, nor were cauda epididymal sperm counts at age 14 months. **Conclusions:** Exposure to concentrated ambient PM_{2.5} significantly decreased ovarian follicle numbers and this was associated with evidence of increased activation of primordial follicles and increased autophagy and apoptosis in follicles at later stages of development. By contrast, there were no statistically significant effects of PM_{2.5} exposure on testicular or epididymal sperm counts or testicular histopathology.

PS 3843 Perinatal E-Cigarette Exposure and Maternal-Fetal Distribution of Nicotine and Its Metabolites

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Background and Purpose: Perinatal exposure to nicotine from electronic cigarettes (e-cigs) continues to raise concerns regarding fetal susceptibility to nicotine and its bioactive metabolites. The impact of gestational e-cig exposure is reported, but limited studies target the metabolite quantification. In the current study, we quantified maternal-fetal nicotine transfer by measuring nicotine and key metabolites in biological samples from e-cig-exposed pregnant mothers and their offspring. **Methods:** Using a well-established E-Cigarette Nicotine Delivery System (ENDS), pregnant Sprague Dawley rat dams (200-250-gram body weight) were exposed to nicotine (E- blue cigarette 2.4% with 50/50 propylene glycol and vegetable glycerol, 144 puffs over 12h daily) from embryonic day 6 (E6) until weaning at postnatal day 21 (PND21). Control dams were exposed to either filtered air or vehicle PG/VG. On E21, some mothers (control and nicotine exposed) were sacrificed for amniotic fluid, placenta and serum collection for the measurement of nicotine and metabolites (cotinine, norcotinine, trans-hydroxycotinine, and cotinine-n-oxide) concentrations via Liquid Chromatography-Mass Spectrometry (LC-MS). Postnatally, pups (N=8-10) were studied for these metabolites at PND1 and PND21 in the serum. **Results:** LC-MS quantification showed significant concentrations of cotinine, norcotinine, trans-hydroxycotinine, and cotinine-n-oxide in maternal serum, confirming systemic nicotine absorption during gestation. Similarly, pups' serum contained increased levels of the same metabolites at PND1, demonstrating efficient placental transmission. Elevated concentrations of nicotine metabolites were also identified in placental tissue (100-200 ng/g tissue) and amniotic fluid (358.22 $\pm$ 56.42 ng/ml), indicating fetal exposure throughout gestation and highlighting nicotine delivery to the developing fetus via these compartments. Substantial levels of these metabolites were detected in offspring serum at PND21 (56.80 $\pm$ 16.42 ng/ml), corroborating neonatal exposure to nicotine during development. Surprisingly, the serum nicotine levels of were significantly higher in female pups as compared to male pups at PND21. **Conclusions:** Taken together, these findings reveal that perinatal e-cigarette exposure results in significant fetal and neonatal accumulation of nicotine metabolites across maternal, placental, and fetal compartments highlighting the potential for adverse developmental effects and long-term health consequences. **Grant Support:** HL151769 (NIH); T32IP5044, T32IR5048, T32IR5365, T291R0737, T34IR8406 (TRDRP); and EDUC4-12837 (CIRM)

PS 3844 Chronic PFUnA exposure induces liver hypertrophy in CD-1 mice

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Background and Purpose: Exposure to per- and polyfluoroalkyl substances (PFAS) during pregnancy is associated with several adverse outcomes including preterm birth, low birth weight, and preeclampsia. PFAS are found in thousands of prod-

ucts, are extremely long-lasting, and are ubiquitous in the environment. Of specific concern to pregnant populations, PFAS have been shown to cross the placental barrier, accumulate in the placenta, and have been detected in cord blood of infants. Prior work has shown that perfluoroundecanoic acid (PFUnA) is significantly associated with adverse pregnancy outcomes, including preeclampsia, yet the mechanisms underlying these effects remain understudied. PFUnA has never been studied in the context of pregnancy outcomes in mice, specifically preeclampsia. The objective of this study was to assess multiple maternal, fetal, and placental endpoints after chronic exposure to PFUnA in drinking water in mice. **Methods:** CD-1 outbred mice were exposed to 0, 0.1, 0.5, or 2.5 mg/kg/day of PFUnA in drinking water from the day of weaning (23 days old) until gestational day (GD) 17.5 of their pregnancy. All mice were mated between 8-9 weeks of age to minimize any differences in exposure length. At GD 17.5, several organs and endpoints were recorded for the dams, including liver, kidney, and aorta. Pups and placentas were collected on GD17.5 with weights and morphological abnormalities recorded. Mass spectrometry was utilized to verify PFUnA accumulation in placental samples. Statistical analyses such as Anova or t-tests were performed between the two exposure groups and the control. **Results:** PFUnA in drinking water was associated with a significant decrease in body weight for the 2.5mg/kg/day group. Within 15-20 days of exposure, mice had lost 20% or more of their body weight, making them no longer eligible for the continuation of the study and subsequent pregnancy assessments. However, upon collection, their livers had visible signs of damage. Liver to body weight ratios at ~5 weeks old averaged 8.66 compared to 20.2 in the 10-week non-pregnant control mice. This shows a significant decrease in liver efficiency and increase in hypertrophy. Body weights for the control, 0.1, and 0.5 mg/kg/day exposure groups showed no significant differences at the end of pregnancy or in the non-pregnant mice at 10 weeks of age. Liver weights were significantly larger in the exposed mice. Body weight adjusted liver weights were on average 46.8% larger in the 0.5mg/kg group and 11.6% larger in the 0.1mg/kg group in comparison to the control. Assessments of PFUnA quantification in the placenta showed 1,321.2 ng/g in the 0.1 mg/kg group and 1.65 ng/g in the control. While there was no significant difference between the placenta weights, mean values show a trending increase as PFUnA exposure increases. **Conclusions:** In this study, PFUnA exposure at 2.5mg/kg/day was shown to be too high for the general health of the mice. This can be informative on the potency of this chemical, as there are several other chronic PFAS studies that utilize this dose. Additionally, our results show that there is a significant effect on the liver, suggesting an attempt to metabolically protect against the effects of this exposure. Lastly, PFUnA has a large potential to accumulate in the placenta, providing evidence that it can efficiently cross the placental barrier and have potential effects on the developing fetus. Future directions for this study include albumin creatinine ratios from GD17.5 to assess proteinuria, which is representative of kidney damage. Renal and liver abnormalities will also be characterized by immunohistochemistry. ELISA assays will be utilized to measure serum levels of placenta growth factor (PLGF) and soluble FMS-like tyrosine kinase-1/ vascular endothelial growth factor receptor (sflt-1/ VEGFR). Understanding the implications of PFUnA in drinking water can give insights on the effects this chemical may be having in the body. Specifically, as this PFAS has been associated with adverse pregnancy outcomes such as preeclampsia, testing for those same associated symptoms can give information on the mechanisms PFUnA uses to dysregulate systems within the body. This work has direct importance and translatability to the UNC-based high risk pregnancy cohort where PFUnA was detected in approximately 50% of placentas. This study may also help to elucidate future biomarkers, pathways, or processes that get altered in relation to PFAS exposure.

PS 3845 CONFOUNDING OF REPRODUCTION ENDPOINTS BY UNDERNUTRITION: A CASE-STUDY

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Background and Purpose: It is well-understood that undernutrition lowers fertility of female rodents and retards the development of their offspring. However, the extent to which this phenomenon confounds the interpretation of reproductive findings in regulatory toxicology studies remains controversial. Here, we present a case study (taken from an ongoing regulatory assessment under EU REACH) exemplifying this problem and providing some answers. **Methods:** A comprehensive programme of GLP-compliant OECD 422 test guideline studies with Han-Wistar rats were conducted via the dietary route on a set of 15 related Rosin (CASRN 8050-09-7) and derivatives substances. Extensive Multiple Linear Regression analyses were conducted across these regulatory studies to reveal relationships between undernutrition and reproductive parameters. Subsequently, bespoke GLP-compliant mechanistic studies based on the OECD 421 test guideline, including a pair-feeding study, were conducted to test whether the relationships had a causal basis. **Results:** The regulatory studies revealed that dietary exposure of rats to several Rosin and derivatives substances lowered mean *Corpora Lutea* counts (reduced ovulation rate). Other sexual function and fertility findings included changes in absolute weight of some sex organs, including ovaries. The only consistent developmental finding was of a retardation of offspring growth, sometimes accompanied by delayed ossification. In these studies, the test substances proved unpalatable and food consumption during the first week of the pre-pairing period was transiently, but severely, restricted in animals administered treatment diet. Consequently, treated rats were undernourished at the point of mating and remained underweight throughout gestation. Multiple Linear Regression analyses revealed highly significant correlations between all the aforementioned

reproductive findings and undernutrition. A subsequent mechanistic OECD 421 pair-feeding study confirmed that growth retardation seen in offspring of dams exposed to Rosin in the diet was caused by the undernutrition and is unrelated to any intrinsic property of the substance *per se*. Surprisingly, no effect of Rosin on sexual function and fertility was seen under pair-feeding conditions. **Conclusions:** The impaired development of rat offspring seen in dams exposed to dietary Rosin and derivatives substances is a non-specific secondary consequence of maternal under-nutrition. Further work is required to establish whether the fertility effects of these substances too are caused by undernutrition. More generally, the data presented here may be of considerable general interest because they clearly demonstrate that undernourishment at levels not uncommonly seen in toxicology studies does indeed affect reproduction in a common regulatory study design.

PS 3846 Overcoming the DART Assessment Bottleneck in Transgenic Mice: Study Design Strategies Informed by Historical Control Data

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Background and Purpose: Following ICH S6(R1) and ICH S5(R3) guidelines, transgenic mice are now a critical relevant species for the developmental and reproductive toxicity (DART) assessment of biotherapeutics, often as an alternative to non-human primates (NHPs). However, a major limitation is the paucity of practical data on their reproductive and developmental performance. This study aims to conduct a systematic review of the historical control data from DART studies accumulated at our facility involving various transgenic mouse models, and to evaluate the utility, inherent challenges, and critical design elements for DART assessment in these models. **Methods:** A retrospective analysis was conducted on 26 DART studies performed at our facility that utilized transgenic mouse models on C57BL/6N background for biopharmaceuticals. The dataset included 6 Fertility and Early Embryonic Development (FEED), 15 Embryo-Fetal Development (EFD), and 5 Pre- and Postnatal Development (PPND) studies. Models targeted key molecules such as IL-1 β , IL-17A, CD22, and hIL6/hIL6R, among others. Reproductive performance parameters—including mating rate, pregnancy rate, early embryonic development, intrauterine embryo-fetal survival, and F1 generation litter size were evaluated in control animals to assess the robustness of these models for DART testing and to inform optimal study design. **Results: Variable Reproductive Performance in Transgenic Mice** Analysis of mating and pregnancy data from 6 FEED and 15 EFD studies revealed that transgenic mice exhibited normal mating behavior at sexual maturity (8-11 weeks of age), with a high mean mating rate (98.02%) and most pairs cohabiting successfully within one estrous cycle (4-5 days). The mean pregnancy rate was 85.90% in FEED studies, which declined to a mean of 72.78% in the larger dataset of EFD studies, with considerable inter-study fluctuation (as low as 37.5%). Consequently, study designs must incorporate an increased number of initial mating pairs to ensure a sufficient number of pregnant animals for a robust evaluation. **Analysis of Early Embryonic and Embryo-Fetal Survival in FEED and EFD Studies.** FEED studies (GD18) identified post-implantation loss (range: 8.58%-18.14%; Mean $\pm$ SD: 12.09% $\pm$ 4.08%) as the primary factor reducing live litter size, substantially exceeding pre-implantation loss rates (mean: 9.79%). Data from EFD studies (GD 18) were consistent, showing post-implantation loss rates from 4.85% to 27.27% (Mean $\pm$ SD: 13.75% $\pm$ 6.6%), primarily driven by early resorptions (mean: 12.15%), with notable variability across studies. Although the incidence of late resorptions and dead fetuses was low (combined mean: 1.11%), it was present in individual studies. Consequently, the mean number of live fetuses per litter in EFD studies ranged from 4.3 to 9 (Mean $\pm$ SD: 7.37 $\pm$ 1.24). **PPND Studies are Challenged by Insufficient and Variable Offspring Numbers.** Data from six PPND studies showed that the mean litter size for transgenic mice at birth (litter size) ranged from 5.9 to 8.5 pups (Mean $\pm$ SD: 7.6 $\pm$ 1.02). While individual litter sizes were highly variable (range: 1-12 pups), frequently accompanied by sex ratio bias. This situation aligns with the challenges reported for the PPND study of the approved drug UPLIZNA (Inebilizumab, an anti-CD19 monoclonal antibody), where a CD19 transgenic mouse model used characterized by "low pregnancy rate, low fecundity, and small litter size" resulting in an insufficient number of F1 offspring and leading regulators to deem the neurobehavioral assessment inadequate. This case underscores the critical need to: 1) Increase the number of initial mating pairs; 2) Implement litter standardization to a lower size (e.g., 6 pups/litter); and 3) Carefully plan the allocation of animals to various postnatal subgroups and assessments. **Conclusions:** The systematic collection of historical control DART data for transgenic mice is a cornerstone for their reliable use in DART assessment. Our data identify inherent limitations, including variable reproductive performance and limited offspring numbers, which must be proactively integrated into study design through optimized mating strategies, increased cohort sizes, and adjusted litter standardization practices. We recommend early regulatory engagement as a prudent strategy to align study designs with scientific and regulatory expectations for DART assessment using transgenic mice.

PS 3848 The Importance of Species Selection in Drug Discovery and Development

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Background and Purpose: Species selection in the discovery and development of a drug is often trivialized. To evaluate the safety profile and toxicology for zalunfiban, a novel small molecule inhibitor of the platelet α IIb β 3 receptor, for use as the first point of medical contact treatment for ST segment elevation myocardial infarction (STEMI), its relative potency was tested across commonly used models in preclinical development to determine which species provided the most translatable data. **Methods:** Platelet aggregation and determination of the zalunfiban IC₅₀ was performed using light transmission platelet aggregation studies at 37°C with stirring using ADP to induce aggregation. **Results:** Zalunfiban caused inhibition of aggregation of platelets in human citrated platelet-rich plasma induced by adenosine diphosphate (ADP) (5 μ M) with an IC₅₀ of 0.057 $\pm$ 0.010 μ M. Approximately 14 times more drug was required to achieve comparable inhibition of the platelet aggregation of cynomolgus monkeys (*Macaca fascicularis*). Approximately 47 times as much zalunfiban was required to inhibit the aggregation of platelets from beagle dogs compared to human platelets. Moreover, because beagle dog platelets have variable responses to ADP alone, the studies with these animals required a combination of epinephrine (10 μ M) followed by ADP (25 μ M). Inhibition of rat platelet aggregation induced by 5 μ M ADP required more than 1,000 times as much zalunfiban compared to human platelets, whereas inhibition of mouse and rabbit platelet aggregation required approximately 200 times as much zalunfiban. Zalunfiban's hepatocyte stability was similar among species evaluated, as were the zalunfiban metabolite identification studies, with all species showing the des-glycine hydrolysis derivative of zalunfiban as the major metabolite. **Conclusions:** Based on the relative potency and translatability of zalunfiban to humans, the mouse and NHP were selected as the rodent and non-rodent nonclinical species for development.

PS 3849 Timing of Artifactual Erythrocyte Morphologic Changes Associated with Storage of Blood in Common Laboratory Animal Species

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Background and Purpose: Artifactual changes in hematologic parameters due to prolonged storage of potassium ethylene diaminetetraacetate (EDTA) blood have been well documented in many species. Corresponding blood smears often yield cellular morphologic changes that could result in misinterpretation of a pathologic condition. Prompt sample processing and proper storage conditions can reduce artifactual morphologic alterations; however, sample stability varies between species and should be considered when establishing laboratory operating procedures. Cellular morphologic changes were evaluated at multiple time points for macaques, dogs, rats, and rabbits in conjunction with the Siemens Advia® 2120i sample stability assessment conducted at Labcorp. **Methods:** Five whole blood EDTA samples were obtained from each of the following species: cynomolgus macaques, dogs, rats, and rabbits. A baseline blood smear slide was prepared for each sample within an hour of collection. Samples were divided into two aliquots; one aliquot was stored at room temperature and one stored refrigerated (2-8°C). A slide was prepared from each aliquot at approximately 4, 6, 24, 30, 48, and 72 hours from time of collection. All slides were stained and changes in leukocyte, erythrocyte and thrombocyte morphology were evaluated by comparison with the baseline slide. Slides were graded by a single technician as either changes seen or no changes seen. Analysis of the Siemens Advia® 2120i instrument stability occurred in conjunction with the slide stability assessment. **Results:** The slide evaluation revealed significant erythrocyte crenation preceded morphologic changes in leukocytes and thrombocytes; therefore, leukocyte and thrombocyte morphology were not assessed. At room temperature, macaque samples showed the longest stability, with no visible erythrocyte changes up to 6 hours. Dog samples maintained cell integrity for up to 4 hours. Rat and rabbit samples began showing changes by 4 hours, with more prominent alterations in rats. Refrigeration consistently improved cell preservation across all species. Rat samples remained the least stable, with erythrocyte changes evident by 6 hours. By 24 hours, all species exhibited notable cellular changes. Erythrocyte morphologic changes aligned with instrument sample stability at room temperature for macaques and rats, while instrument sample stability exceeded slide stability for dogs and rabbits. Refrigerated sample stability for the instrument was longer for macaques and rabbits but was comparable to slide stability for rats and dogs. **Conclusions:** The data demonstrated that blood smear slides should be prepared as soon as operationally possible upon arrival in the lab and within the accepted instrument stability in order to maintain optimal integrity of erythrocyte morphology. Sample refrigeration prolonged erythrocyte stability. In addition, since species were affected at different rates, priority for slide generation should be given first to rats, second to rabbits and dogs, and then third to macaques.

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Background and Purpose: The aim of this study was to localize and measure lacrimal glands (LG) from laboratory animals. **Methods:** Both LGs were collected from Sprague Dawley (SD) rats (n=12), New Zealand White (NZW) rabbits (n=8), Beagle dogs (N=8) and Cynomolgus Macaques (CM) (n=20). LGs were dissected, weighed (mg) and measured (width x length in mm). **Results:** Exorbital LG in SD rats was localized over the masseter muscle, mean weight of 117.4±3.1 mg, and measured 7.9 mm ±0.14 mm by 12 mm ±0.31 mm. Intraorbital LG underneath the lower temporal part of the orbit, mean weight was 13.5±1.9 mg, and measured 7.6 mm ±0.27 mm by 3.3 mm ±0.06 mm. In NZW rabbits the LG was embedded posteriorly in the superotemporal part of the orbit, mean weight was 328.4±23 mg, and measured 8.4 mm ±0.17 mm by 20.2 mm ±0.09 mm. In Beagle dogs the LG was in the superotemporal aspect of the globe and underneath the orbital ligament, mean weight was 337.9±89.5 mg, and measured 11.1 mm ±0.16 mm by 4.59 mm ±0.23 mm. LG in CM were located superotemporal aspect of the globe and was easily assessed by rotating the eye nasally and elevating the superior palpebra. The mean weight was 50.4±2.6 mg and measured 11.73 mm ±0.41 mm by 4.25 ±0.12 mm. **Conclusions:** LG location and measurements are essential information as new therapies and administration routes are being explored for treatment of dry eye disease.

PS 3851 Histopathological Assessment of Purkinje cells in the Cerebellum of Japanese Medaka (*Oryzias latipes*) Fish Exposed to Graphene Oxide

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Background and Purpose: Previous studies from our laboratory have demonstrated that graphene oxide (GO), a widely used carbon-based nanomaterial, exhibits minimal endocrine-disrupting effects in Japanese medaka (*Oryzias latipes*). The observed alterations in reproductive organs, interrenal glands, endocrine pancreas, and thyroid appear to result primarily from the physical aggregation and accumulation of GO rather than from direct endocrine interference. Although some histopathological changes, such as agglomeration in the gonads, liver, and kidney, have been reported, these are largely attributed to the physical characteristics of GO, not its endocrine-disrupting potential. In the present study, we extended our investigation to the central nervous system, focusing specifically on the cerebellum of adult Japanese medaka. Our objective was to examine the potential effects of GO exposure on Purkinje cells (PKCs), a critical neuronal population involved in cerebellar function and motor coordination. **Methods:** Adult Japanese medaka were exposed to GO *in vivo* via two routes: immersion (IMR; 20 mg/L GO in balanced salt solution, BSS) and single intraperitoneal (IP) injection (100 µg/g body weight). Control groups were maintained in BSS (for IMR) or injected with nanopure water (for IP). Following exposure, brains were dissected and processed for histological analysis. Paraffin-embedded brain sections were stained with hematoxylin and eosin (H&E), and the cerebellum was visualized using an Olympus CKX53 inverted microscope equipped with a DP22 camera and CellSens imaging software. A minimum of two cerebellar images per fish were analyzed using ImageJ software. **Results:** Purkinje cells (PKCs) were readily distinguishable from other cerebellar layers due to their large, drop-like morphology and localization within the Purkinje layer—situated between the molecular and granular layers. In Japanese medaka, the cerebellum is anatomically divided into three main lobes: valvula cerebelli (VC), corpus cerebelli (CC), and lobus caudalis cerebelli (LC). PKCs were exclusively distributed in the VC and CC, with no detectable presence in the LC. The Purkinje cell zone was observed to originate at the caudal end of the VC, extend through the CC, and terminate at the ventral margin of the CC. PKC density varied depending on the sectioning plane (dorsoventral, rostrocaudal, and lateral), with the highest concentration located in the central region of the CC and fewer PKCs found at the dorsal and ventral extremes. Exposure to GO via IP injection significantly reduced the number of PKCs in the CC of both male and female fish. In contrast, immersion exposure to GO did not elicit any measurable effect on PKC numbers in either sex. Additionally, morphometric analyses of the nucleus and soma of PKCs, including their area and nucleus-to-soma ratios, revealed inconsistent patterns of alteration, dependent on both the route of exposure and sex of the fish. **Conclusions:** These findings suggest that GO has the potential to alter brain morphology in Japanese medaka, likely through penetration of the blood-brain barrier and subsequent disruption of cerebellar structure. The observed reduction in PKC numbers, particularly following IP exposure, indicates a route- and sex-specific neurotoxic effect that may impair cerebellar function. Further studies are warranted to elucidate the mechanistic basis of GO-induced neurotoxicity and its potential implications for vertebrate neurodevelopment and behavior.

PS 3852 Risks of Microplastic Exposure for Vertebrate Immunity: Insights from *Xenopus*

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Background and Purpose: Small plastic debris, or microplastics (MPs; 1 µm-5 mm), have become ubiquitous in the environment and within animal and human tissues, including the brain, liver, placenta, and breast milk. However, their specific health effects remain poorly defined. Using tadpoles of the amphibian *Xenopus laevis*, we applied a comparative biology approach to investigate how early-life exposure to microplastics affects macrophage homeostasis, activation, and defense against pathogens such as nontuberculous mycobacteria (NTMs) commonly found in plastic-associated biofilms. *Xenopus* tadpoles offer an excellent model because their external post-embryonic development—including the immune system—makes them highly sensitive to waterborne pollutants, while their immune architecture shares strong conservation with mammals. Instead of using uniform microspheres at unrealistically high concentrations, we exposed tadpoles to low concentrations (0.1-10 mg/L) of environmentally relevant irregular microplastics (I-MPs; 1-20 µm), including polyethylene terephthalate (PET) and nylon. **Methods:** The biodistribution of fluorescently labeled I-MPs (Nile Red) was assessed by fluorescence microscopy on whole-mount tissues and isolated peritoneal macrophages, as well as by enzymatic digestion and silicon nanomembrane filtration. Tadpole colonization and immune responses to ranavirus (FV3) and NTMs were quantified by qPCR. **Results:** Even at concentrations as low as 0.1 mg/L, I-MPs accumulated rapidly in tadpole intestines, liver, kidneys, brain, and peritoneal macrophages, where they persisted for over a week. This accumulation led to macrophage dysfunction, impaired antiviral responses, and decreased resistance to FV3 infection, without inducing overt inflammation. Moreover, we found that mycobacteria pathogens detected in biofilms associated with environmental MPs such as *M. marinum*, can tightly bind to I-MPs *in vitro*, which may promote their colonization into tadpoles. **Conclusions:** Environmentally relevant microplastics accumulate and persist in *Xenopus* tissues at low concentrations, disrupting macrophage function and compromising immunity. The association of NTMs with I-MPs may further enhance pathogen colonization, highlighting potential immunological risks of microplastic exposure in vertebrates.

PS 3853 Insights into Sleep Architecture and EEG Spectral Profiles During Postoperative Recovery in Group-Housed Mice

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Background and Purpose: Studying physiological parameters during post-surgical recovery is critical for gaining insights into animal health. However, technological restrictions pose practical challenges when conducting experiments with group-housed animals. **Methods:** To address this, we developed a telemetry system, SoHo™, capable of simultaneously collecting electroencephalogram (EEG), electromyogram (EMG), temperature, and activity in group-housed small animals. This study evaluates the efficacy of the SoHo™ to wirelessly monitor these parameters in group-housed mice. C57BL/6 female mice (22 weeks old; N=5) were surgically instrumented (subcutaneously) with the SoHo™ telemetry transmitters (model X02) and group-housed under a standardized 12-hour dark/light cycle. Synchronized EEG, EMG, body temperature, gross locomotor activity, and video data were continuously collected during 12 days of post-operative recovery. **Results:** Following surgery (day 0), the average percentage of slow wave sleep (SWS) was 32.9±15.5% and paradoxical sleep (PS) was 11.2±6.1%. By day 12, SWS decreased to 26.2±9.7% and PS decreased to 5.2±0.9%. EEG Delta power was 4693±276 µV2 (dark) and 3843±742 µV2 (light) post-surgery and decreased by 45% (dark) and 21% (light) on day 12. Post-surgery, the subcutaneous temperatures were 35.9±0.4 °C (dark) and 35.7±0.2 °C (light) and did not change on day 12. Average gross locomotor activity (calculated as counts per minute; cpm) was 13181±509 cpm (dark) and 81707±8161 cpm (light) post-surgery and increased by 1360% (dark) and decreased by 29% (light). **Conclusions:** The data demonstrates that SoHo™ telemetry is a robust tool for evaluating EEG, EMG, temperature, and activity in group-housed mice. Future studies are needed to further characterize EEG dynamics during recovery.

PS 3854 Evaluation of blue light exposure from LED lighting on lifespan and sleep in mice

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Background and Purpose: LED lighting is widely used because its lifespan is more than three times longer than that of fluorescent lighting, and it consumes only one-third the power. LED lights emit a relatively high proportion of blue light (350-500 nm) compared to fluorescent lights, and nighttime exposure to blue light has been linked to sleep disturbances, although whether daily and lifetime exposure to blue light affects sleep is not well understood. Additionally, previous studies have shown that blue light exposure shortens lifespan in *C. elegans* and *Drosophila*. In this

study, we aimed to evaluate whether blue light exposure from LED lighting affects sleep or reduces lifespan in mice, as a representative mammalian model. **Methods:** Regarding evaluating the effect of lifespan by blue light exposure, senescence-accelerated mouse prone 8 (SAMP8) mice were used due to their short lifespan (approximately 1 year) and reported disruption of circadian rhythm, suggesting high sensitivity to blue light exposure. SAMP8 mice (N = 25 per group) were purchased at 3 weeks of age. After acclimation, mice were exposed to each light condition from 5 weeks of age: 1) 5,000K (high blue light component), 2) 3,500K (low blue light component), 3) blue light cut (no blue light component), and reared until the end of life. Room illuminance was maintained between 150 and 300 lux, and the lights were turned on from 7:00 AM to 7:00 PM. For measuring sleep parameters, we used SAMP8 and senescence-accelerated mouse resistant 1 (SAMR1) (as a control strain) (N = 8-10 per group). Each sleep parameter, including sleep duration (as a measure of sleep quantity), sleep episode duration (as a measure of sleep quality), and the circadian rhythm of sleep, was measured continuously using the non-invasive sleep monitoring system PiezoSleep at 27, 41, and 55 weeks of age. To ensure that changes in sleep parameters were due to light effects and not individual differences among groups, we measured sleep parameters at 4 weeks of age under a 5,000K light condition prior to exposure to specific light conditions. The data were analyzed using SleepStats v4 software. **Results:** The median life expectancy of SAMP8 mice was 50 weeks (5,000K), 71 weeks (3,500K), and 58 weeks (blue light cut). However, there were no significant differences among the groups. This result suggests that blue light exposure did not induce the severe shortening of lifespan reported in *C. elegans* and *Drosophila*, when tested in SAMP8 mice under standard rearing conditions. Regarding sleep, the duration of individual sleep episodes (sleep quality) showed age-dependent differences among strains. However, there were no significant differences among the light exposure groups. In terms of total sleep duration (sleep quantity), the data of SAMR1 mice showed that sleep during the dark period was significantly decreased in the 5,000 K group, which led to an increased light/dark (L/D) sleep ratio compared with the other groups at 27 and 55 weeks of age. Consistent with these observations, the circadian rhythm of sleep in SAMR1 mice differed between the 5,000 K group and the blue light-cut group, particularly at the beginning of the dark period. These changes were not observed in SAMP8 mice. **Conclusions:** In terms of evaluating the effect of blue light on lifespan, the current study using SAMP8 mice found no significant differences among groups with different blue light ratios. Regarding the effect of blue light on sleep, the sleep response to blue light exposure varied among strains. Blue light exposure reduced sleep duration at the beginning of the dark period and increased the L/D sleep ratio in a particular strain, suggesting that blue light exposure affects sleep patterns in certain populations.

PS 3855 Investigating the Role of the Aryl Hydrocarbon Receptor in Diet-Modulated Energy Metabolism and Weight Regulation in Male Mice

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Background and Purpose: Obesity is a major global health issue and a key risk factor for cardiovascular disease, type 2 diabetes, and cancer. However, many of the molecular mechanisms regulating body weight remain poorly understood. The aryl hydrocarbon receptor (AHR) has recently emerged as a contributor to energy balance. AHR is a ligand-activated nuclear receptor initially identified for mediating the toxicity of dioxins and polycyclic aromatic hydrocarbons, but it also regulates numerous genes with critical physiological functions. Emerging evidence suggests that AHR may influence energy metabolism, yet its precise role and significance remain unclear. The present study aimed to shed further light on the role of AHR in energy balance by examining the effects of global AHR deficiency in mice fed with either a standard diet (SD) or a high-fat diet (HFD). Previous studies have shown that a 30 % reduction in dietary tryptophan (TRP) increases energy expenditure in obesity-prone rats fed on a HFD, without affecting body composition. We hypothesized that this effect might be mediated by reduced AHR activity. To test this, we included a modified HFD with a 30 % lower TRP concentration (HFDtrp) in our study design. **Methods:** A total of 56 male AHR knockout (AHRKO; on C57BL/6J background) or wildtype (WT) mice (derived from C57BL/6J × AHR^{-/-}). These mice were fed either SD (10% of energy from fat), HFD (60% of energy from fat), or HFDtrp (60% of energy from fat), and were randomly assigned to six groups: WT-SD, AHRKO-SD, WT-HFD, AHRKO-HFD, WT-HFDtrp, and AHRKO-HFDtrp. All diets were purchased from Research Diets (USA), and the exposure lasted for 24 weeks. During weeks 16-17, the mice were subjected to glucose and insulin tolerance tests (GTT and ITT, respectively), and during weeks 18-20, their energy expenditure was determined by an indirect calorimetry. Whole-body composition measurements were carried out by a mini-spec analyzer on week 20. Upon study termination, total RNA was extracted from relevant tissues and reverse-transcribed to cDNA for real-time RT-qPCR analysis. **Results:** Body weight analysis showed no significant differences between genotypes on the same diet. AHRKO-HFD mice did not resist weight gain, similar to WT-HFD mice. Interestingly, the AHRKO-HFDtrp group showed the greatest weight gain, although no significant differences were observed among the HFD-fed groups. Relative liver weight was lower in AHRKO mice compared to WT, while epididymal white adipose tissue (EpiWAT) weights did not differ. Minispec analysis revealed no differences in body fat percentage among the HFD groups. Energy expenditure measured by

indirect calorimetry was also comparable across HFD-fed mice. Plasma chemistry analysis showed that all AHRKO groups had lower cholesterol and glucose levels than their WT counterparts. In liver tissue, *PPARα* expression was relatively lower in AHRKO mice compared with WT, and a similar trend was observed for brown adipose tissue *Ucp1* (uncoupling protein 1) expression. Histological analysis showed no significant differences in hepatic steatosis among the HFD-fed groups in either genotype. As expected, hepatic *Cyp1a1* mRNA abundance an index of AHR activity was significantly higher in WT compared with AHRKO mice. **Conclusions:** In contrast to previous reports, AHRKO mice did not resist weight gain when fed with HFD. Reduced dietary tryptophan also failed to attenuate weight gain. Energy expenditure was similarly unchanged across genotypes and diets. Hepatic steatosis levels were comparable across all HFD groups, indicating no genotype- or diet-specific effects. As expected, hepatic *Cyp1a1* expression was markedly lower in AHRKO mice, confirming reduced AHR activity. Together, these findings indicate that AHR deficiency does not protect against diet-induced weight gain under the present conditions and highlight a complex interaction between AHR signalling, dietary tryptophan, and metabolic regulation. Further investigation is needed to better understand the underlying mechanisms.

PS 3856 Loss of the Suppressor of Cytokine Signaling 1 (SOCS1) leads to progressive systemic inflammation in a SOCS1 inducible knockout adult mouse model

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Background and Purpose: The Suppressor of Cytokine Signaling 1 (SOCS1) is a negative regulator of the immune system constraining the response of IFN γ signaling making it a potential target for therapeutic strategies aimed at immunomodulation. SOCS1 constitutive knockout mice are normal at birth but exhibit stunted growth and die within three weeks after birth, with a syndrome characterized by severe lymphopenia, activation of peripheral T cells, fatty degeneration and necrosis of the liver, and macrophage infiltration of major organs. Furthermore, SOCS1 haploinsufficiency is associated with patients presenting immune dysregulation with multisystem autoimmunity and autoimmune disease such as systemic lupus erythematosus. To evaluate potential liabilities related to postnatal loss of SOCS1, we conducted 1- and 4- month long phenotype studies in a tamoxifen inducible SOCS1 knock-out adult mice. **Methods:** Mice carrying floxed SOCS1 gene, either in one allele (HET SOCS1) or in both (HOM SOCS1) were treated with tamoxifen (75 mpk) daily for 5 consecutive days to induce excision of SOCS1. Vehicle-treated mice with same genotype or tamoxifen-treated mice not carrying the Rosa26-CreERT2 transgene were used as negative controls. Groups contained 4-10 mice each, both females and males. Mice were terminated either 1 month or 4 months after tamoxifen administration. Study endpoints included clinical observations, body weight, blood chemistry and hematology, histopathology, T-cell activation by flow cytometry, serum cytokines and anti-dsDNA autoantibody levels measurement. **Results:** Phenotypic evaluation of HOM SOCS1 mice at 1 and 4 months revealed a progressive organ inflammation, with HET SOCS1 mice exhibiting only minimal findings, supporting a gene dosage effect. At 1 month, in the HOM SOCS1 mice, severe histopathological changes were primarily confined to the gastrointestinal tract, however, by 4 months, the pathology extended to multiple organs with mixed-cell and/or mononuclear cell infiltrates observed in bone marrow, spleen, liver, and skeletal muscle. HOM SOCS1 mice showed lack of body weight gain or mild weight loss 4 months post-tamoxifen, consistent with gastrointestinal inflammation. Clinical blood pathology profiles supported inflammatory histopathology findings, showing decreased A/G ratios, elevated neutrophils and eosinophils and reduced lymphocytes. HOM SOCS1 mice showed also increased T-cell activation both at 1 and 4 months post-tamoxifen. Cytokine analysis indicated progressive increases of IFN γ levels, a negative regulator of SOCS1, IL6 and TNF α . Additionally, a modest elevation in dsDNA autoantibody levels was observed in HOM SOCS1 mice at 1 month and in both HET and HOM SOCS1 mice 4 months post-tamoxifen. Metabolic changes were also evident, with sustained reductions in glucose levels and transcriptional alterations in insulin signaling, including decreased expression of the liver gluconeogenic rate-limiting enzyme phosphoenolpyruvate kinase (PCK1), potentially related to liver inflammation. Overall, HET SOCS1 iKO mice displayed only minor changes compared to the pronounced phenotypic and metabolic alterations observed in HOM SOCS1 iKO mice. **Conclusions:** In this study, postnatal SOCS1 knockout demonstrated a gene-dosage effect: homozygous mice exhibited progressive multi-organ inflammation, with the gastrointestinal tract showing the earliest onset and increasing regional spread and severity, whereas heterozygous iKO mice displayed only minimal changes.

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Background and Purpose: B lymphocyte-induced maturation protein-1 (BLIMP-1) is a transcriptional repressor that plays a pivotal role in the differentiation and homeostasis of immune cells. Given its central function in regulating B cell maturation, toxicological liabilities associated with lymphoma development were a significant concern. Notably, research suggests that loss of BLIMP-1 function may contribute to the pathogenesis of diffuse large B-cell lymphoma. In addition to its immunological roles, BLIMP-1 has been implicated in ocular development, specifically in the formation of the retina and the shaping of the corneal lens. However, its function in adult tissues remains largely unexplored. In light of these potential toxicities, we utilized a BLIMP-1 inducible knockout mouse model to systematically investigate the consequences of sustained loss of BLIMP-1, with a particular focus on lymphomagenesis and ocular pathology. **Methods:** To investigate on-target toxicities resulting from chronic BLIMP-1 suppression, a tamoxifen-inducible BLIMP-1 knockout mouse model was utilized. This model, obtained from the Walter and Eliza Hall Institute, enables conditional deletion of the floxed BLIMP-1 gene upon tamoxifen administration. Mice (n=10-20 mice/group/sex) at approximately 7 weeks old received daily tamoxifen dosing for five consecutive days. Clinical observations for all mice were recorded daily, and body weights were measured every other week. Due to declining animal health, humane euthanasia was performed between 5- and 7-months post tamoxifen-induction. At necropsy, blood samples were collected for flow cytometric analysis and target organ tissues were collected for gross and histological examination. **Results:** Female homozygous inducible knockout mice began exhibiting adverse clinical signs—including corneal ulcers, cervical lymphadenopathy and impaired weight gain—approximately 10 weeks after tamoxifen administration, and males showed similar signs beginning at week 19. Histopathological analysis revealed corneal lesions characterized by inflammation and squamous metaplasia. Neoplastic lesions were identified in the liver, lungs, mesenteric and mandibular lymph nodes, and spleen. Immunohistochemistry confirmed the presence of metastatic B-cell lymphoma and correlated with significant reductions in circulating B cells in both male and female inducible knockout mice. **Conclusions:** Histological findings corroborated the clinical observations, demonstrating that sustained loss of BLIMP-1 expression in adult mice was not tolerated and results in the development of metastatic B-cell lymphoma and ocular pathology that would likely result in blindness in humans. *All procedures were conducted in accordance with Bristol Myers Squibb institutional animal care and use protocols.*

PS 3858 Establishment of a mouse model of ischemic stroke and recurrence for preclinical studies

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Background and Purpose: Stroke is the second largest cause of death in the world. It is one of major diseases to cause a long-term disability of work. It has been estimated for stroke patients that about eighty percent of patients are ischemic stroke and the others are hemorrhagic stroke or other type. The economic costs or social burden are very huge after stroke. For three out of four patients a stroke is the first event, while one in four patients can experience a recurrent stroke. The preclinical and clinical data showed the potential contribution of environmental toxins to an increased risk of stroke. Several performed ischemic or hemorrhagic stroke animal models have been established. However, few animal models of recurrent ischemic stroke have been established because of their complicated procedures and high mortality rate. The aims of this study were to establish an improved two-stage middle cerebral artery occlusion (MCAO) protocol for ischemic stroke and recurrence. We also provided an example for investigating the effect of non-invasive low-intensity pulsed ultrasound (LIPUS), a therapeutic ultrasound, combined with aerobic exercise on ischemic recurrent stroke. **Methods:** Male C57 BL6J mice with six-week-old were used. An intraluminal filament technique was used to determine transient focal cerebral ischemia induced by MCAO. Mice were anesthetized. The common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were carefully exposed in the neck region. A 6-0 nylon filament was then inserted through the CCA into the ICA and advanced to the origin of the middle cerebral artery (MCA). The filament was left in place for about 40 min to block blood flow in the MCA. A second stroke surgery was performed 14 days after the first MCAO. A 6-0 nylon filament was reinserted through the external carotid artery into the MCA via the original CCA opening. This procedure could significantly reduce the mortality rate. The filament was again left in place for approximately 40 min to induce MCA occlusion, during which laser Doppler was used to monitor blood flow. LIPUS (100 mW/cm², 20 min daily to the cortical of brain) + exercise (treadmill aerobic training, 10-20 m/min, 10 min/2 times/day) were applied for 12 days after the first MCAO, and then the 2nd MCAO operation was carried out. All mice were euthanized on day 16 after initial MCAO. **Results:** We first observed mortality rates. In 1st MCAO, the survival rate was 80-100% at day 4 (n=10; two repeats). The survival rate reaches

up to 90% after the 2nd MCAO at day 16 (n=10). In 1st and 2nd MCAO mice, open field testing revealed marked reductions in movement patterns and exploratory behavior, and exhibited significantly decreased total travel distance and movement speed compared to original controls (n=8-10, p<0.05). The brain pathological changes in 1st and 2nd MCAO mice displayed a slight to moderate local neuronal cell necrotic loss with neuropil vacuolation, especially observed in the hippocampus and cortex areas; these severe lesions with hemorrhage were observed in 2nd MCAO (recurrent) mice (n=8-10, p<0.05). Moreover, we tested the effect of combined LIPUS with aerobic exercise rehabilitation on ischemic recurrent stroke. LIPUS + exercise significantly improved post-stroke mobility loss and significantly improved brain damage scores, including neuronal damage, vascular changes, and immune inflammatory infiltration (n=5, p<0.05). **Conclusions:** A two-stage MCAO mouse model for recurrent ischemic stroke, which significantly reduced locomotor activity, decreased movement speed, and diminished neurobehavioral scores compared to the first-time stroke and control, was established that may be applied to investigate the contribution of environmental toxins to an increased risk of stroke/recurrence or pharmacological studies for stroke/recurrence therapy. Using this model, we demonstrated that LIPUS + aerobic exercise rehabilitation offered potential benefits for ischemic stroke and its recurrence, laying the groundwork for future clinical applications.

PS 3859 Development and validation of a repeated exposure elastase-induced murine model for COPD and acute exacerbation of COPD

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Background and Purpose: Despite being a leading cause of death internationally, targeted treatment options for Chronic obstructive pulmonary disease (COPD) are limited. The long-term objective of this research is to evaluate the role of pathogen recognition receptors (PRRs) in COPD and acute exacerbation of COPD (AECOPD) to test a new therapeutic approach. To achieve this goal, this study aimed to establish and validate single-dose versus repeated-dose porcine pancreatic elastase (PPE)-induced murine models of COPD and AECOPD. Viral infections are among the most observed etiologies for AECOPD. A successful model is expected to exhibit an emphysematous phenotype, as evidenced by changes in lung function, increased lung immune cell infiltration, and alterations in lung gene and protein expression related to inflammation and extracellular matrix remodeling. **Methods:** Three different experimental designs of PPE exposure in C57BL6 mice were tested. Group 1 received 2IU PPE intranasally once per week for 3 weeks, followed by 3 weeks of rest. Group 2 received a single intratracheal dose of 1IU PPE and was sacrificed 3 weeks later. Group 3 received 0.2IU PPE intratracheally once per week for 4 weeks and was given two weeks of rest after the final dose. A portion of Group 3 also received 4mg/kg of TLR7 agonist, imiquimod, intranasally 24 hours prior to sacrifice. Healthy, unexposed mice were used as controls. Lung function was monitored over time with Whole Body Plethysmography. At the end of the experimental period, detailed lung function assessments were performed using FlexiVent Systems that included both forced oscillation-based and negative pressure-driven forced expiratory assessments. Cytology of bronchoalveolar lavage (BAL) was performed on all mice, and lung tissue was collected for mRNA expression analysis by qPCR. **Results:** Mortality within 48 hours following a PPE exposure occurred in 45.8% and 72.2% of mice exposed to 2IU of PPE or 1IU of PPE, respectively. No mortality was observed in mice receiving 0.2IU PPE with or without imiquimod. In all exposure schemes, PPE increased the number of macrophages, neutrophils, and lymphocytes in the BAL. Relative to controls, respiratory elastance and the ratio of forced expiratory volume to forced vital capacity (FEV:FVC) significantly decreased in mice exposed to 2IU PPE and 0.2IU PPE. Additional exposure to imiquimod in 0.2IU PPE-exposed WT mice further decreased the FEV:FVC ratio. **Conclusions:** The dosing schemes using 2IU PPE and 0.2IU PPE induced a COPD-like phenotype by pulmonary immune infiltration and respiratory dysfunction. However, the dosing scheme of four intratracheal exposures to 0.2IU PPE is a better model for our experimental needs due to animal mortality. Following imiquimod exposure in PPE-exposed mice, the further decrease in lung function by the FEV:FVC ratio suggests that this may be a promising model for AECOPD. Going forward, this model will be helpful for mechanistic assessment of the role of PRRs on COPD and AECOPD.

PS 3860 Identification of Liver-Specific Extracellular Vesicle (EV) RNAs as Biomarkers of Hepatotoxicity in CD9-EGFP Reporter Mice

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Background and Purpose: Extracellular vesicles (EVs), including exosomes, are secreted from cells and circulate in the bloodstream. EVs contain nucleic acids such as mRNA, microRNA (miRNA), and DNA, and function as mediators of inter-cellular communication. In recent years, miRNAs derived from human tumor-spe-

cific EVs have been reported as biomarkers with diagnostic accuracies exceeding 90%. Furthermore, we and others have demonstrated that EV-derived miRNAs in body fluids are useful biomarkers of chemical-induced toxicity [1,2]. We generated a Cre-inducible CD9-EGFP knock-in mouse line targeting the Rosa26 locus and established a mouse model that expresses human CD9-EGFP specifically in the liver. The aim of this study was to isolate liver-derived EVs using this model and to characterize the liver-specific EV miRNA profile by transcriptomic analysis. **Methods:** Male CD9-EGFP knock-in (flox/WT) mice were crossed with female Alb-Cre mice to generate flox/Alb-Cre (liver-specific CD9-EGFP expression) and WT/Alb-Cre mice. At 10 weeks of age, mice were administered a single oral dose of carbon tetrachloride (CCl₄, 70 mg/kg) or vehicle (corn oil). Twenty-four hours after dosing, serum was collected and liver-derived EVs were isolated by immunoprecipitation using an anti-human CD9 antibody. Total RNA was extracted from these EVs and subjected to transcriptomic analysis. **Results:** By using an CD9-EGFP knock-in mouse, we successfully isolated EVs restricted to those secreted from the liver, enabling us to determine whether the candidate biomarker EV RNAs truly originate from hepatotoxic injury or instead from other organs. Furthermore, RNA-seq-based expression profiling of liver-derived EVs allowed us to identify 20 highly sensitive biomarkers of hepatotoxicity. **Conclusions:** By using this mouse line, we successfully isolated EV RNAs that serve as bona fide liver-derived biomarkers of hepatotoxicity. These findings are expected to greatly contribute to the refinement of toxicity testing and to the 3Rs (replacement, reduction, and refinement) of animal welfare. Furthermore, by crossing this animal model with other organ-specific Cre driver mice, the same strategy can be used to efficiently isolate toxicity biomarkers for a variety of organs. **References:** [1] Ono R., et al. : Novel hepatotoxicity biomarkers of extracellular vesicle (EV)-associated miRNAs induced by CCl₄. *Toxicol Rep.* 2020; 7: 685-692. [2] Ono R., et al. : Extracellular vesicle small RNAs secreted from mouse amniotic fluid induced by repeated oral administration of VPA to pregnant mice. *Fundam. Toxicol. Sci.* 2024; 11(1): 37-56.

PS 3861 A Machine-Learning Virtual Histology Tool Reduces Animal Use and Captures Injury Dynamics in Toxicology Studies

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Background and Purpose: Heterogeneity in preclinical toxicology studies produces noisy data requiring large sample sizes and the desired endpoint histology can often only be obtained by terminal cull. Serial histology without sacrifice would reduce variability and animal use. We hypothesized that circulating biomarkers quantitatively correlate with tissue injury severity over time, enabling non-invasive histological injury quantification. However, biomarker-injury relationships are often non-linear and time-dependent, requiring flexible modelling approaches **Methods:** Under local ethical approval (093-LFR-24), fifty 12-week male C57BL6/J mice (Charles River, UK) received 350mg/kg intraperitoneal acetaminophen. Serial 40microl tail-vein microsamples (n=6 timepoints over 48h) were obtained to quantify alanine aminotransferase (ALT) and microRNA-122 (miR-122). Pre-defined groups were culled for tissue correlates at each timepoint. A necrosis prediction model was derived using Generalized Additive Modelling with Leave-One-Out Cross-Validation to integrate time and biomarker kinetics to predict individual mouse hepatic necrosis fractional area. **Results:** Cull hepatic necrosis ranged from 0.02-0.80 (median 0.32, IQR 0.23-0.48). The serial microsampling group showed consistent variability across timepoints (mean longitudinal ALT AUC CV 35.6±1.8%). In contrast, terminal sample necrosis showed high inter-timepoint variability (mean CV 38.7±28.9%), reflecting between-animal heterogeneity that serial cull designs cannot capture. Virtual histology derived from longitudinal biomarker kinetics achieved a Mean Absolute Error (MAE) of 7.88% (ALT) and 9.41% (miR-122). Power analysis demonstrated that use of the ALT model would allow equivalent statistical power to be achieved with 64-89% fewer animals depending on sampling frequency and desired effect size. Use of a machine learning approach improved the MAE by 13.6% compared to linear regression approaches. **Conclusions:** This virtual histology approach enables non-invasive, serial quantification of hepatic necrosis in individual mice, directly supporting 3Rs principles. The approach is potentially applicable to any frequently used animal model where there is a tissue injury of interest and a known tissue biomarker. Replacing terminal culls with longitudinal monitoring provides a more ethical, efficient, and statistically robust framework for preclinical toxicity and efficacy testing, without loss of the biological complexity captured by animal models. Additionally, capturing the injury trajectories from onset to resolution provides crucial insights into individual response heterogeneity and builds a clearer translational bridge to the serial monitoring designs used in clinical practice. The high-resolution datasets produced also provide a more confident basis for critical 'go/no-go' decisions in preclinical development, de-risking candidate selection and accelerating timelines.

PS 3862 Modulatory effects of glutamate transporter enhancers in alcohol-related liver injury in alcohol preferring rats

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Background and Purpose: Prolonged ethanol use can induce behavioral and physiological alterations, along with affecting liver's capacity to metabolize ethanol, leading to injury and damage to hepatocytes. MC-100093, a beta-lactam non-antibiotic compound, possesses glutamate transporter-1 (GLT-1) modulatory capabilities, and demonstrated a reduction in ethanol consumption. **Methods:** Male and female P rats were exposed to ethanol for six weeks and were divided into four groups. On week six, rats received intraperitoneal injections of saline or MC-100093 (100 or 150 mg/kg) for five days consecutively. **Results:** MC-100093 effectively attenuated ethanol-induced liver injury by decreasing ethanol consumption and normalizing the expression of GLT-1, cystine/glutamate antiporter (xCT) and lipid and collagen type 1 (COL-1) in liver of male and female rats. Moreover, MC-100093 reversed ethanol-induced inflammation, and reduced triglyceride contents in the liver. **Conclusions:** Our data demonstrate that MC-100093 is effective in mitigating ethanol-induced liver injury.

PS 3863 Cytological Composition of Bone Marrow in Athymic Nude RH-FoxN1^{tmu} Rats

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Background and Purpose: Long-term pharmacology and safety evaluation of innovative human-derived cell therapy products require the use of immunocompromised animals to avoid immune-mediated destruction of injected human-derived cells. Athymic Nude rats represent an interesting alternative to broadly used Nude mice, particularly when implantation surgery is required and animals of larger size are preferred. **Methods:** Cytological composition of the bone marrow of 28 clinically healthy RH-FoxN1^{tmu} nude rats (11 males, 17 females) was determined as follows: complete differential counts, morphological assessment and calculation of myeloid:erythroid ratios. **Results:** Percentages of each cell type were obtained and myeloid:erythroid ratios were calculated (0.66 to 3.16, mean 1.62). Morphological characteristics of erythroid, granulocytic and lymphoid populations were obtained. **Conclusions:** Increase in the proportion of eosinophils (all stages), increase in emperipolesis of neutrophils on megakaryocytes and decrease in lymphocyte proportion were observed, in comparison to other commonly used rat strains. These results provide preliminary data of cytological bone marrow findings in athymic nude RH-FoxN1^{tmu} rats.

PS 3864 Interaction between pyrethroid exposure and Shank3 genotype on neurodevelopmental disorder-relevant behaviors in prairie voles

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Sponsor: J. Burkett

Background and Purpose: Developmental pesticide exposure has been linked to an increased risk of neurodevelopmental disorders such as autism spectrum disorder and attention deficit hyperactivity disorder (ADHD). Developmental pyrethroid pesticide exposure in mice and prairie voles causes a broad behavioral phenotype relevant to autism and ADHD. SHANK3 is among the strongest candidate genes for autism, with brain mechanisms overlapping with the effects of developmental pyrethroid exposure in animal models. Whether SHANK3 genotype interacts with developmental exposure is unknown. **Methods:** In this study, prairie vole mothers were administered one of four doses of the pyrethroid deltamethrin (0, 0.3, 1, or 3 mg/kg Q3D PO) throughout pre-conception, gestation, and lactation. We used a litter-based design with heterozygous breeding to produce one wild-type and one Shank3 +/- mutant offspring per litter. Adult offspring were assessed in hyperactivity, repetitive behaviors, and circadian rhythms. **Results:** Results related to differences in dose-response between siblings of different genotypes will be interpreted to evaluate both direct pesticide effects and genotype-by-environment interactions. **Conclusions:** These findings have the potential to inform on genetic susceptibility to developmental pesticide exposures that are a risk for autism and ADHD.

PS 3865 A comparative study of ocular anatomy in 3 different laboratory Swine strains

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Background and Purpose: The use of laboratory swine in ophthalmic research has grown considerably due to their anatomical and physiological similarities to humans. This study was designed to confirm quantitatively the similarity of ocular structures,

both in size and weight, between Sinclair Nanopigs (SN) comparable to those of the well-established Gottingen Minipigs (GM) and Yucatan pigs (YP). **Methods:** A total of 32 animals (15 SN, 12 GM, and 5 YP), with ages ranging from one- to thirty-months old, were used in this study. Eyes were either dissected (24 SN eyes, 10 GM eyes, and 6 YP eyes) or fixed in 10% formaldehyde (6 SN eyes, 14 GM eyes, and 4 YP eyes) overnight for evaluation. Dissected eyes had the following tissues collected and weighed: aqueous and vitreous humor volumes, cornea, iris/ciliary body, lens, trabecular meshwork, retina, choroid/RPE and sclera. Fixed eyes were sectioned sagittally for measurement of anteroposterior (axial) and transverse (horizontal) globe lengths, cornea diameter, anterior and posterior segment depths, and lens (thickness and diameter). All measurements were analyzed using ANOVA and Tukey-Kramer Test. **Results:** Within our preliminary results, no significant differences were observed in axial globe length, lens width/depth, and anterior segment depth between SN, GM, and YP. Although minimal, statistically significant differences were observed in transverse globe length (21.9 ± 2.22 mm SN, 20.3 ± 0.74 mm GM, 23.3 ± 0.82 mm YP), corneal diameter (13.0 ± 1.46 mm SN, 13.4 ± 0.67 mm GM, 15.3 ± 0.52 mm YP), and posterior segment depth (9.3 ± 0.82 mm SN, 10.5 ± 0.85 mm GM, 11.0 ± 0.82 mm YP). **Conclusions:** In conclusion, while statistically significant differences were observed in some globe measurements among all 3 species, eyes from all 3 species are comparable in weight and size and equally suitable for use in ocular studies.

PS 3866 Comparative Assessment of Oral Gavage, Intravenous, and Subcutaneous Dosing Routes on Maternal Body Weight and Food Consumption in Rabbit Embryo-Fetal Development Studies

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Background and Purpose: The rabbit embryo-fetal development (EFD) study is a cornerstone of reproductive toxicology assessment, yet its execution is often complicated by the rabbit's intrinsic sensitivity to handling and dosing procedures. Although oral gavage (PO), intravenous (IV), and subcutaneous (SC) administration are the most commonly employed dosing routes, systematic comparisons of their impact on maternal body weight (BW) and food consumption (FC) - two critical indices of maternal tolerance - are limited. **Methods:** To address this gap, we retrospectively analyzed aggregated data from 172 rabbit EFD studies (2,390 animals), focusing on control group animals dosed via PO, IV, or SC routes. Dosing occurred during gestational days (GD) 6-19, followed by an undosed observation period (GD20-29). **Results:** Results demonstrated no statistically significant differences among dosing routes in maternal BW or FC during either the dosing (GD6-19) or post-dosing (GD20-29) periods. Across all routes, BW exhibited an upward trajectory from GD6-29, with PO animals showing the smoothest BW profile during the dosing period, followed by SC and IV. Mean daily FC followed a "U-shaped" pattern during GD6-20, with the nadir at GD12-16. SC-dosed animals exhibited the highest mean FC, while PO-dosed animals had the lowest. During GD20-29, mean daily FC declined progressively across all routes, reaching the lowest levels at GD25-29. Median FC trends paralleled the means, while PO-dosed animals demonstrated lower values with sharper mid-gestation (GD11-17) declines. **Conclusions:** In conclusion, these dosing routes did not materially influence maternal BW outcomes in rabbit EFD studies. However, FC was modestly affected, with PO exerting the greatest impact, followed by IV and SC. These findings support the interpretation that dosing route exerts minimal influence on maternal tolerance parameters and reinforce the robustness of rabbit EFD studies across standard administration routes.

PS 3867 Cytological Bone Marrow Cell Differential Counts and Morphological Findings in the Common Marmoset (*Callithrix jacchus*)

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Background and Purpose: Cytological bone marrow evaluation is performed in nonclinical toxicological studies to identify potential effects of tested compounds on hematopoiesis. The common marmoset (*Callithrix jacchus*) is a non human primate species used in biomedical research for over 30 years. Due to its small size and limited supply of other NHP species, the number of marmosets used in toxicological studies is expected to increase. The purpose of this poster is to present baseline bone marrow cytological quantitative and qualitative preliminary data. **Methods:** Bone marrow smears were prepared from the proximal femur from 26 healthy control marmosets (13 males and 13 females, 2-4 years old). Total population numbers and percentages of each individual cell type and myeloid:erythroid ratios were obtained and calculated following microscopic examination. **Results:** Bone marrow smears were of high quality. M:E ratios ranged from 0.61 to 1.77. Percentages of granulocytic (31% to 58%), erythroid (32% to 58%) and lymphocyte (5%-22%) series were obtained. Morphologic characteristics of each series were described and included: large to giant neutrophilic metamyelocytes and band neutrophils and very rare ring-shaped nuclei in the granulocytic series, and nuclear blebbing or lobulation with variable number of shapes and binucleated metarubricytes in erythroid series. Quantitative and morphological findings were similar to those observed in Cynomolgus monkeys. **Conclusions:** Bone marrow smears from the proximal femur are adequate for cytological bone marrow assessment in marmo-

sets. These results demonstrate technical and scientific appropriateness of cytological bone marrow evaluation in the common marmoset and provide preliminary data regarding background morphological findings.

PS 3868 Comparison of baseline levels of biomarkers commonly used to assess drug candidate safety, efficacy, and mechanism of action in Cynomolgus Monkeys of Cambodian, Chinese, or Cambodian-Chinese Mixed Origin bred in a US-based Research Facility

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Background and Purpose: Cynomolgus monkeys (*Macaca fascicularis*) are a commonly used nonhuman primate model in preclinical toxicology research. Preclinical drug programs predominantly utilize cynomolgus monkeys of a single origin (Southeast Asian or Mauritian) to reduce experimental variability; however, a restriction in origin poses supply challenges. Here, we explore the use of mixed origin cynomolgus monkeys born at a US breeding facility by (1) characterizing standard biomarkers used to evaluate drug candidates' effects (clinical pathology, peripheral blood lymphocytes [by immunophenotyping], complement protein fragments, and cytokines) in Cambodian-Chinese cynomolgus monkeys, and (2) comparing the biomarkers to cynomolgus monkeys of Cambodian alone or Chinese alone origin. **Methods:** A total of thirty, research naïve, juvenile cynomolgus monkeys (n=10 per origin) were sampled. Clinical pathology parameters were quantified using standard diagnostic equipment; and peripheral blood lymphocytes, complement protein fragments, and cytokine levels were quantified using immunoassays. **Results:** Preliminary data suggests that there are no significant differences in baseline levels of biomarkers between cynomolgus monkeys of Cambodian, Chinese, or Cambodian-Chinese mixed origin bred in a US-based Research Facility. **Conclusions:** These findings are important because they support the use of mixed-origin cynomolgus monkeys born at a US breeding facility in preclinical toxicology research.

PS 3869 African Green Monkey Nonhuman Primate Periodontal Exam Scoring System (NHP-PESS)

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Background and Purpose: Comprehensive assessment of the periodontium is essential for accurate evaluation of oral health and disease progression. In translational research, nonhuman primates (NHPs) allow modeling of periodontal pathology and interventional testing given clinically relevant anatomy and physiology and naturally incident periodontitis. The St. Kitts African green monkey (*Chlorocebus sabaeus*) represents a particularly valuable species for this purpose, offering accessibility and limited genetic diversity. **Methods:** To establish a standardized approach periodontal scoring a "Nonhuman Primate Periodontal Exam Scoring System" (NHP-PESS) was developed by adapting the human "Periodontal Screening and Recording" (PSR) system. The scoring relies on a color-coded periodontal probe with 3 - 6 - 8 - 11 mm reference bands, as described by Miller [1950, Textbook of periodontia (oral medicine). Blakiston], allowing rapid visual assessment of periodontal pocket depth. Additional criteria were incorporated to increase precision and cross-study comparability, including the modified Gingival Index (MGI) for inflammation, Rustogi Plaque Index (RPI) for plaque distribution, and mobility classification. **Results:** The NHP-PESS employs a five-code structure (Codes 0-4) derived from the PSR methodology. Each code corresponds to specific ranges of probing depths, gingival bleeding, calculus presence, gingival inflammation, and tooth mobility, providing a rapid visual and concrete scoring framework: Code 0: Probing depth 1-3 mm, no calculus, healthy gingiva (MGI 0), mobility 0. Code 1: Probing depth 1-3 mm, mild inflammation, bleeding after probing, mobility 0. Code 2: Probing depth 1-3 mm, supragingival or subgingival calculus, mild to moderate inflammation, mobility 0. Code 3: Probing depth 3-6 mm, calculus present, moderate inflammation, mobility I-II. Code 4: Probing depth ≥ 6 mm, calculus present, severe inflammation, mobility II-III. The NHP-PESS framework provides an efficient method for classifying periodontal health and disease states in nonhuman primates. By prioritizing clinically relevant parameters and alignment to human PSR methodology, the system supports rapid disease staging while maintaining inter-observer consistency for translationally relevant data development. Incorporating the MGI and RPI further supports the integration of standard oral evaluation measurements in translational oral health outcome focused studies. The structured coding minimizes the need for extensive charting and reduces examination time per subject, improving reproducibility and workflow standardization. **Conclusions:** The "Nonhuman Primate Periodontal Exam Scoring System" provides a concise, objective, and reproducible framework for assessing periodontal health in different preclinical studies settings, bridging the gap between veterinary and clinical periodontal diagnostics. This method enhances welfare monitoring, supports longitudinal assessments of disease progression, and establishes a foundation for integrating clinical, histopathological, and molecular endpoints in translational research. Future validation will focus on correlating NHP-PESS scores with systemic inflammatory biomarkers and histopathological outcomes.

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Background and Purpose: Approximately 18,000 individuals in the US sustain a spinal cord injury (SCI) every year (National Spinal Cord Injury Statistical Center - NSCISC) with over 300,000 people living with long-term impairments. Despite medical advances there are no effective treatments for SCI. This highlights the importance of a clinically relevant nonhuman primate for translational research. Such a model is essential for bridging the gap between rodent studies and human trials, as it more accurately replicates the complexities of human SCI. African green monkeys (AGM) provide a better test system for spinal injury preclinical studies as they better approximate human SCI than rodent models, and they accommodate assessment of multiple recovery variables and rehabilitative therapy. The current contusion devices on the market provide standardized impact forces that may be sufficient for rodent models due to their relatively uniform spinal cord dimensions; however, this approach is inadequate for larger species like nonhuman primates (NHP), which exhibit significant variability in spinal cord size and anatomy. Here, we present a novel SCI cervical hemiconusion model to accurately mimic human SCI using the novel *High-precision in vivo tissue test system* (HITT) developed by the Neurospine lab (SFU). This system allows for individualized impact parameters based on pre injury MRI measurements of each subjects spinal cord. **Methods:** Twenty-six African Green Monkey (4 - 12 years, 5-8 kg for males; 3.5-7 kg for females) underwent a pre injury MRI one week before contusion. The subjects were placed in two different cohorts based on preclinical safety assessment timelines. Cohort 1 (n=11, 6 males and 5 females), three months recovery, and cohort 2 (n=15, 7 males and 8 females) nine month recovery. The subjects received a cervical spinal cord hemiconusion at the C5 vertebral level (C7 spinal level) on the right side. During the injury surgery we performed a C5 laminectomy to expose the spinal cord. The spinal column was then stabilized by clamping the C3/4 and C6/7 spinous processes (Large Animal Spinal Clamps, Kopf Scientific) to isolate the mechanical loading to the spinal cord. The impactor rod was placed on midline and moved from 0.1 to 0.5 mm over the midline (depending on spinal cord size). The impactor rod was lowered to displace the dorsal and ventral CSF and to trap the spinal cord at the bottom of the spinal canal. Then a controlled hit with set displacement was achieved. Post injury, an acute intraoperative ultrasound was performed to visualize the unilaterality and extent of the injury. The animals underwent recovery for 3 and 9 months post injury as part of a GLP, IND-enabling study. **Results:** All animals survived the contusion surgery and underwent post-surgery recovery. Of the 26 animals studied, 24 exhibited rapid post-operative recovery, sitting upright within hours of surgery and presenting with a contained uniform motor deficit localized to the right arm and hand. In contrast, two animals remained recumbent for approximately 48 hrs: in one monkey this was due to more severe impact forces that delayed but did not prevent their ability to sit upright, and in the second case preference for the recumbent position despite a mild impact (the monkey subsequently sat up). All of the lesions were restricted to the right side, as unilateral injuries are important for monkey well-being and long term care. Measurements from the acute intraoperative ultrasounds revealed that injury extent ranged from 35.1% ± 2.5% (SE) to 44.0% ± 2.2% of the entire spinal cord width. Functional observations showed a consistent deficit in all animals at 14 days post injury, with a right flaccid hand held in a flexed posture with minimal to no voluntary movement two weeks post-injury. **Conclusions:** Here we present a novel NHP primate hemiconusion injury model in the African green monkey population. Subject-specific injury parameters that are based on MRI measurements of lesion laterality and extent lead to a functional deficit that is consistent. This rigor and consistency of this model is useful for developing and testing candidate therapeutic interventions for SCI.

PS 3871 Spondylosis and intervertebral disc degeneration in African green monkeys (*Chlorocebus sabaeus*)

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Background and Purpose: Intervertebral disc degeneration (IVDD) is a major cause of lower back pain in aged individuals and has significant negative impact on quality of life and productivity. Over one hundred million lost workdays and over 100 billion total costs are estimated per annum in the United States due to low back pain, making it a major clinical and public health problem with significant economic consequences. While the entire pathogenesis is unclear, IVDD in humans is multifactorial with age, gender, smoking, and nutrition being contributing factors. Disc degeneration also occurs naturally in the rhesus macaque (*Macaca mulatta*) in the thoracolumbar region, mimicking many aspects of human IVDD; in rhesus as well as humans, age is an important factor for lesion development and typical lesions include disc narrowing, degeneration, and osteophytosis. We have defined similar findings in African green monkeys (AGMs, *Chlorocebus sabaeus*). **Methods:** Three aged female AGMs (all greater than 17 years old) from our colony were incidentally noted to have lesions consistent with spondylosis and intervertebral disc disease at necropsy (e.g. thickened/enlarged and bridging bone at the ventral aspect of

thoracolumbar vertebral bodies), supporting relevance as a translational species for age associated pathologies and significant overlap with human and other NHP models in anatomy and pharmacology. One of these three was reportedly kyphotic, and another had experienced an episode of difficulty ambulating three years prior to necropsy. Otherwise, there were no relevant clinical signs or findings. The spinal columns were collected, fixed, decalcified, sectioned, and stained with hematoxylin and eosin (H&E). **Results:** Microscopic examination of two animals confirmed the gross findings of bridging ventral osteophytosis and dense fibrous connective tissue (spondylosis), as well as degeneration of the intervertebral disc with characteristic disc thinning, disc tears, and bulging. **Conclusions:** Aged AGMs represent a potential naturally occurring model of spondylosis and intervertebral disc disease, with similar gross and microscopic findings to those reported in humans and other NHPs. Further characterization will focus on the incidence and spectrum of disease in this population, with attention to age and sex, as well as MRI evaluation of, and screening for, IVDD in this species.

PS 3872 Male rhesus macaques fed a Western diet are susceptible to bone loss caused by both chronic binge alcohol and infection with simian immunodeficiency virus with subsequent antiretroviral therapy

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Background and Purpose: People living with HIV (PWH) are at an increased risk of low bone mineral density (BMD), osteoporosis, and bone fractures. HIV infection, antiretroviral therapy (ART), and high alcohol consumption are factors negatively affecting bone health. We and others have established that alcohol exerts a plethora of toxic effects on the skeleton by inhibiting bone formation and stimulating bone resorption, resulting in diminished BMD and bone strength. A further confounding effect on the skeletal health of PWH is the impact of diet, such as a high-fat Western diet. In this study, the aim was to address how these factors interact on parameters of bone health in a non-human primate model. **Methods:** Adult male rhesus macaques (*Macaca mulatta*) were fed a high-fat Western diet (42% carbohydrates (27% sucrose), 42% fat, 16% protein in % kcal) throughout the length of the study. After 4 months on the Western diet, chronic binge alcohol (CBA) at 13 - 14 g ethanol/kg/week or vehicle (VEH) were administered via intragastric catheters and continued for the remainder (1 year) of the study. 4 months after initiation of CBA/VEH infusion, macaques were either infected with simian immunodeficiency virus (SIV) or mock infected. All SIV-infected macaques received ART at a daily intragastric dose of 50 mg bictegravir, 200 mg emtricitabine, and 25 mg tenofovir alafenamide, starting 10 weeks after SIV infection. There were four experimental groups: VEH/SIV-; VEH/SIV+, CBA/SIV- and CBA/SIV+ (N = 7, 5, 5, and 6, respectively). The macaques were sacrificed after an additional 6 months. BMD of the whole body, humerus, thigh, and abdominal area was measured by DXA at four time points (pre-alcohol, pre-SIV infection, pre-ART, and at sacrifice). Whole body bone turnover was assessed by ELISA of serum bone formation marker P1NP and bone resorption marker CTX-1. The tibiae were collected at necropsy, and bone microstructure and geometry determined by microcomputed tomography (μCT). Gene expression in the fibula shaft was analyzed by qRT-PCR. **Results:** Over the course of the experiment, DXA revealed a significant decrease in BMD with time (P < 0.001 for all regions). The decrease was smallest for the VEH/SIV- group, especially between the pre-SIV and necropsy time point, for which the BMD decrease of the thigh area was significantly lower (P < 0.05) in the VEH/SIV- group than in the VEH/SIV+ group. μCT of the tibia revealed a significant decrease in the relative bone volume of the trabecular compartment (BV/TV) by both CBA and SIV+/ART (P < 0.001) without any apparent additive effects. The differences in BV/TV were explained, in part, by increased trabecular separation induced by CBA in non-infected macaques (P < 0.01) and the small but not significant changes in trabecular number. The changes measured by μCT were limited to the trabecular compartment, since no cortical bone parameters were changed by any treatment. The between-subject variation in serum concentrations of P1NP and CTX-1 was too large to yield statistically significant group differences. Gene expression measured in the midshaft area of the fibula showed a significant increase in RANKL mRNA expression by both CBA and SIV+/ART (P < 0.05) without additive effects, and a trend of increased expression of the osteoclast marker Ocstamp by SIV/ART (P = 0.07 for the main effect). **Conclusions:** Both CBA and SIV/ART contributed to decreased bone mass in the trabecular compartment of the tibia. Increased expression of RANKL by both CBA and SIV/ART likely increased osteoclast activation and bone resorption contributing to loss of bone mass. No clear additive or synergistic effects of CBA and SIV/ART were observed on any of the measured parameters. Support: NIH P60AA009803 (PM), R37 AA018282 (MR).

PS 3873 Perfluorooctanesulfonamide-induced epiboly delay is associated with decreased ATP production within zebrafish embryos

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) have been widely used within various industrial processes and consumer products, such as food packaging and fire-fighting foams. PFAS persistence within environmental media has led to ubiquitous exposure within humans and wildlife. We previously conducted a high-content toxicity screen of 38 different PFAS within the first 72 h of zebrafish development. This screen identified perfluorooctanesulfonamide (PFOSA) as the most potent developmental toxicant among the 38 PFAS tested. At non-lethal concentrations, exposure to PFOSA from 0.75 to 6 h post-fertilization (hpf) - a developmental window that includes cleavage, blastulation, and early-gastrulation - resulted in a concentration-dependent delay in epiboly (blastoderm migration and spreading) by 6 hpf. Therefore, for this study, we determined whether 1) PFOSA delays epiboly by inhibiting actin polymerization within the yolk, as epiboly progression is reliant on yolk-associated actin networks, and/or 2) PFOSA-induced effects are associated with a decrease in embryonic ATP, as PFOSA is a potent uncoupler of oxidative phosphorylation *in vitro*. **Methods:** To accomplish this objective, we first tested whether there was an association between the uptake of PFOSA into the developing embryo, and whether the timing and magnitude of PFOSA-induced epiboly delay were similar to CCB-induced epiboly delay. Next, we employed phalloidin-based staining of F-actin and high-resolution laser-scanning confocal microscopy to determine if CCB and PFOSA inhibited yolk-associated actin polymerization throughout the entire embryo. Third, using mRNA-sequencing, we tested whether the potential impacts of PFOSA on the transcriptome were similar to CCB during epiboly progression. Finally, we determined whether 1) PFOSA-induced epiboly delay was associated with a decrease in embryonic ATP and 2) co-exposure to exogenous ATP mitigated PFOSA-induced delayed epiboly. **Results:** Although PFOSA and CCB induced a similar magnitude of epiboly delay beginning at 4 hpf following initiation of exposure at 0.75 hpf, PFOSA did not, contrary to CCB, significantly decrease yolk-associated actin within embryos. Using mRNA-sequencing, we also found that, consistent with chemical-specific differences in effects on actin polymerization, PFOSA-exposed embryos were also transcriptionally different from CCB-exposed embryos. Moreover, phenotypically matched, PFOSA-exposed embryos at 6 hpf were transcriptionally similar to vehicle-exposed embryos at 5 hpf, and PFOSA delayed the maternal-to-zygotic transition (MZT) beginning at 5 hpf. Finally, PFOSA-induced delayed epiboly was associated with decreased ATP levels *in vivo*, an effect that was partially mitigated by co-exposure to exogenous ATP. **Conclusions:** Overall, our findings suggest that PFOSA exposure during early embryonic development decreases ATP production in the absence of effects on actin polymerization *in vivo*, an effect that is associated with PFOSA-induced delays in epiboly and the MZT.

PS 3874 Perfluorononanoic acid (PFNA) causes hepatomegaly while inhibiting hepatocyte proliferation independently of PPAR α and CAR in mice

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Background and Purpose: Per- and polyfluoroalkyl substances (PFASs), including perfluorononanoic acid (PFNA, C₉), are a group of synthetic chemicals once widely used for industrial purposes and in consumer products. Because of their extraordinary resistance to degradation, PFASs are universally detected in the environment and human body, thus posing toxicological concerns. Once inside the body, PFASs accumulate in the liver, interrupting hepatic functions. Among PFASs, PFNA causes hepatomegaly in the mouse liver, but the underlying mechanism is not well understood. The present study aimed to investigate how PFNA affects liver homeostasis with respect to hepatocyte proliferation by employing PPAR α -null and CAR-null mouse models. **Methods:** A single dose of PFNA (0.1 mmol/kg) was intraperitoneally administered to WT, PPAR α -null, and CAR-null mice (n = 5/treatment/genotype). Two weeks later, mouse livers were collected for gross assessment, histology, gene expression analysis, and immunoblotting. In addition, human Huh7 hepatocellular carcinoma (HCC) cells were chosen as the *in vitro* model to complement the *in vivo* mouse studies. Huh7 cells, in quadruplicates, were treated with 10, 30, and 100 μ M PFNA for 7 days, followed by assessment of cell viability, proliferation state, cell size, gene and protein expression. **Results:** PFNA increased the liver-to-body weight ratio by 2.6-, 2-, and 1.8-fold, respectively, in WT, PPAR α -null, and CAR-null mice. The H&E staining analysis of liver sections showed that PFNA increased the size of hepatocytes (WT, 1.4-fold; PPAR α -null, 2.9-fold; CAR-null, 2-fold; of respective controls) but decreased the number of hepatocytes within the same microscopic field (WT, 52%; PPAR α -null, 49%; CAR-null, 67%; of respective controls). The hepatomegaly effect seemed to be due to cellular hypertrophy rather than hyperplasia of hepatocytes in all three genotypes of mice. Such a hypertrophic effect was also observed in PFNA-treated Huh7 cells. PFNA concentration-dependently decreased Huh7 cell number, with more than 70% decrease at 100 μ M, but increased cell size (10 μ M, 1.2-fold; 30 μ M, 1.8-fold; 100 μ M, 2.7-fold; compared to control). In addition, in the mouse liver, PFNA altered the expression of genes involved in the regulation of hepatocyte proliferation and/or hypertrophy. Specifically, PFNA induced the mRNA expression of growth arrest and DNA damage-inducible beta (Gadd45b) by 3.5-, 5.9-, and 5.4-fold, respectively, in WT, PPAR α -null, and CAR-null mouse livers.

PFNA increased the mRNA expression of cyclin-dependent kinase inhibitor 1 (p21) by 1.4-, 4.2-, and 3.6-fold, respectively, in WT, PPAR α -null, and CAR-null mouse livers. Both Gadd45b and p21 inhibit cell cycle progression. Furthermore, PFNA decreased the mRNA expression of yes-associated protein 1 (Yap1) (WT, 40%; PPAR α -null, 70%; CAR-null, 82%; of respective controls) and increased phosphorylated Yap1 protein (WT, 2.1-fold; PPAR α -null, 1.1-fold; CAR-null, 2.2-fold; of respective controls). This suggests that PFNA reduced Yap1 level, inhibited Yap1 activity, and thus contributed to suppressed cell proliferation. High-mobility group protein B2 (Hmgb2) regulates the cell size of hepatocytes. PFNA decreased the Hmgb2 mRNA expression by 17%, 54% and 93%, respectively, in WT, PPAR α -null, and CAR-null mouse livers. **Conclusions:** Our findings indicate that PFNA inhibits hepatocyte proliferation by modulating the expression of Gadd45b, p21, and Yap1, and PFNA increases hepatocyte hypertrophy, which is mediated, at least partially, by Hmgb2 suppression in a manner independent of PPAR α or CAR.

PS 3875 Preserved but Not Lost: Quantifying PFAS in Formalin-Fixed and Paraffin-Embedded Tissues for Retrospective Exposure and Dose Assessment

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Background and Purpose: Formalin-fixed (FFT) and formalin-fixed paraffin-embedded (FFPET) tissues represent an immense, underutilized resource for reconstructing historical chemical exposures. For persistent contaminants such as per- and polyfluoroalkyl substances (PFAS), these archived specimens could enable retrospective biomonitoring and tissue-level dosimetry, linking exposure history to disease outcomes. However, fixation and embedding may alter analyte stability and recovery, creating uncertainty in quantification. **Methods:** We developed and validated a liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS) method to quantify six environmentally relevant PFAS (PFHxS, PFOA, PFOS, PFNA, PFDA, PFUnA) in mouse liver, kidney, ileum, and brain. Tissues from exposed mice were divided into matched sets of cryopreserved (FT), formalin-fixed (FFT), and formalin-fixed paraffin-embedded (FFPET) tissue samples. PFAS recovery and loss were tracked through fixation, storage, and deparaffinization steps. **Results:** All PFAS were detectable in each matrix. The total tissue load quantified in the cryopreserved liver tissue was 22.6 μ g/g; in the kidney, 2.69 μ g/g; in the ileum, 1.05 μ g/g; and in the brain, 0.28 μ g/g. Formalin fixation largely preserved analytes, with recoveries of 77-84% relative to cryopreserved tissue, although 12-20% of PFAS were detected in the storage solution. Paraffin embedding resulted in further losses, yielding 57-75% recovery, with 6-16% loss during xylene and ethanol processing. Long-chain PFAS (PFNA-PFUnA) exhibited greater retention than short-chain species (PFHxS, PFOA), and tissues with higher PFAS burdens, such as liver, showed better recovery than low-burden tissues like brain and ileum. **Conclusions:** This work demonstrates the feasibility of quantifying PFAS in archived tissues and provides the first systematic insight into compound- and matrix-specific recovery patterns. Further validation is needed for short and ultrashort PFAS (C1-C4). Human tissues with much lower PFAS loads may experience proportionally greater analytical losses, requiring higher analytical sensitivity and application of correction factors for accurate dose estimation.

PS 3876 Perfluorooctanoic Acid and Trifluoroacetic Acid Exposure Impacts Doxorubicin Uptake and Doxorubicin-Induced DNA Damage Repair in Mammalian Liver and Ovary Cells

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Background and Purpose: Our genomes are under constant threat of DNA damage from exogenous and endogenous sources. If DNA repair is disrupted by environmental chemical exposures, then the risk of cancer-driving mutations is heightened. Per- and polyfluoroalkyl substances (PFAS) are a widely distributed class of synthetic fluorinated chemicals. Exposure to some PFAS is associated with cancer development, yet PFAS impacts on DNA damage repair remains unclear. Given that human exposure to PFAS is widespread, understanding the dynamics of DNA repair under a PFAS + exogenous carcinogen co-exposure scenario is critical. Limited prior studies suggest exposure to perfluorodecanoic acid (PFDA) or perfluorooctanoic acid (PFOA) inhibits double strand DNA break repair, but this work is limited in scope - assessing effects of individual PFAS only and not considering the impact of PFAS exposure on DNA damaging agent uptake. We investigated whether exposure to two PFAS, perfluorooctanoic acid (PFOA) and trifluoroacetic acid (TFA), impacts the repair of DNA breaks induced by the chemotherapeutic doxorubicin. We hypothesized; if PFAS exposure interferes with baseline DNA repair processes, then exposing mammalian cells to PFAS prior to and during DNA damage will result in reduced DNA damage repair rate. **Methods:** We exposed human hepatoblastoma cells (HepG2) and Chinese hamster ovary cells (CHO-K1) to PFOA (0.78 μ M or 100 μ M), TFA (6 μ M or 200 μ M), or vehicle control (water) for 24 h, then co-exposed cells to vehicle control or PFAS + Doxorubicin (0.125 μ M - 1 μ M) for 24 hours. Cells were then maintained in vehicle control or PFAS and collected over a 0-24 h recovery period. DNA damage

(comet tail intensity, TI) was quantified using the 96-well HT alkaline Comet Chip assay (CellArray™) and Comet Analysis Software (Trevigan). Doxorubicin uptake after 24 h PFAS exposure was assessed by fluorescence spectroscopy of cell lysate using a microplate reader at 470/560nm ex/em. PFAS stock concentrations were quantified using liquid chromatography-mass spectrometry. **Results:** Exposure to PFOA or TFA alone at tested concentrations did not induce DNA damage in CHO-K1 or HepG2 cells. CHO-K1 cells exposed to PFOA or TFA prior to and throughout doxorubicin (1 µM) treatment exhibited increased initial doxorubicin-induced DNA damage (i.e. at recovery timepoint 0 h). At 0 h recovery the median TI of vehicle control + doxorubicin exposed cells was 40.5%, while the median TI after PFOA (0.78/100 µM) + doxorubicin co-exposure was 59.4%/64.5% and median TI after TFA (6.5/2000 µM) + doxorubicin co-exposure was 59.1%/70.0%. After 24 h, TFA-exposed CHO-K1 cells reduced TI less efficiently than vehicle control or PFOA treated cells. The median TI of vehicle control + doxorubicin exposed cells was 20.1%, median TI of PFOA + doxorubicin exposed cells was 20.9%/23.5%, while median TI of TFA + doxorubicin exposed cells was 32.3%/35.4%. HepG2 cells exposed to PFOA prior to and throughout doxorubicin treatment exhibited higher TIs at recovery timepoint 0 h compared to vehicle control exposure, but the effect was less pronounced than in CHO-K1 cells (9.7% to 19.4%/19.1% with PFOA, 9.7% to 13.3%/8.4% with TFA). After 24 h, PFOA and TFA exposed HepG2 cells recovered to vehicle-control TI levels. As DNA repair fidelity may vary even if TI is similar, follow up mechanistic assessments of repair pathways utilized in PFAS-exposed cells are needed. Experiments assessing PFAS impacts on doxorubicin uptake are ongoing. **Conclusions:** This study assesses whether PFOA and TFA exposure alter DNA repair in mammalian cells. Our results show two PFAS (TFA and PFOA) enhance initial doxorubicin-induced DNA damage and TFA impairs DNA repair efficiency in mammalian cells. Incorporating doxorubicin uptake measurements will clarify potential mechanisms and improve translatability of this work. We will expand this experimental framework to additional individual PFAS and PFAS mixtures [hexafluoropropylene oxide dimer acid (HFPO-DA), perfluoro-3,5,7,9,11-pentaaxadodecanoic acid (PFOSDoA), and perfluoro-2-methoxyacetic acid (PFMOAA)], and to other DNA-damaging agents to investigate environmentally relevant co-exposure scenarios.

PS 3877 Investigating the neurodevelopmental effects of PFAS mixtures in a chicken embryo model

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are a large class of synthetic compounds widely used in manufacturing, firefighting foams, nonstick coatings, and many consumer products. Their strong carbon-fluorine bonds make these pollutants persistent in the environment, allowing them to accumulate in water, soil, and living organisms. In Minnesota, PFAS contamination has been detected in drinking water across multiple communities, where individuals are often exposed to complex mixtures of individual compounds. While previous research has shown that individual PFAS compounds can affect growth and development, the effects of PFAS mixtures remain poorly understood. In this study, we use chickens (*Gallus Gallus*), which have a developmental pathway comparable to humans, to investigate the early developmental effects of exposure to environmentally relevant PFAS mixtures representative of those found in Minnesota drinking water (PFOA, PFOS, PFBA, PFBS, and PFHxS). **Methods:** Fertilized chicken eggs were incubated for 48 hours to reach Hamburger-Hamilton stage 15. At 48 hours the embryos were dissected, and neural tube sections were plated to isolate neural crest cells, which play an essential role in early embryonic development. After 24 hours of neural crest cell migration, we used fluorescence microscopy to analyze cell health and mitochondrial density. **Results:** Exposure to PFAS mixtures caused dose-dependent developmental effects on chick neural crest cells. Preliminary findings suggest that higher concentrations result in reduced neural crest cell survival and visible alterations in mitochondrial abundance. Qualitatively, we noticed visible differences in embryo growth, albumin consistency, and yolk fragility, suggesting general developmental stress at higher PFAS concentrations. **Conclusions:** These findings demonstrate that PFAS mixtures, at levels comparable to those found in Minnesota drinking water, can affect early embryonic development. The results highlight that evaluating single PFAS in isolation does not consider real-world exposure risks. Understanding how mixtures change cellular and mitochondrial processes is crucial for informing environmental health policies and supporting stronger regulations to protect both wildlife and human populations. Further work is necessary to determine the influence of PFAS mixtures on long-term developmental and health outcomes.

PS 3878 Evaluating the Effects of Environmentally Relevant PFAS Mixtures on Human Cell Viability and Mitochondrial Density

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are synthetic, long-lasting compounds widely used since the 1940s in commercial and industrial products. Because of their strong carbon-fluorine bonds, PFAS persist in the environment and accumulate in living organisms, leading to contamination and associated health risks such as cancer, immune disruption, and hormonal imbalance. While most toxicological studies focus on individual PFAS, real-world exposure occurs as

mixtures. This study aimed to evaluate the toxicity of environmentally relevant PFAS mixtures on human cells, with a focus on cell viability and mitochondrial density, to better understand combined chemical effects. **Methods:** Human colorectal carcinoma (HCT 116) cells were exposed for 24 hours to seven treatment groups: two PFAS mixtures and five individual PFAS compounds (PFOA, PFOS, PFBA, PFBS, PFHxS) at environmentally relevant concentrations ranging from 0.1 nM to 1 µM. Mixture 1 consisted of equal proportions of the five PFAS, while Mixture 2 reflected realistic blood-level ratios based on Minnesota biomonitoring data. Cell death and mitochondrial density were quantified using fluorescence microscopy and analyzed with CellProfiler software to assess dose- and compound-specific toxicity. **Results:** Preliminary analyses show high variability in cellular responses across all exposures. Trends in mitochondrial density were variable by compound. PFBS and PFOS exposure led to the highest mitochondrial density at 100 nM. PFBA exposure led to the highest mitochondrial density at 0.1nM, whereas PFHxS exposure was associated with a reduction in mitochondrial quantity at 10nM. Exposure to both mixtures was associated with a reduction in mitochondrial density, with mixture 2 (Minnesota biomonitoring-based mixture) exhibiting a more pronounced effect at 10nM. **Conclusions:** While results were not significant, the variation in effect by compound mixture is indicative of the importance of studying biologically relevant exposure in addition to individual compounds and equimolar mixtures. Understanding these effects is critical for assessing potential risks to human health, particularly during sensitive periods of neurodevelopment. Further work is ongoing to resolve the high replicate variability and examine the effects of these exposures in neuroblastoma cells to assess their effects on neurodevelopmental health.

PS 3879 Impact of PFAS on Prostate Cancer Metabolism

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Background and Purpose: Prostate cancer is the second most frequent cause of cancer among men in the world. Prostate cancer progresses into metastatic castration-resistant prostate cancer (mCRPC) after resistance to treatment is acquired. Per- and polyfluoroalkyl substances (PFAS) are found in everyday items as they are designed to be resistant to water, stains, heat, and grease. Previous studies have investigated hundreds of different PFAS and their impact on the body but two PFAS that are more prevalent and the oldest are perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS). The International Agency for Research on Cancer has listed these two as Class 2B carcinogens that are capable of increasing risks of kidney and testicular cancer which leads to the potential risk for other cancers such as Prostate Cancer. Preliminary data collected in our lab utilized RWPE-KRAS cells exposed to old, long chained PFOS or new, short chained perfluorobutane sulfonic acid (PFBS) with or without dihydrotestosterone (DHT) and found PFOS exposure alters regulation of metabolites in key metabolic pathways. PFOS was chosen as the primary PFAS for this study. This study aimed to examine the impact PFOS has on metabolic pathways associated with the activated nuclear receptor PPARα. **Methods:** Vehicle and PFOS groups were treated with media and 1x10⁻⁸ M PFOS respectively, then analyzed using Metabolomic profiling and Western blots. **Results:** Metabolic pathway analysis indicated disruptions in key metabolic pathways including TCA cycle and galactose metabolism, contributing to altered energy production and disease progression. Two-way t-tests revealed non-significant results (p>0.05) for both trials of the Western blots. **Conclusions:** These findings emphasize the need for further research on specific implications of PFAS exposure on metabolic pathways and overall increased risk of mCRPC through protein analyses and metabolite quantifications.

PS 3880 Per- and Polyfluoroalkyl Substances in Enterohepatic Bile Acid Homeostasis

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS), or “forever chemicals,” are environmental pollutants with high resistance to degradation. An estimated 98% of the U.S. population has detectable levels of PFAS in their bodies (Coulson, 2024). Nuclear receptors, such as Farnesoid X Receptor (FXR), Peroxisome Proliferator-Activated Receptor α (PPARα), Constitutively Active Receptor (CAR), and Pregnane X Receptor (PXR), are ligand-activated transcription factors that play a crucial role in regulating lipid and bile acid synthesis, transport, and detoxification. The purpose of this study was to investigate the effects of PFAS on the activation of CAR, PPARα, FXR-FGF15/19 axis, and PXR in mice and their regulation of gene expression in lipid and bile acid homeostasis. **Methods:** Eight-week-old CD-1 female mice received 0, 0.4, 1.2, and 4 µg/mL of Perfluoronanoic acid (PFNA) via drinking water for 8 weeks. Gene expression at mRNA levels was quantified using RT-qPCR. **Results:** Ileal Fgf15 shows dose response inhibition; however, both classic and alternative bile acid synthesis pathways show no significant change. CYP2B10 and Cyp3a11 were dose-dependently induced, indicating activation of CAR- and PXR-mediated detoxification pathways. Cyp4a10, a PPARα target gene involved in fatty acid oxidation, showed up to 16-fold induction. Cyp36 and Fabbp1, PPARα target genes associated with lipid transport and metabolism, showed upward regulation,

indicating PFAS-induced PPAR α activation and increased intracellular lipid load. **Conclusions:** These changes imply PFAS exposure disrupts bile acid detoxification and hepatic lipid metabolism. This study offers insight into how forever chemicals disrupt lipid and bile acid homeostasis, potentially contributing to the pathogenesis of liver diseases.

PS 3881 Cardiac Remodeling as a Novel Target of Short-Chain PFAS Toxicity

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are a class of 15,000+ synthetic chemicals that persist in environmental and human blood, urine, and drinking water. Long-chain PFAS, perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), are associated with immune dysfunction, metabolic disturbances, and cardiovascular toxicity. Cardiac macrophages are key regulators of tissue homeostasis and repair; these macrophages coordinate immune responses and extracellular matrix (ECM) turnover. However, their dysregulation contributes to chronic inflammation, oxidative stress, and myocardial fibrosis, hallmarks of diastolic dysfunction and heart failure with preserved ejection fraction. Short-chain PFAS, including pentafluoropropionic acid (PFPrA), are replacing long-chain PFAS compounds and are increasingly detected in surface and drinking water. Although considered less bioaccumulative, emerging data indicate that short-chain PFAS retain biological activity and immune and metabolic signaling. Despite growing concern, their impact on cardiac health, particularly macrophage-mediated inflammation and fibrosis, remains largely uncharacterized. Defining whether PFPrA promotes profibrotic cardiac remodeling is essential to evaluate cardiovascular risk from environmentally relevant exposures. We hypothesize that PFPrA activates cardiac macrophages and profibrotic signaling pathways, leading to ECM remodeling and early fibrosis. **Methods:** Male (M) and female (F) C57BL/6 mice were orally administered PFPrA (0, 0.5, 5.0, or 50 mg/kg/day) for 30 days. Livers were weighed, and frozen for assessment of peroxisome proliferation using acetyl-CoA oxidase-1 (ACOX-1) activity. Relative liver weight and ACOX-1 activity, biomarkers of PFAS exposure, were compared to PFOA (7.5 mg/kg/day; relative liver weight increases: ~210% F, ~138% M; peroxisome proliferation [ACOX-1]: ~200% M/F). Hearts were weighed, and the left ventricles were fixed in 4% paraformaldehyde for histological evaluation. For histological evaluation, Masson's trichrome staining will be used to assess collagen deposition and ECM remodeling. The remaining hearts were snap-frozen, for future analysis of inflammatory and fibrotic gene expression (IL-1 β , MMP-12, TGF- β 1, Nrf2, and PPAR γ). Macrophage pro-inflammatory M1 type will be characterized through CD11b immunohistochemistry staining. Macrophage infiltration will be quantified by F4/80 immunohistochemistry. Sex-specific differences will be evaluated across exposure groups. **Results:** PFPrA exposure did not alter terminal body or relative heart weights compared to controls. However, relative liver weights increased significantly in males at 5 and 50 mg/kg (~71%) and in F at 50 mg/kg (~69%). Peroxisome proliferation also increased in a dose-dependent manner (M: 82% at 5 mg/kg and ~230% at 50 mg/kg; F: 68% at 50 mg/kg), consistent with early hepatic response to PFAS exposure. We anticipate that cardiac macrophage proinflammatory polarization and infiltration will increase with PFPrA exposure. Our preliminary results indicate CD11b positive macrophage area increased in F heart ventricular epicardium tissue: 5 and 50 mg/kg/day, quantitative analysis is ongoing. Upregulation of IL-1 β , MMP-12, and TGF- β 1 is expected to indicate activation of ECM remodeling and early fibrotic signaling, even in the absence of overt myocardial damage. **Conclusions:** Although PFPrA did not induce systemic toxicity based on changes in body weight, early hepatic activation was evident through increased liver weight and peroxisome proliferation. These findings indicate sufficient internal exposure to PFPrA, such that we must evaluate downstream immunotoxic and cardiotoxic effects. This study addresses a critical gap in PFAS toxicology by defining whether short-chain PFAS such as PFPrA contribute to macrophage-driven cardiac remodeling and fibrosis. By linking immune activation to structural cardiac changes, this work advances understanding of PFAS-induced cardiovascular dysfunction and will inform risk assessment for exposed human populations. Future phases will incorporate echocardiography to evaluate cardiac function and enhance translational applicability to human health outcomes. Additional mechanistic studies are needed to characterize chronic exposure effects, interspecies variability, and long-term cardiovascular risk of emerging short-chain PFAS.

PS 3882 Comparative Effects of Per- and Polyfluoroalkyl Substances Exposure on Lifespan and DJR-1 Expression in *Caenorhabditis elegans*

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Background and Purpose: Parkinson's disease (PD) is a progressive neurodegenerative disorder marked by tremors, rigidity, and bradykinesia. Its primary pathology involves the loss of dopaminergic (DA) neurons in the substantia nigra, with elevated reactive oxygen species (ROS) playing a central role in neuronal death. Environmental contaminants that promote oxidative stress may contribute to the neurodegenerative processes. Among these are per- and polyfluoroalkyl substances (PFAS), a class of

synthetic chemicals used both industrially and commercially. These environmentally persistent organic compounds are associated with various health concerns. With over 14,000 existing variants, PFAS have become major contaminants, particularly in drinking water. In 2024, the Environmental Protection Agency (EPA) established drinking water regulations for six PFAS chemicals to reduce human exposure; in 2025, these only applied to two perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA). Prior research has shown that PFOS and PFOA reduce the lifespan of *Caenorhabditis elegans* (C. elegans) and may exacerbate oxidative stress, disrupting DA neuron function. This study investigates whether exposure to two previously regulated PFAS compounds, a short-chain perfluorobutanesulfonic acid (PFBS) and a long-chain perfluorononanoic acid (PFNA), alters the lifespan of C. elegans while also examining the impact of these substances on DJR-1 expression, a gene involved in the oxidative stress response. With this study, we aim to understand the influence of environmental PFAS contamination on aging and risks to human health. **Methods:** Age-synchronized populations of C. elegans were collected and exposed to 0 (control group), 1, 10, 50, and 100 μ M concentrations of PFNA and PFBS separately. Approximately 40 hours after treatment, lifespan assays were conducted to identify any changes in the longevity of C. elegans. Worms were monitored daily, their deaths scored, and mortality rates were analyzed over a 26-day period. To assess DJR-1 expression, worms were treated with 0 (control group), 50, 100, and 150 μ M concentrations of PFNA or PFBS, followed by real-time PCR analysis. **Results:** After 26 days of exposure, the lifespan assays indicate that the survival rate of C. elegans treated with PFNA began to decline at Day 9 post-treatment; the highest mortality rate of each concentration was reached between Days 20 and 23. Lifespan reduction in C. elegans exposed to PFBS was initiated on Day 10, revealing a similar trend to PFNA. Data collection and statistical analysis for these treatment groups are underway. Previously collected data on gene expression indicate that PFOS exposure modulates DJR-1 expression, suggesting a potential mechanism for PFAS-induced oxidative stress. Ongoing experiments are determining whether PFNA and PFBS elicit comparable or distinct effects on DJR-1 regulation, potentially linking lifespan reduction to oxidative stress dysregulation. Comparative analysis will clarify the differential impact of individual PFAS congeners on oxidative stress pathways in C. elegans. **Conclusions:** This study aims to elucidate the role of PFAS in modulating oxidative stress-related gene expression during neurodevelopment and reducing organismal lifespan. A connection between these environmental pollutants and their effects on a model organism is highlighted, and our findings contribute to a broader understanding of environmental risk factors in neurodegenerative disease etiology, particularly PD.

PS 3883 Assessment of Developmental and Endocrine Pancreatic Toxicity of Aqueous Film Forming Foams (AFFF) in Larval Zebrafish (*Danio rerio*) and its Potential Modulation by the Nrf2 Pathway

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Background and Purpose: The use of aqueous film-forming foams (AFFF) as fire-fighting agents at military bases and fire training facilities is a well-documented source for drinking water contamination by per- and polyfluoroalkyl substances (PFAS). The major constituent of AFFFs are PFAS, a class of chemicals that are neither metabolized nor degraded and are known to persist in the environment. While the developmental and pancreatic toxicity of individual PFAS has been reported, the adverse effects of PFAS mixtures are less understood. Nfe2l2 (or Nrf2) is a transcription factor which regulates the cells' antioxidant responses and has been shown to modulate toxicity of some PFAS. In this study, we aim to assess the developmental and pancreatic toxicity of a mixture of 27 PFAS that were detected in legacy AFFF samples, in comparison to a mixture of 6 PFAS that are currently regulated, using transgenic zebrafish (*Danio rerio*) larvae. **Methods:** A mixture of 27 PFAS chemicals found in legacy AFFF and a mixture of 6 regulated PFAS chemicals, both comparable to the proportions present in 3% AFFF were synthesized by the Massachusetts Pesticide Analysis Lab. Homozygous Nrf2a wild-type and mutant (nrf2a^{th318}) Tg(ins:GFP) embryos were exposed to either mixture at 0.0005%, 0.001%, and 0.0025% AFFF, with 0.1% methanol as a solvent control at 3 hours post fertilization (hpf). Exposures were static up to 100 hpf when larvae were collected and imaged under brightfield and fluorescence microscopy. Images were blinded and fish length, yolk sac area, pericardial area, and islet area were measured using ImageJ. The toxicity of the PFAS mixtures was then compared to the most abundant PFAS in the mixtures, perfluorooctane sulfonate (PFOS), using previous data from our lab. **Results:** No difference was observed in morphology between the Danieau's control group and solvent control group (0.1% methanol). Our results for the 27 PFAS mixture show that fish length was significantly decreased in wildtype larvae exposed to 0.001% and 0.0025% AFFF, and all concentrations for mutant larvae. Yolk sac area and pericardial area were significantly increased in both wildtype and mutant larvae exposed to 0.001% and 0.0025% AFFF. Islet area was significantly decreased in wildtype and mutant larvae exposed to 0.001% and 0.0025% AFFF, but the effect was not as severe in the mutant larvae. The 6 PFAS mixture with comparable PFOS concentrations was markedly less toxic than the mixture of 27 PFAS, with impaired swim bladder inflation and a small decrease in growth, but no pericardial edema or overt deformities. Comparable exposures to PFOS alone at its concentration within the mixtures showed no morphological changes. **Conclusions:** Larvae exposed to

the 27 PFAS mixture showed developmental and pancreatic toxicity, with differences between Nrf2a mutant and wildtype larvae for fish length and yolk sac area, and a partial decrease in severity of islet toxicity in the Nrf2a mutant fish, as shown previously for PFOS. The Nrf2 pathway may therefore play a role in modulating PFAS mixtures toxicity and should be studied further. The results of this study show that the total sum of PFAS in a mixture exposure is more predictive of toxicity than either PFOS alone or PFOS in combination with five other commonly measured PFAS. This suggests current water monitoring and regulatory approaches for PFAS likely underestimate toxicity and risk. This work was supported by R01ES025748

PS 3884 The Effect of PFAS on Microbial Communities in South Carolina Estuaries of Varying Urbanization

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Background and Purpose: Estuaries are dynamic and biologically rich ecosystems that provide crucial ecological services such as nutrient cycling, contaminant filtration, and habitats for juvenile organisms. However, these ecosystems are threatened by anthropogenic pressures, especially pollution from coastal urbanization. Among the most concerning pollutants are per- and polyfluoroalkyl substances (PFAS), which are synthetic, heat- and water-resistant compounds that persist and bioaccumulate in the environment. PFAS contamination has been detected in water systems worldwide and locally, including South Carolina in urban estuaries such as Charleston Harbor (CH). Microbes play essential roles in estuarine health by driving biogeochemical cycling, yet PFAS exposure is disrupting these communities by reducing diversity, altering metabolic function, and interfering with key processes. Though it is thought some microbial taxa may possess the metabolic capacity to transform or degrade PFAS, offering a natural avenue for bioremediation. This study compares PFAS concentrations and microbial community composition between an urbanized (CH) and non-urbanized (St. Helena Sound, SHS) estuary to understand how contamination affects microbial diversity and function. We hypothesize that (1) PFAS concentrations in surface water and sediment are higher in CH than SHS and (2) microbial communities in PFAS-enriched sites will be dominated by taxa more resistant to PFAS or capable of transformation. **Methods:** Surface water and sediment samples were collected from three sites within each estuary between October 2024 and May 2025. High-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) quantified PFAS concentrations. Sediment components were characterized by pipette analysis to determine the relative proportions of sand, silt, and clay, given that finer sediments may facilitate PFAS sorption. 16S rRNA sequencing was used to characterize microbial community structure by assessing alpha diversity (Shannon index, Pielou's evenness), and beta diversity (Bray-Curtis and UniFrac distances). Statistical analyses tested for site and seasonal differences across PFAS profiles. **Results:** Preliminary data indicates that PFAS concentrations in sediment samples were not significantly different between CH and SHS, however overall PFAS concentrations were significantly higher in CH than SHS in water samples, supporting our hypothesis. These elevated concentrations in CH appear to be driven primarily by perfluorooctanesulfonic acid (PFOS), which was the most abundant compound detected in both sample types from CH. The total PFAS concentrations showed significant differences in both sediment and water samples, indicating that localized environmental factors influence PFAS concentration. Microbial community analyses revealed site- and season-specific differences in diversity and composition. CH exhibited lower microbial evenness and overall diversity relative to SHS, consistent with greater environmental stress in the more urbanized estuary. Alpha and beta diversity analyses showed clear clustering by estuary and season, suggesting that PFAS exposure, salinity, and sediment properties together shape microbial assemblages. Dominant phyla included taxa commonly linked to contaminant metabolism and stress tolerance. Although these findings highlight associations between PFAS concentrations and microbial community composition, controlled experiments will be explored in the future. **Conclusions:** To directly test microbial tolerance and transformation capacity, controlled culture experiments will be conducted using sediment-derived microbial communities under aerobic and anaerobic conditions. Cultures will be exposed to environmentally relevant and elevated PFOS concentrations (up to 10mM) for 30 days to monitor potential degradation and community shifts. Analytical techniques such HPLC-MS/MS will be used to quantify PFOS loss and detect transformation products. Concurrent 16S rRNA sequencing of these cultures will identify taxa that persist under PFOS stress, potentially capable of PFAS degradation. These experiments will clarify whether microbes from estuarine sediments can actively metabolize PFAS, provide microbially mediated bioremediation strategies, and further our understanding of how urban pollution alters microbial communities in coastal environments.

PS 3885 Unveiling PFAS-Induced Cardiovascular Endothelial Toxicity: Differential DNA Damage and Oxidative Stress Responses

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS), or "forever chemicals," are synthetic, persistent compounds widely used in consumer and industrial products, and PFAS exposure has been linked to vascular dysfunction and an increased risk of hypertension. Human coronary artery endothelial cells (HCAEC) are responsible for regulating vascular tone and endothelial integrity, and their functional disruption is marked by increased permeability and elevated reactive oxygen species (ROS). While most research has focused on their effects in hepatic, immune, and reproductive systems, the impact of PFAS on HCAEC remains largely unexplored. In particular, the individual and mixed effects of (i) legacy PFAS (e.g., PFOS, PFOA, PFNA, PFDA), and (ii) emerging short-chain PFAS (e.g., PFHxA, PFHpA) on genetic alterations in HCAEC have not been investigated. This study aims to evaluate selected PFAS (PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, and their mixture) on HCAEC, specifically to 1) assess ROS formation and 2) determine the genotoxic potential of individual and mixed PFAS. **Methods:** HCAEC were treated with lower (10 nM) and higher (10 µM) doses of individual PFAS (PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, and their mixture) for 24 hours. Following exposure, cell viability and ROS formation were measured to assess oxidative stress, while DNA damage biomarkers were evaluated to characterize genotoxicity. **Results:** At a low dose, 10 nM, PFAS activated the ATR-Chk1/Chk2 DNA damage checkpoint, upregulating stress response proteins p21 and p53. In contrast, high-dose PFAS (10 µM) induced cytotoxicity and increased ROS in HCAEC. The extent of toxicity correlated with chain length, with longer-chain PFAS (PFNA, PFDA, and the mixture) producing stronger cytotoxic effects and greater ATR pathway activation. **Conclusions:** PFAS exhibit both dose and structure-dependent effects on endothelial cells. Low-dose PFAS triggers a protective DNA damage response, leading to growth arrest rather than cell death. Conversely, longer-chain PFAS exacerbate oxidative stress and checkpoint activation, contributing to endothelial toxicity and genomic instability. Understanding how PFAS structure and exposure level influence these cellular responses is crucial for assessing cardiovascular toxicity risks.

PS 3886 Low-dose PFAS Exposure Disrupts ABC and OATP Transporter Activity in Human Cell-based Models

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Background and Purpose: PFAS are bio accumulative chemicals which have been consistently detected in the serum of humans. Recent work has suggested relevant exposure to low doses of many common short chain PFAS alters the expression of the ABC transport protein ABCG2, one of many ABC implicated in drug resistance and critical metabolic processes. In this work, the protein expression of ABCG2 in commonly used cell lines, the gene expression of many ABC in HepaRG, and the functional ability of HepaRG liver cells to uptake and efflux prototypical fluorescent substrates, such as Hoechst, rhodamine, and fluorescein, has been investigated following PFAS exposure in order to understand the potential impact to overall human health following PFAS exposure, and the potential mechanisms involved. **Methods:** In this work, nine cell lines were first evaluated for ABCG2 protein expression treated with PFBS, PFHxA, PFHxS, 6:2FTOH, or DMSO control. Cells were grown on glass slides, dosed with 1 nM or 1 µM PFAS for 48 hours, and an anti-ABCG2 alexafluor antibody used to allow for confocal imaging and quantification of RFU per cell. For gene expression experiments, HepaRG cells were treated with 1 µM doses of PFAS in low serum media for 48 hours, and qPCR performed using TaqMan ABC array plates. For transport assays, HepaRG were pre treated for 48 hours with PFAS or control media. Cells were then changed to PFAS treated media containing fluorescent substrates CMFDA, Hoechst33342, Rhodamine 123, Bodipy Cholesterol, or fluorescein for 45 minutes. For fluorescein, a substrate of OATP uptake transporters, cells were then washed and fluorescence read on a plate reader. For ABC efflux substrates, cells were washed and changed to plain media for 45 minutes to allow for efflux. Cells were then washed and read to determine remaining retained fluorescence per well. **Results:** All cell lines investigated here expressed ABCG2, with the highest expression seen in liver and kidney cell lines. HepaRG, which have robust transporter expression, were then evaluated further, demonstrating significantly increased protein expression following both 1 nM and 1 µM PFAS treatments. Likewise, 1 µM exposures in HepaRG significantly increased log fold gene expression of many ABC, including ABCA4, ABCB6, ABCB2, ABCG2, and ABCA1. ABCD2 displayed the largest relative change to expression, with PFBS, PFHxA, and PFHxS all leading to an 8 fold or greater increase. In addition to changes to the gene and protein expression of ABC, the ability of these transporters to move prototypical substrates was also significantly altered. For all substrates, short term 45 minute PFAS exposure caused little effect, while 48 hour PFAS pre-treatment significantly altered both ABC efflux, and OATP uptake. Relative to DMSO controls, fluorescein uptake was decreased by 12% or more for all 1 nM treatments, with the greatest decrease seen following exposure to 1 µM PFBS, with a 35% decrease in relative retained fluorescence. All ABC Substrates had significant decreases to retained fluorescence following PFAS exposures, indicating increased substrate efflux. Bodipy cholesterol was most significantly altered by PFAS exposure, with all 48 hour treat-

ments significantly decreasing relative retained fluorescence by 30% or more. The most significant change was seen with Bodipy cholesterol following exposure to 1 μ M PFHxA, with a 66% decrease in relative retained fluorescence, and a p value less than 0.0001. **Conclusions:** ABCG2 protein is expressed throughout the body, and is significantly altered by PFAS exposure, with a greater potential impact in the liver, where ABC play a major role. This effect was not limited to ABCG2, with many ABC being upregulated, and the overall ability of ABC and OATP to transport their substrates in a robust liver transport model ultimately being affected. These significant and widespread results suggest that exposure to short chain PFAS at concentrations currently found in the serum of the US population may alter the ability of cells to function normally, and impact human health. The notable changes to transport of the substrate Bodipy cholesterol were particularly alarming, highlighting the potential for low dose human PFAS exposures to alter cholesterol transport by ABC in the liver.

PS 3887 Integrative Multi-Scale Approach Using Zebrafish Transcriptomics and Molecular Docking to Determine the Sulfonamide-Specific Mechanisms of Developmental Toxicity of Four-Carbon PFAS

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are a structurally diverse class of contaminants characterized by a hydrophilic headgroup and a fluorinated tail. Differences in chain length or headgroup influence biological uptake, protein binding, and gene expression, giving each PFAS a unique toxicity profile. As regulatory pressure against long-chain PFAS increases, production has shifted toward short-chain alternatives. However, evidence shows many short-chain PFAS still cause physiological effects, suggesting headgroup may impact biological activity beyond chain length. Understanding how structural variations drive protein interactions and downstream regulation is critical to predicting PFAS toxicity. In an embryonic zebrafish screen of four four-carbon PFAS, only perfluorobutane sulfonamide (FBSA) caused malformations. To investigate the molecular basis of this headgroup-specific activity, we performed transcriptomic, molecular docking, and miRNA analyses comparing FBSA to perfluorobutane sulfonate (PFBS), which has similar uptake but distinct outcomes. **Methods:** To examine the effect of headgroup in four-carbon PFAS, we compared two compounds, FBSA and PFBS. Embryonic zebrafish exposure started at 8 hours post fertilization (hpf) to each compound at identical concentrations (47 μ M); the morphological EC₅₀ for FBSA and a matching PFBS concentration for direct comparison. Morphological malformation was evaluated at 120 hpf, and transcriptomic profiling at 48 hpf to capture gene expression changes preceding visible effects. To investigate the molecular basis of the divergent outcomes, AutoDock was used to screen both compounds across a library of human ligand-binding sites to identify proteins uniquely predicted to bind FBSA. To test if FBSA-repressed transcriptional changes may be mediated by miRNA-based post-transcriptional regulation, genes downregulated by FBSA were cross-referenced with TargetScanFish potential miRNA targets. **Results:** At 48 hpf, FBSA and PFBS had similar uptake (11 $\pm$ 1.5 ng/mg and 8.9 $\pm$ 0.99 ng/mg, respectively), but only FBSA caused malformations at 120 hpf, while PFBS was inactive. Transcriptomic profiling revealed that FBSA misregulated 1,909 genes at its EC₅₀, compared to only 5 DEGs for PFBS. Molecular docking identified 114 proteins with zebrafish orthologs uniquely predicted to bind FBSA at $\leq$ -7.0 kcal/mol that may underlie the divergent transcriptional and morphological responses between the two compounds. Integration of transcriptomic and TargetScanFish analyses found several FBSA-downregulated genes also predicted as miRNA targets. **Conclusions:** By integrating developmental toxicity screening, transcriptomics, molecular docking, and miRNA prediction, this study reveals a mechanistic basis for divergent toxicity of short-chain PFAS with distinct headgroup chemistries. To investigate this divergence, we examined interaction potential across protein and post-transcriptional regulatory networks. Molecular docking identified proteins predicted to uniquely bind FBSA, indicating the sulfonamide headgroup enables interactions not accessible to the sulfonate. Integration of transcriptomic and miRNA analyses suggests that FBSA-responsive downregulation may involve both direct protein-ligand interactions and miRNA-mediated post-transcriptional regulation, providing a molecular explanation for the contrasting developmental outcomes of FBSA and PFBS.

PS 3888 PFAS Terminology and Fit-For-Purpose Risk-Based Grouping

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Background and Purpose: Accurate and consistent PFAS terminology is essential to ensure effective PFAS communication. But not all PFASs have the same risk. The tremendous diversity across 14,000+ PFASs complicates risk management communication. Recently, PFAS risk management actions globally illustrate this terminology-grouping debate: PFAS bills by Health Canada, some US states and the current proposed EU PFAS restriction exclude some substances (e.g. fluoropolymers or PFAS as pesticide active ingredients) meeting the terminology definition of a PFAS from risk management actions. Risk management actions are separate from the PFAS definition. These two concepts of *terminology* and *risk-based grouping* are not,

however, mutually exclusive. *Terminology* is essential to ensure consistent and accurate PFAS communication occurs. *Risk-based grouping* is essential to put the PFAS terminology into human and environmental protection perspective, beyond chemical and molecular structure. Agreement upon that PFAS terminology and diligent application thereof then enables the establishment of *fit-for-purpose working scopes* or *risk management-based grouping* of PFAS. **Methods:** Through two examples of polymeric PFASs, a fluoropolymer (polytetrafluoroethylene, PTFE) and a perfluoropolyether (PFPE, 1,1,1,2,2,3,3,3-heptafluoro-3-(1,1,2,2,2-pentafluoroethoxy) propane ether), the implementation of a consistent PFAS *terminology* is applied in five elements of risk management-based groupings: grouping by *hazard*, *exposure*, *risk* (as a function of hazard and bioavailable exposure), *socioeconomics*, and ultimately *risk management grouping*. Criteria are proposed for each *fit-for-purpose risk-based grouping* founded on already established and globally implemented consensus criteria such as the UNECE GHS categories, the U.S. TSCA, the EU REACH regulation, and the UNEP Stockholm Convention. **Results:** Fluoropolymers and PFPEs are distinctly different from non-polymeric PFASs such as perfluoroalkyl acids (e.g., PFOA), though the OECD 2021 *terminology* identifies them all as PFASs. But their *hazard-based grouping* across the life cycle differs (e.g., toxicity, molecular weight, vapor pressure, water solubility, bioaccumulation, environmental partitioning, etc.). *Exposure-based grouping* results in many uses across a wide range of high technology/high value products (e.g., defense, energy, communication, medical devices, lubricants to critical industrial processes, etc.) while highlighting control technology effectiveness and limitations. The potential for bioavailable exposure of the fluoropolymer and PFPE is low. *Socioeconomic-based grouping* illustrates the significant and broad impact of these polymeric PFAS informing risk management decision-making. Residual risks, identified after these fit-for-purpose groupings, are integrated into a *relative risk spectrum* from which risk management measures are proposed. The value of a relative risk versus the all-or-nothing "P-sufficient" approach is discussed. Risk management actions proposed focus on emissions control during manufacturing and use and highlight responsible end of life options for PTFE and the PFPE. **Conclusions:** While no risk assessment is ever without uncertainty, an overall PFAS risk management process using relative risk (based on hazard, bioavailable exposure and control technologies, and socioeconomic risk) is as comprehensive an approach as possible while consistently applying correct PFAS terminology. Public and environmental health protection, promotion of responsible manufacturing and innovation, and harnessing fluorinated chemistry to meet societal expectations within economic realities is achievable.

PS 3889 Environmental Monitoring of PFAS in Himachal Pradesh: Contamination Levels and Human Health Implications.

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are contaminants of increasing concern owing to their persistence in the environment, their propensity to accumulate in living organisms, and their links with negative health outcomes. The current research is an analysis of the PFAS pollution in different water bodies in Himachal Pradesh with a particular focus on the potential health hazards. **Methods:** Thirty-one samples were taken in rivers, groundwater, and drinking water supply sources in five districts, Una, Kangra, Chamba, Solan, and Bilaspur and subjected to solid-phase extraction and LC-MS/MS in compliance with the USEPA Method 537.1. **Results:** PFOS and PFOA were found to be high, particularly in Solan and Una districts and some of the results exceeded the advisory levels set by the United States Environmental Protection Agency (70 ng/L) and the European Commission. Non-carcinogenic and carcinogenic risks attributed to Long-term exposure to contaminated drinking water were assessed using health risk assessment methods based on hazard quotient (HQ) and lifetime cancer risk estimates. Industrial areas like Baddi in the Solan district had a greater variety of both established and novel PFAS compounds, which led to an increase in the exposure risks. **Conclusions:** The paper points to a significant requirement of PFAS-specific components of controlling actions, systematic observation, and directing health care in India to reduce the long-term impact and ensure water purity. **Acknowledgement:** Financial support for this work was provided under the Core Research Grant (CRG) Scheme, Earth and Atmospheric Division, ANRF (Grant No: CRG/2021/007791), for the project titled "Initiative to estimate the presence of perfluoroalkyl substances (PFAS) in rivers and ground/drinking water in Himachal Pradesh: Emission and implications for human exposure."

PS 3890 Astrocyte Dysfunction as a Mechanism of PFAS-Induced Neurotoxicity: Implications for Neuroinflammation and Neurodegeneration

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS), including perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS), are persistent environmental contaminants linked to multiple health effects. While neuronal toxicity has been well characterized, the impact of PFAS on astrocyte function remains poorly understood. Astrocytes play essential roles in maintaining neuronal homeo-

stasis, regulating glutamate clearance, modulating neuroinflammation, and supporting mitochondrial metabolism. This study aimed to elucidate how PFAS exposure disrupts astrocyte function and contributes to neuroinflammatory and neurodegenerative processes. **Methods:** Human iPSC-derived astrocytes were exposed to environmentally relevant concentrations of PFOA and PFOS. Transcriptomic and proteomic analyses were performed to assess PFAS-induced molecular alterations. Cytokine profiles were validated by ELISA, while glutamate uptake and phagocytosis assays were used to evaluate astrocyte functionality. Lipid peroxidation was quantified to assess oxidative stress responses. **Results:** PFAS exposure significantly altered cytokine expression profiles in astrocytes, with several key pro-inflammatory mediators upregulated and confirmed by ELISA. Functional assays revealed decreased glutamate uptake capacity, indicating impaired neurotransmitter regulation, along with increased astrocytic phagocytosis, suggesting maladaptive activation under stress. Elevated lipid peroxidation further indicated enhanced oxidative stress and mitochondrial dysfunction. **Conclusions:** These findings reveal that PFAS exposure induces astrocyte dysfunction characterized by disrupted glutamate regulation, oxidative stress, and heightened inflammatory signaling. The data highlight astrocytes as critical mediators of PFAS-induced neurotoxicity and suggest that astrocytic impairment may contribute to the pathogenesis of neuroinflammatory and neurodegenerative disorders. Targeting astrocyte pathways may therefore represent a promising strategy to mitigate PFAS-related neurological risks.

PS 3891 Impact of Developmental Exposure to Environmentally Relevant Levels of PFAS and PFAS Mixtures on Multi-tissue Transcriptomic Profiles in Adult Zebrafish

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Background and Purpose: Concern over the potential effects of per- and polyfluoroalkyl substances (PFAS) commonly present in the environment (including drinking water) have risen since the early 2000s. Although certain PFAS, including perfluorosulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) are no longer used in manufacturing in the United States, they are ubiquitous in the environment due to their strong carbon fluorine bonds rendering them effectively indestructible. PFOA and PFOS have been reported to induce immunotoxicity, endocrine toxicity, and developmental toxicity. Epidemiological studies have associated PFAS with multiple adverse health outcomes, including altered immune function, liver disease, lipid metabolism dysregulation, kidney disease, miscarriages, and cancer. **Methods:** In this study, we sought to determine the effect of an environmentally-relevant levels of exposure (70ng/L) of PFOA, PFOS (240 ng/L), and a mixture (35 ng/L of PFOA + 120 ng/L of PFOS) of each on larval zebrafish from 0-5 days post-fertilization (dpf) that were then raised to adulthood (~18 months). RNA-Seq (3' mRNA-seq) transcriptomic analyses was then used to analyze extracted RNA from brain, gonad, liver, and kidney tissue of male and female control and exposed fish (n=2-4/treatment; 4-8/respective control). Libraries were quantified using a Qubit 2.0 fluorometer and run on an Agilent TapeStation 2200 for quality control. The samples were sequenced on an Illumina HiSeq 2500 and analyzed using a combination of the Lexogen Bluebees Genomics Platform and Lexogen in-house data analysis. Readswere aligned to *D. rerio* (Build GRCz11). Differentially expressed genes (DEGs) were determined via DESeq2 and defined by absolute log2 fold change value $\geq |1|$; $p < 0.05$. DEGs were compared for overlap using UpSetR and were uploaded into Ingenuity Pathway Analysis (IPA). **Results:** Previously, we reported that developmental PFAS exposure altered lipid transcriptomics and light/dark swim distance in larval fish (5 dpf) and impacted fecundity in PFOA-exposed fish raised to adulthood (4-6 months). Comparisons between larval and adult-onset differential gene expression revealed significantly higher effect later in life. The number of DEGs was low (ranging from 1-106) in larval fish, whereas adult fish exposed during development were found to have anywhere from 2-9360 DEGs. IPA analysis of DEGs revealed significant enrichment of several disease pathways, including lipid metabolism disruption (p-value = 7.02×10^{-25} PFOS female liver), endocrine disruption (p-value = 6.11×10^{-85} mixture male gonad), organismal survival (p-value = 3.11×10^{-34} mixture female liver), cancer (p-value = 3.90×10^{-142} PFOA female liver), and reproduction (p-value = 4.12×10^{-66} PFOA male gonad) in PFAS exposed fish. Outcomes in the brain were highly elevated in the mixture exposure. More tissue-specific outcomes were identified including motor development (p-value = 1.35×10^{-66} mixture male brain) and seizure markers (p-value of up to 7.24×10^{-33} mixture male brain) in the brain and inflammation (p-value = 4.39×10^{-10} PFOS female liver), steatosis (p-value = 6.95×10^{-11} mixture male liver) and liver tumor (p-value = 1.19×10^{-18} PFOS male liver) in the liver. Several upstream regulators including beta-estradiol (p-value 7.02×10^{-57} mixture female gonad), BDNF (p-value = 2.71×10^{-25} mixture female liver), TGF β 1 (p-value = 1.74×10^{-14} PFOA male gonad), HNF4A (p-value of 8.01×10^{-35} mixture female gonad) and PPAR α (p-value - 1.99×10^{-33} PFOS female liver) were identified to be altered regulators of observed diseases. **Conclusions:** Early life PFAS exposure at environmentally relevant levels has multi-systemic effects months after the initial exposure. Ongoing studies are supporting the associated links of PFOA and PFOS in human populations with animal toxicology studies including zebrafish. Several theorized molecular mechanisms of PFAS were observed to be dysregulated, potentially leading to the diseases observed. Animal studies such as this are important for confirming findings of epidemiologic and cell line toxicology studies. This research is important to determining the effect of PFAS on public health and the relative safe level of PFAS in drinking water.

PS 3892 Characterization of PFAS in Vermont public water systems

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Background and Purpose: Per- and poly-fluorinated alkyl substances (PFAS) have gained considerable attention due to their persistence, widespread environmental detection, and frequent presence in drinking water systems. While certain regions are impacted due to local PFAS manufacturing facilities, the State of Vermont makes for an interesting case study because it has no known primary manufacturing sources of PFAS, yet many PFAS congeners are present in Vermont drinking water. The Vermont Water Supply Rule requires public water systems to routinely test for PFAS, including 18 individual congeners and the sum of PFHpA, PFNA, PFOS, PFOA, and PFHxS. Vermont regulations state that the sum of these 5 congeners must be below 20 ppt. Although the testing results are publicly available, the database format is not easily accessible for analysis. Here, we systematically transformed Vermont's public PFAS drinking water database into a usable format and conducted a quantitative analysis characterizing PFAS occurrence and temporal and spatial patterns across the state. This analysis provides insight into "background" PFAS drinking water concentrations in regions with no known primary PFAS sources. **Methods:** PFAS drinking water results were obtained from the Vermont Drinking Water Database on August 19, 2025. The PDF file was imported into RStudio to parse and extract data, and the resulting dataset was exported into Microsoft Excel for further manual data tidying and quality assurance due to the inconsistent formatting of the PDF output. Because the limit of detection (LOD) was not provided for results reported as non-detects, a value of 0.1 ppt was used for imputation to enable further quantitative analysis. Each town was mapped to its county for broader geographical analysis. Summary statistics were calculated for each congener, and the sum of the five Vermont-regulated PFAS was used to characterize temporal and geographical trends. **Results:** A total of 631 unique public water systems across 231 towns and all 14 counties were sampled between July 28, 2019, and August 4, 2025 (n=2,656). Most (94.1%) systems were sampled on at least 3 separate occasions. 503 systems (79.7%) and 148 towns (64.1%) exhibited no detectable PFAS in any sample. Six of the 18 congeners assessed were not detected in any sample. The long chain legacy PFOA and PFOS congeners were the most frequently detected analytes, observed in 16.3% and 13.6% of samples, respectively, and had the highest average concentrations among all congeners (1.15 and 1.59 ppt, respectively). They were primarily detected simultaneously, wherein 306 samples had detectable levels of both PFOA and PFOS, and only 186 samples resulted in concentrations of either compound alone. The detection frequencies of the shorter-chain replacement compounds were lower: PFHxS (12.2%), PFBS (8.6%), PFHxS (4.9%), and HFPO-DA (0.2%), indicating dominance of legacy long-chain PFAS. Years with the highest number of samples had the lowest average annual PFAS concentrations. The average sum of the 5 Vermont-regulated PFAS was 3.63 ppt, which is lower than the regulated sum of 20 ppt. Rutland County exhibited the highest average sum value (9.83 ppt). The ten highest sum concentrations were all observed in a single water system in Mount Holly, suggesting a potential localized secondary source of PFAS. Other towns with relatively elevated sum concentrations compared to the rest of the state included Barre Town, Arlington, and Killington. PFOS and PFHxS were the primary contributors to elevated sum concentrations. **Conclusions:** The majority of public water systems in Vermont do not have detectable levels of PFAS, which is consistent with the absence of primary point sources within the state. Although long-chain congeners were phased out more than a decade ago, they continue to persist in higher concentrations and detection frequency than the short-chain replacements, indicating historical legacy contamination rather than ongoing inputs. The annual average trend with sample size shows expanded monitoring found smaller overall statewide impacts. PFAS concentrations were elevated in several specific areas, possibly due to secondary sources that historically used PFAS in their production. Overall, the data show that PFAS detections in Vermont drinking water supplies are low and the congener profile is indicative of historical widespread use, rather than continued ongoing contamination.

PS 3893 Sex-specific hepatic mitochondrial and peroxisomal bioenergetic adaptations following PFOA (C8) exposure

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Background and Purpose: Perfluorooctanoic acid (PFOA) is a persistent per- and polyfluoroalkyl substance (PFAS) that accumulates in the liver and is consistently associated with hepatotoxicity. In humans, elevated PFOA exposure correlates with increased liver enzymes, including ALT. Animal studies show PFOA induces hepatomegaly, peroxisome proliferation, altered fatty-acid oxidation, and triglyceride accumulation. Very-long-chain fatty acids (e.g., C24) undergo initial partial oxidation in peroxisomes before mitochondrial β -oxidation, whereas medium-chain (e.g., C8) and long-chain (C16) substrates follow primarily mitochondrial pathways with varying peroxisome-mitochondria interplay. Despite these mechanistic insights, sex-specific mitochondrial and peroxisomal bioenergetic responses to PFOA, especially in the context of chain-length-specific oxidation is poorly characterized. This study evaluated hepatic mitochondrial respiration across multiple substrate chain lengths and peroxisomal acyl-CoA oxidase-1 (ACOX-1) activity in male and female mice following sub-chronic PFOA exposure. **Methods:** Adult male and female C57BL/6 mice (n = 6/group) received oral PFOA (7.5 mg/kg/day) for 15 days. Liver tissue was

harvested, homogenized (tissue mg $\times$ 20 μ L buffer), and assayed for mitochondrial and peroxisomal bioenergetics. Mitochondrial oxygen consumption was measured using substrates selected to probe chain-length-specific oxidation: lignoceric acid (C24) for peroxisome-mitochondria coordination; palmitoyl-carnitine (C16) for long-chain mitochondrial β -oxidation; octanoyl-carnitine (C8) for medium-chain mitochondrial oxidation; glutamate/malate for TCA-linked respiration; pyruvate/malate for carbohydrate-linked oxidation; and pyruvate alone for anaplerotic (pyruvate carboxylase-driven) flux. Peroxisomal ACOX-1 activity was quantified as a biomarker of enhanced peroxisomal fatty-acid flux. Data were analyzed by two-way ANOVA (sex $\times$ exposure). **Results:** PFOA exposure induced significant substrate- and sex-specific hepatic bioenergetic alterations. Liver weight increased by approximately 116% in males and 142% in females versus controls. ACOX1 activity increased $\sim$ 254% in male PFOA-exposed mice and $\sim$ 194% in females, indicating enhanced peroxisomal fatty-acid flux. In male PFOA-exposed mice, liver homogenate C24 oxidation doubled; C16 oxidation increased $\sim$ 3.6-fold; C8 oxidation $\sim$ 2.7-fold; glutamate/malate-supported respiration $\sim$ 1.2-fold; pyruvate/carboxylase-stimulated (anaplerotic) respiration $\sim$ 3.2-4.7-fold; and pyruvate/malate (carbohydrate-linked) respiration $\sim$ 2-fold. In female PFOA-exposed mice, liver homogenate C24 and C8 oxidation increased $\sim$ 1.6-4.3-fold, with selective ADP + malate-stimulated respiration indicative of a shift toward fatty-acid-preferential β -oxidation. Glutamate/malate respiration nearly doubled, while pyruvate/malate and pyruvate/carboxylase responses were modest. Two-way ANOVA revealed significant main effects of sex and exposure and sex $\times$ exposure interactions for most substrates ($p < 0.0001$ to 0.05). **Conclusions:** Sub-chronic PFOA exposure drives sex-specific hepatic metabolic reprogramming, evidenced by elevated peroxisomal ACOX-1 activity and enhanced mitochondrial fatty-acid oxidation. In males, broad mitochondrial activation aligns with increased peroxisomal chain-shortening flux, whereas in females a selective fatty-acid-preferential oxidation pattern emerges. These findings underscore the functional interplay between peroxisomes and mitochondria in chain-length-specific fatty-acid metabolism and highlight the critical importance of including sex as a biological variable in mechanistic toxicology and PFAS risk-assessment frameworks.

PS 3894 PFOS toxicity in yeast cells: the upregulation and downregulation of genes by RNAseq

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are characterized by their strong carbon-fluorine bonds, which makes it highly resistant to degradation. A notable member of the PFAS group is perfluorooctane sulfonate (PFOS), which is widely used in products like non-stick cookware. It is used because of its unique ability to repel water and oil. However, PFOS's high environmental persistence and long half-life poses significant risks to human health and natural biogeochemical processes. Several studies about PFOS and its toxicity to bacteria and certain eukaryotic cells have been reported; however, studies about PFOS and its toxicity within yeast cells remain to be accomplished. **Methods:** A budding yeast strain was cultured and grown in yeast extract peptone dextrose (YPD) media exposed to PFOS with varying concentrations. Cell viability and mitochondria structure alterations were examined to determine any proliferation rate changes and/or energy depletion within the cell. To determine which genes are being expressed differently when exposed to PFOS, an RNAseq analysis was performed where total RNA from control and treated cells were isolated. The RNA samples were converted to cDNA for the next generation sequencing. A bioinformatic approach was employed to reveal global gene expression in PFOS-treated cells, which was helpful in finding specific biological pathways that are being downregulated or upregulated in response to PFOS. **Results:** PFOS exposure decreased yeast cell viability in a dose-dependent manner. The treatment groups were as follows: no treatment (control), 8 μ M, 80 μ M, 160 μ M, 800 μ M, and 1600 μ M with 800 μ M being the inhibitory concentration (IC50). Due to decrease in cell viability, PFOS exposure impaired mitochondrial function in yeast by disrupting and decreasing energy production, leading to reduced cellular respiration and growth. Finally, PFOS exposure induced differential gene expression in yeast, particularly affecting genes involved in stress response, metabolism, and mitochondrial function. **Conclusions:** Showcasing the toxicity of PFOS in budding yeast cells will offer new insights into the underlying molecular mechanisms of PFOS-mediated toxicity in fungal cells in relation to mammalian and bacterial cells. It will also provide potential impacts in raising public awareness about the risk of PFOS exposure as well as strategies to decrease the presence of the toxic compound within the environment.

PS 3895 Mechanisms driving Per and Polyfluoroalkyl Substances (PFAS) Disposition in Human Breast Milk: Roles of Efflux Transporters, Permeability, and Binding

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are widespread environmental pollutants linked to neurodevelopmental disorders in children. Maternal transfer of PFAS through breastfeeding represents a critical exposure path-

way during early life. This study investigated the toxicokinetic mechanisms driving PFAS transfer into breast milk by evaluating interactions with the efflux transporter Breast Cancer Resistance Protein (BCRP), passive membrane permeability, and PFAS binding in human serum and breast milk. **Methods:** PFAS binding in human serum and breast milk was quantified using solid-phase microextraction with C18 fibers. Passive permeability and BCRP-mediated transport were assessed in trans-well *in vitro* assays using transporter knock-out and BCRP knock-in cell lines and the results were compared to molecular docking simulations using the Molecular Operating Environment (MOE) software. **Results:** PFAS binding in breast milk increased linearly with molecular weight for sulfonic acid and sulfonamide groups. In contrast, carboxyl and fluorotelomer PFAS followed this trend only up to 500 g/mol, after which binding declined. BCRP-mediated transport increased with molecular weight, with efflux ratios ranging from 10- to 50-fold higher in BCRP-expressing cells compared to inhibited controls, agreeing with molecular docking simulations. Similarly, passive permeability increased with molecular weight, with the highest permeability observed for PFAS above 500 g/mol. **Conclusions:** Our preliminary results show that molecular weight strongly influences PFAS transfer into breast milk, with higher molecular weight compounds exhibiting increased binding, greater passive permeability, and enhanced BCRP-mediated transport. The mechanistic data will be integrated into a physiologically based toxicokinetic model to simulate PFAS transfer into breast milk under human physiological conditions, accounting for inter-individual variability such as BCRP polymorphisms and hypoalbuminemia as potential risk factors for disproportionate transfer of PFAS to the breastfeeding infant during lactation.

PS 3896 Understanding the Molecular Impact of PFOS on Protein Structure and Function: A Biophysical and Functional Assessment

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Background and Purpose: Perfluorooctane sulfonate (PFOS), a widespread and highly stable perfluoroalkyl substance, persists in the environment and accumulates in biological systems, posing serious toxicological risks. Its molecular interactions with proteins are suspected to disrupt structural integrity and cellular processes, yet these mechanisms remain poorly defined. This study aimed to investigate how PFOS alters protein structure and function and to link these molecular alterations with cytotoxic and neurotoxic outcomes. **Methods:** Model proteins, including bovine β -lactoglobulin (BLG) and hen egg white lysozyme (HEWL), were incubated with PFOS under physiological conditions. Ultraviolet-visible (UV-Vis) absorption, fluorescence, and Fourier-transform infrared (FTIR) spectroscopy were employed to assess tertiary and secondary structural changes. Morphological and aggregation alterations were visualized by atomic force microscopy (AFM). Fibril formation kinetics were examined using thioflavin T (ThT) fluorescence assays. SH-SY5Y neuroblastoma cells were used to assess cytotoxicity and oxidative stress through cell viability and fluorescence microscopy analyses. Finally, *Caenorhabditis elegans* served as an *in vivo* model to evaluate behavioral and neurodevelopmental toxicity following different concentrations of PFOS exposure. **Results:** Spectroscopic analyses showed distinct PFOS-induced conformational changes, including reduced fluorescence intensity and red-shifted emission maxima, indicating partial unfolding of the protein. FTIR spectra revealed a shift from α -helix to β -sheet structures, consistent with aggregation confirmed by AFM and ThT assays. In SH-SY5Y cells, PFOS exposure caused concentration-dependent decreases in viability, increased reactive oxygen species (ROS) production, and morphological changes. In *C. elegans*, exposure led to reduced locomotor activity and delayed neurodevelopmental milestones, consistent with systemic neurotoxicity. Together, these findings demonstrate that PFOS destabilizes protein structure and function, leading to downstream cellular and organismal effects. **Conclusions:** PFOS exerts a dual molecular and biological impact by inducing protein misfolding and aggregation while impairing neuronal cell health and organismal behavior. The combined use of biophysical, cellular, and *in vivo* approaches establishes a mechanistic framework linking PFAS-induced structural perturbation to neurotoxicity. These results enhance our understanding of PFOS toxicity and highlight the importance of integrating molecular and functional endpoints to develop predictive biomarkers for environmental and human health risk assessment.

PS 3897 Persistent albuminuria in type 2 diabetes is associated with lower circulating PFAS levels: implications for kidney research

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Background and Purpose: The prevalence of diabetic kidney disease (DKD) in the US is rising, highlighting the importance of identifying modifiable risk factors that may accelerate DKD progression, such as exposure to per- and polyfluoroalkyl substances (PFAS). PFAS are nephrotoxic in animal models, yet some epidemiological studies on DKD have identified an inverse association with circulating PFAS levels, making PFAS appear protective against DKD. This unexpected relationship may be due to loss of albumin through the urine (albuminuria), a defining feature of

DKD. Albumin, the most abundant protein in blood, binds strongly with PFAS, and albuminuria has been associated with lower blood PFAS concentrations. We hypothesized that persistent albuminuria, driven by increasing diabetes duration and subsequent progression to DKD, leads to increased urinary PFAS clearance and a decline in circulating PFAS concentrations. **Methods:** The role of albuminuria on PFAS levels was explored through three analyses using data from 1773 participants with diabetes from the National Health and Nutrition Examination Survey 2005-2020 (NHANES) and 137 participants with chronic kidney disease from the Kidney Precision Medicine Project (KPMP). Serum PFAS were measured through liquid chromatography and mass spectrometry; albuminuria was assessed via urinary albumin-to-creatinine ratio (uACR). Analysis 1 used NHANES data to evaluate the relationship between diabetes duration, uACR, and PFAS concentration to test whether albuminuria mediates the association of diabetes duration with PFAS. Analysis 2 applied a one-compartment toxicokinetic model to the NHANES data, integrating physiological and biochemical data to separate albuminuria-mediated from baseline/estimated glomerular filtration rate (eGFR) associated clearance and quantifying the albuminuria-mediated contribution to PFOA variance. Analysis 3 used KPMP data to compare the uACR-to-PFAS association in chronic kidney disease (CKD) versus acute kidney injury (AKI) patients. **Results:** In NHANES, longer diabetes duration was associated with a statistically significant decrease in PFOA levels. Mediation analysis identified a significant negative indirect effect, indicating that some of the reduction in PFOA at longer diabetes durations was ascribed to differences in uACR; 54.3% of the variance in PFOA in people with diabetes was explained by uACR. The direct effect, indicating the remaining difference in PFOA between diabetes groups after accounting for uACR, was not statistically significant ($p > 0.05$). The toxicokinetic model indicated that a 10-fold increase in albumin clearance reduced PFOA levels by 30% across the observed range of albuminuria; when accounting for the range of albuminuria observed across people with diabetes, this translated to a projected 76% reduction in serum PFOA levels. In comparison, differences in estimated eGFR-associated PFOA clearance were not associated with differences in serum PFOA levels. Results were generally consistent across NHANES cycles. KPMP results indicated that lower circulating PFOA is associated with albuminuria in CKD (persistent albumin loss), but not in AKI (short-term albumin loss; interaction p -value = 0.019). **Conclusions:** Results suggest that progressive diabetic nephropathy, marked by persistent continuing albuminuria, increased urinary excretion of albumin-bound PFAS and decreased blood PFAS concentration. This decrease can be explained by the mediating role of albuminuria. These findings help explain the previously reported paradoxical protective association between blood PFAS and DKD risk. Results highlight concerns about the reliability of using blood PFAS measurements for assessing PFAS exposure in populations with albuminuria, particularly DKD.

PS 3898 A bioinformatics approach identifies emerging PFAS associated with amino acid and lipid metabolic pathways during early pregnancy

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Background and Purpose: Exposures to dozens, if not hundreds/thousands, of environmental chemicals can occur during pregnancy, yet only a handful of chemicals, like some per- and polyfluoroalkyl substances (PFAS), are routinely biomonitored. For instance, although there are >14,000 different PFAS of known production, the exposures and associated toxic effects of only a handful (<1%) of PFAS are known. Some of these emerging PFAS are marketed as less toxic and bioaccumulative, yet they may have similar metabolism disrupting potential and impacts to maternal and child health as known legacy PFAS (e.g., PFOS and PFOA). Targeted approaches are routinely conducted to monitor some legacy PFAS, yet nontargeted high-resolution mass spectrometry (HRMS)-based approaches coupled with bioinformatics tools may hold benefits over these approaches. These HRMS approaches can simultaneously measure complex, real-world mixtures of both legacy and emerging environmental pollutants and metabolic products, offering to better determine disease risk during pregnancy and the breadth of chemical exposure. **Methods:** We aimed to use a nontargeted HRMS approach that monitors both endogenous metabolites and environmental pollutants to identify associations between mixtures of emerging and legacy PFAS with metabolic pathway in serum of 100 pregnant women from MARCH (Michigan Archive for Research on Child Health), a cohort of the Environmental influence on Child Health Outcomes (ECHO) program. A discovery-based bioinformatics approach, FluoroMatch 3.0, was used to identify nonlegacy/emerging PFAS in these same samples. Metabolic pathways of interest were determined using MetaboAnalyst software. Linear models adjusted for variables known to impact circulating PFAS levels and metabolic profiles during pregnancy (diet, alcohol consumption, maternal age, smoking, body mass index, income, and race/ethnicity) were used to assess associations between PFAS exposure and metabolic pathways. **Results:** 105 PFAS were identified with high identification confidence from matching retention time, exact mass, and fragmentation patterns to the library database, with 86 chemicals showing >90% detection across all maternal serum samples. Only 9 identified compounds can be considered a legacy or a routinely-monitored emerging PFAS, while the remaining are emerging PFAS with no previously reported detection or toxicity assessments in the current literature. Of the list of 105 FluoroMatch-identified PFAS, 51 showed significant associations with at least one metabolic

pathway at $FDR < 0.05$. 21 emerging PFAS also showed a significant positive ($p < 0.05$) association with legacy PFAS (e.g., PFOA, PFOS). Pathways significantly associated with these 21 emerging PFAS were largely similar as legacy PFAS and were near-exclusively related to amino acid metabolism, including taurine and hypotaurine metabolism, glycine, serine, and threonine metabolism, arginine and proline metabolism, and valine, leucine, and isoleucine biosynthesis (at $FDR < 0.05$). **Conclusions:** Dozens of emerging PFAS not previously reported in the current literature with potentially similar metabolism-disrupting potential as legacy PFAS were detected with high detection frequency in Michigan mothers during early pregnancy. These results will inform future biomonitoring studies that utilize targeted approaches to include and prioritize these additional PFAS of emerging concern. Moreover, these results may lead to potential new biomarkers of exposure and identify biomechanisms of toxicity to aid in early intervention and mitigate maternal-child health burdens.

PS 3899 Parkin regulates PFOS-induced apoptosis via BAX/BAK ubiquitination in rat dopaminergic neuronal cells

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are a group of synthetic compounds known for their high chemical stability and resistance to degradation. Perfluorooctane sulfonate (PFOS) is one such PFAS that has been found to produce mitochondrial stress and neurotoxicity. Mitochondrial dysfunction is a critical early event in neuronal injury that may lead to apoptotic cell death. Despite growing evidence on the involvement of PFOS exposure in neurotoxicity, the precise molecular mechanisms governing this process remain incompletely understood. Parkin, an E3 ubiquitin ligase, has a central role in maintaining cellular homeostasis by ubiquitinating substrate proteins involved in crucial cellular processes, including protein catabolism, immune response, and cellular apoptosis. Dysregulation of parkin activity has been implicated in mitochondrial damage and neuronal vulnerability. Considering the critical role of parkin in mitochondrial quality control and apoptosis, this study aimed to test the hypothesis that PFOS-induced apoptosis in dopaminergic neuronal cells would be modulated through Bax, Bak, and Bcl-2 ubiquitination. **Methods:** N27 cells were exposed to 0-650 μ M of PFOS for different durations (0-72 hrs) to evaluate dose- and time-dependent effects. Cell viability was assessed using MTT. Mitochondrial ROS generation and mitochondrial membrane potential ($\Delta\Psi_m$) were assessed by MitoSOX Red and TMRM fluorescence, respectively, to assess mitochondrial function. Based on these results, toxic and subtoxic concentrations and corresponding time points were selected for further analysis of gene and protein expression of apoptosis- and mitochondrial-related markers. **Results:** PFOS exposure resulted in a dose- and time-dependent elevation of mitochondrial reactive oxygen species (ROS), accompanied by a marked reduction in TMRM fluorescence. During the early phase of stress, both parkin gene expression and protein levels were increased, suggesting an adaptive cellular response aimed at maintaining mitochondrial integrity and suppressing apoptosis. Increased parkin activity enhanced substrate ubiquitination, including Bax/Bak/Bcl2, leading to reduced expression of pro-apoptotic factors and a relative increase in anti-apoptotic Bcl-2 levels, indicating a transient protective effect. However, at higher PFOS concentrations and prolonged exposure, parkin expression declined, coinciding with a significant increase in Bax and caspase-3 expression and a reduction in Bcl-2 levels, consistent with the activation of the intrinsic apoptotic pathway and loss of mitochondrial homeostasis. **Conclusions:** Our data indicate that PFOS induces mitochondrial dysfunction and apoptosis in dopaminergic N27 cells, potentially through disruption of parkin-mediated ubiquitination of apoptosis-related proteins. The resulting imbalance between pro- and anti-apoptotic signalling may contribute to PFOS-associated neurotoxicity. The findings are expected to provide insight into how environmental toxins may disrupt parkin-dependent mitochondrial surveillance mechanisms and thereby contribute to dopaminergic neurodegeneration.

PS 3900 Environmental fate and behavior studies of polytetrafluoroethylene (PTFE) - results and application to risk assessment

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) may be non-polymeric (per- or polyfluoroalkyl substances) or polymeric (fluoropolymers, perfluoropolyethers, side-chain fluorinated polymers). Although peer-reviewed publications have demonstrated that PTFE has low toxicity, high stability, high chemical resistance, and is not bioaccumulative, the literature does not contain environmental fate and behavior testing. The environmental fate and behavior for a well characterized PTFE-FA (high molecular weight, fine powder, microparticulate PTFE meeting the ASTM D4895 specification) are presented here. Physicochemical properties, biotic and abiotic degradation (in air, water, soil and sediment), transformation, mobility, partitioning, long range transport potential, and application of this data to the state-of-the-science polymer risk assessment are discussed. This addresses what would happen to PTFE-FA if found in the environment: would it degrade to and subsequently release substances of concern like non-polymeric PFAS? **Methods:**

OECD test guidelines (TG) 100 (physical chemical properties) and 300 (environmental fate and behavior) series were performed at CRL utilizing validated analytical methods. An ultra-high molecular weight, fine powder PTFE (CAS no. 9002-84-2) that met the ASTM D4895-18 2018 specification for PTFE made from dispersion (referred to as PTFE-FA), with an average particle size (D 50) of 529 µm, was tested. Analytical methods, based on UPLC with MS/MS, were developed for the quantitative analysis of 33 PFAS in aqueous samples, natural sediment, natural soil, and activated sludge. Spike recovery was done in each matrix tested for all analytes resulting in acceptable values validating that they could be detected if they were present in these matrices above the LOD. These methods were validated with respect to specificity, calibration, accuracy and repeatability, limit of quantification, stability of analytical system as well as solutions, and storage stability of samples. These analytes and analytical method development were selected based on best available analytical methods (EPA Methods 533 and 537) in 2019 (study start), rigorous review, adherence to scientific principles and knowledge of fluoropolymer chemistry. **Results:** Using DSC (TG 102), the melt transition was observed at ~350°C. No further melting/decomposition below 400°C was observed. Thermal stability (TG 113) was confirmed by minimal weight loss (5%) at 549°C. The vapor pressure, $< 1 \times 10^{-10}$ of mm Hg at 20°C (TG 104) confirmed the lack of volatility and low likelihood of partitioning to air and long-range transport potential, as a gas or vapor. Although solubility prevented GPC testing (TG 118), molecular weight was $> 500,000$ through Standard Specific Gravity tests. Results of OECD TG 105, 120 and the published literature confirm PTFE-FA is insoluble in water and is highly unlikely to be a water-soluble contaminant or be mobile in water. PTFE-FA was not corrosive, caustic, or ionizable (TG 122). It was not readily biodegradable (TG 301B) and had no inherent biodegradability (TG 302C). Aerobic and anaerobic biodegradation testing in soil (TG 307) and aquatic sediment (TG 308) showed PTFE-FA to be completely stable. It was stable and non-inhibitory in aerobic sewage (TG 303A). It was completely photolytically stable (TG 316 and Phototransformation of Chemicals on Soil Surfaces). PTFE-FA was hydrolytically stable (TG 111). Insolubility prevented the performance of TG 105, 107, 117, 121, 306, Henry's Law Constant and log K_{ow} . **Conclusions:** These results demonstrate PTFE-FA's absence of mobility, degradation, adsorption to soil/sediment, partitioning to air (as a gas or vapor) from water or organic matter, long-range transport in air (as a gas or vapor), water or soil, transformation to and subsequent release of non-polymeric PFAS. Therefore, the persistence of PTFE-FA does not imply future degradation, transformation and subsequent release of substances of concern. As the EU PFAS restriction proposal evolves, this data reduces uncertainty in this fluoropolymer risk assessment based on the lack of degradation, transformation and subsequent release of substances of concern.

PS 3901 PFAS Exposures in Military Firefighters Enrolled in the Millennium Cohort Study

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are major constituents of aqueous film-forming foams used in firefighting operations, and previous research has found that firefighters have higher body burdens of PFAS compared with the general population. The objective of this study was to conduct a comprehensive analysis of PFAS congeners measured in existing serum collected from military firefighters and a comparison group of construction workers enrolled in the Millennium Cohort Study, a large prospective health study that has enrolled over 260,000 US service members since 2001. **Methods:** Existing serum specimens from the Department of Defense Serum Repository were analyzed to quantify short- and long-chain PFAS congener concentrations via a validated assay using UHPLC-coupled electrospray ionization tandem mass spectrometry. PFAS concentrations were compared among 830 firefighters and 616 construction workers. PFAS concentrations were log-transformed and the interquartile range was calculated. Multivariable logistic regression models were used to investigate associations between demographics, military characteristics, and interquartile range increase in log total PFAS concentrations and cardiometabolic conditions. **Results:** The geometric mean concentrations of total PFAS, total perfluorohexane sulfonic acid, perfluorooctanoic acid, perfluorooctanoic acid, and total perfluorooctane sulfonate were elevated among firefighters compared with construction workers (eg, total PFAS: 40.7 vs 26.3 µg/L; $p < .0001$). These differences mostly persisted in analyses stratified by service branch. By Service branch, Air Force personnel consistently had higher geometric mean concentrations of PFAS congeners than those in the Army (eg, total PFAS: 38.3 vs 22.7 µg/L; $p < .0001$). Cross-sectional analyses indicated a 30% higher risk of cardiometabolic conditions per interquartile range increase in log total PFAS concentration (OR 1.29; 95% CI, 1.04-1.60). **Conclusions:** Findings from this study are critical for establishing historical baselines for PFAS body burden among military firefighters. The observed association between total PFAS exposure and cardiometabolic conditions suggests the potential for long-term health consequences among exposed military personnel and veterans.

PS 3902 Perfluorooctanoic Acid (PFOA) Exposure Induces Oxidative and Inflammatory Stress Markers in C57/BL6 mouse Ocular Tissues

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are persistent environmental contaminants linked to immune suppression, hepatotoxicity, and dysregulation of lipid metabolism. The prototypical PFAS, perfluorooctanoic acid (PFOA), structurally resembles fatty acids and interacts with lipid-binding proteins such as fatty acid-binding protein 1 (FABP1), potentially driving oxidative stress through lipid peroxidation pathways. The retina and optic nerve are particularly susceptible to such damage due to their high lipid content and oxygen consumption. Separately, high-fat diets (HFD) have been shown to disrupt retinal lipid metabolism and induce oxidative and inflammatory injury. Together, these findings suggest that chemical (PFOA) and dietary (high-fat diet) lipid stressors may compromise ocular health through similar mechanisms. We hypothesized that PFOA exposure increases oxidative and inflammatory stress while impairing ocular defense pathways, thereby contributing to an elevated risk for eye disease. **Methods:** Male and female C57BL/6 mice were administered PFOA (0 or 7.5 mg/kg/day) by oral gavage for 15 days. A separate cohort was placed on a 60% high-fat diet (HFD) without PFOA (no combined exposure group) to serve as a dietary lipid stress comparison group. Pre- and post-exposure serum and whole eyes (excluding the HFD group) were collected and stored at -80 °C. Ocular tissues were sonicated in RIPA buffer, and supernatants were analyzed by ELISA for Interleukin-6 (IL-6), 8-isoprostane, and Tumor necrosis factor-α (TNF-α) to assess inflammatory and oxidative stress responses. Remaining samples were prepared for future analyses. Ocular expression of apoptotic marker p53 and protective proteins pigment epithelium-derived factor (PEDF) and lactoferrin will be evaluated by Western blotting to assess cellular stress and defense mechanisms. Serum PFOA concentrations will be quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Sex-specific differences will be evaluated using one-way ANOVA followed by Tukey's honestly significant difference (HSD) test. **Results:** Preliminary findings indicate a ~37% reduction in serum IL6 levels following PFOA exposure compared to pre-exposure levels, although, these differences between treatment groups were not statistically significant. No significant IL-6 changes were observed in ocular homogenates. Based on complementary assays, we anticipate elevated 8-isoprostane and TNF-α concentrations in both serum and ocular tissue, consistent with enhanced lipid peroxidation and proinflammatory signaling. Western blot analyses are expected to show increased p53 expression, reflecting oxidative stress-induced apoptosis, along with reduced PEDF and lactoferrin expression, indicating compromised ocular defense capacity. These findings suggest that PFOA suppresses systemic inflammatory signaling while promoting oxidative and apoptotic stress within ocular tissues. The observed IL-6 reduction may reflect PFOA-mediated immune suppression consistent with prior reports of lymphocyte inhibition. Ongoing integration of LC-MS/MS exposure data with biochemical and protein endpoints will clarify the link between PFAS burden, oxidative injury, and ocular health, providing mechanistic insight into early molecular indicators of PFAS-associated eye disease. **Conclusions:** Although changes in IL-6 were not statistically significant, the observed reduction in serum IL-6 levels may reflect PFOA-mediated immune suppression and lymphocyte depletion. Further quantification of oxidative stress (via 8-isoprostane) and inflammatory markers (TNF-α and lactoferrin) will further define the oxidative and inflammatory burden imposed by PFOA exposure. Assessing ocular expression of p53 and PEDF will provide mechanistic insight into how PFAS disrupt redox balance and compromise ocular defense pathways. Due to the structural similarities between PFOA and long-chain fatty acids, these studies may also help uncover shared mechanisms of action between PFAS exposure and high-fat diet-induced metabolic stress. Integrating these findings with serum PFOA concentrations will clarify the molecular cascade linking PFAS exposure to ocular oxidative and inflammatory injury and may identify early biomarkers of PFAS-associated eye disease.

PS 3903 Immuno-proteomic profiling of neural antigens links short- and long-chain PFAS exposure to neuropathological pathways in adolescents from Taranto, Italy

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) exposure has been linked to various neurological disorders, including neurodegenerative and neurodevelopmental pathologies. Early life and chronic exposure to PFAS among individuals born and living near contaminated industrial sites may elevate the risk of neurological disorders. However, the biological mechanisms and molecular pathways through which PFAS disrupt neurological function remain poorly understood. This study aimed to identify PFAS-associated alterations in circulating neural proteins among 286 adolescents living in different zones of exposures near a large steel plant in Taranto, Italy. **Methods:** Four long-chain (PFOS, PFOA, PFNA, PFHxS) and two short-chain (PFBA, PFHxA) PFAS were measured using liquid chromatography-mass spectrometry (LC-MS/MS) in the serum of the study participants. Serum antibodies specific to neural antigens were measured using a multiplex protein microarray. The microarray targeted 64 neuronal and glial antigens associated with

neuroinflammation and cognitive decline. Antibody reactivity was used to assess a targeted neural proteomic profile. Associations between PFAS exposure and neural antibody reactivity were evaluated using correlation heatmaps and volcano plots in RStudio. Functional enrichment of significantly ($p < 0.05$) altered proteins was performed using the STRING database to identify Gene Ontology (GO) pathways. **Results:** Distinct immuno-proteomic profiles of neural antigens associated with short- and long-chain PFAS exposures were observed among the study participants living in different exposure zones of the steel plant. Based on one-way ANOVA with Tukey's post hoc comparisons, short-chain PFAS showed significantly higher concentrations in zones closer to the steel plant (Zones 1 and 2), whereas long-chain PFAS (PFOS, PFOA, PFHxS, and PFNA) were significantly higher in the distal zone (Zone 3). Unlike the ubiquitous long-chain PFAS, the short-chain PFAS detected near the plant likely originate from facility-specific processes or waste streams, such as PFAS-containing industrial materials, or firefighting foams used during operational incidents. The top three proteins upregulated by long-chain PFAS were $\alpha 2B$ -adrenergic receptor (ADRA2B), Beta-Amyloid (25-35), and dopamine β -hydroxylase (DBH), while the most downregulated ones were 5-Hydroxytryptamine (5HT), amphiphysin (AMPH), and acetylcholine receptor. In contrast, short-chain PFAS exposure upregulated 5HT, AMPH, and glutamate decarboxylase 2 (GAD2), and downregulated Beta-Amyloid (25-35), heat shock protein 5 (HSPA5), and Neurofascin proteins. The functional analysis using the STRING database showed that proteins upregulated by long-chain PFAS were significantly enriched in pathways regulating neurotransmitter signaling and dopamine metabolism. Downregulated proteins linked to long-chain PFAS were primarily involved in dopaminergic signaling and synaptic function, indicating potential suppression of neurotransmitter receptor activity and postsynaptic processes that may impair neuronal communication. On the other hand, short-chain PFAS enhanced inhibitory synaptic signaling activity by upregulating proteins involved in inhibitory neurotransmission and postsynaptic receptor function. There was no significant enrichment for proteins downregulated by short chain PFAS. **Conclusions:** The findings reveal that short- and long-chain PFAS produce distinct immuno-proteomic signatures of circulating neural proteins in adolescents. Long-chain PFAS appear to disrupt dopaminergic and excitatory pathways, whereas short-chain PFAS enhance inhibitory neurotransmission. These contrasting molecular patterns suggest short- and long-chain PFAS-specific mechanisms that may alter synaptic balance and contribute to neurological dysfunction. Together, they provide preliminary molecular evidence that both individual PFAS and their mixtures exert type-specific neuropathological effects among adolescents living near the steel plant in Taranto, Italy.

PS 3904 Rapid Non-target Detection of Per- and Polyfluoroalkyl Substances (PFAS) in Artificial Turf

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Background and Purpose: Artificial turf (AT) fields are widely used across the United States, with over 130,000 fields and 1,200-1,500 new fields installed annually. This has raised toxicological concerns regarding potential exposure to per- and polyfluoroalkyl substances (PFAS), a diverse group of >14,000 anthropogenic compounds known for environmental persistence and bioaccumulative potential. PFAS are used in polymer coatings and plastic manufacturing associated with AT production. However, little is known about which PFAS are present, their sources within AT components, or their environmental transformation during AT lifetime. This study will identify multiple PFAS and their concentrations in AT fields to contribute to evidence-based environmental decision-making. **Methods:** AT field samples were collected, ranging from weathered to non-weathered synthetic grass fibers, traditional crumb rubber infill and "eco-friendly" alternative infill, and backing and shock pad materials. Following collection, we performed a non-target analysis using paper spray mass spectrometry (PS-MS), an innovative rapid ionization technique that enables direct analysis from minimal extraction to detect PFAS in each AT field component. After PFAS detection (i.e., presence/absence), tandem mass spectrometry was used to confirm the identity and structure of PFAS species. **Results:** PS-MS revealed multiple PFAS compounds distributed across different AT fields. Over twenty PFAS species were identified, including perfluoropentanoic acid (PFPeA) and perfluorohexanoic acid (PFHxA), in weathered and non-weathered samples, suggesting that PFAS is produced during manufacturing in addition to environmental transformation over AT lifetime. **Conclusions:** This study demonstrates that PS-MS provides rapid, highly sensitive, highly specific, and accurate analysis of PFAS in artificial turf fields. Overall, multiple PFAS were detected in synthetic grass fibers of AT fields, which may contribute to human exposure and environmental contamination. These findings highlight the importance of applying rapid analytical methods in exposure assessment and toxicological evaluation of artificial field components to better inform risk assessment and regulatory monitoring strategies.

PS 3905 PFOS induces cellular damage in HepG2 and THLE-2 cell models

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Background and Purpose: Perfluorooctane sulfonate (PFOS) is a persistent environmental pollutant that has posed potential hepatotoxic effects and associated health risks. Despite its widespread distribution, the underlying mechanisms remain poorly

understood. To investigate the molecular mechanisms behind toxicities of PFOS, our study utilized HepG2, and THLE-2 hepatic cell models to replicate conditions reflecting PFOS accumulating in the liver. **Methods:** Cell viability, cell stress, and cell death assays were conducted to assess the toxicological interactions of the chemical on both cell lines. Total RNA extraction was performed, followed by cDNA sequencing, quantification using rt-qPCR. **Results:** The XTT viability assay revealed a dose-dependent decrease in number of viable cells during treatments with increasing PFOS concentrations. The inhibitory concentration (IC50) values were approximately 100 micromolar, which elevated reactive oxygen species (ROS) production and apoptotic level in HepG2 cells. Based on the transcriptomic analysis for HepG2, mitochondrial genes involved in oxidative phosphorylation were downregulated, including COX, ND, and ATP synthase family. Additionally, there were about 30 up-regulated genes implicated in the cAMP signaling pathway. CYP7A1 and HSPA6 were chosen candidate genes for rt-qPCR validation. **Conclusions:** Our future goals involve exploration of mitochondrial damage under PFOS exposure, providing comprehensive insights into the chemical's toxic effects on hepatic cellular metabolism.

PS 3906 Subchronic 90-Day Oral Toxicity Study With Perfluoropentanoic Acid In Sprague Dawley Rats

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Background and Purpose: Perfluoropentanoic acid (PFPeA) is a perfluorinated alkyl substance (PFAS) that is used in aqueous film forming foams and is also used as a surfactant and stain repellent. PFPeA has been found in surface and drinking water and biosolids in several countries. However, there is limited publicly available toxicity data for PFPeA. A subchronic 90-day oral toxicity study in Sprague-Dawley rats was performed to provide toxicity information. **Methods:** Subchronic (90-day) guideline (OPPTS 870.3100) studies were conducted in male and female Sprague Dawley rats ($n=10/\text{sex}/\text{dose}$) exposed to PFPeA via gavage in 100mM citrate buffer adjusted to pH 4.5 ± 0.2 (5 ml/kg). The dose range was based on repeat dose 14-day studies and were 62.5, 125, 250, 500 and 1,000 mg/kg/day. Ophthalmological examinations were performed after arrival but before dosing and again before termination. Body weights and clinical observations were performed weekly with daily cage-side observations. After ≥ 11 weeks of exposure, animals were screened for neurotoxicity with a functional observational battery (FOB). After ≥ 90 days of exposure, animals were humanely euthanized. Animals were fasted overnight before termination. A full screen necropsy with collection of selected tissues was performed. Tissues were fixed by appropriate methods and microscopically evaluated. Samples were collected for clinical pathology analysis. **Results:** Body weights were decreased at 1000 mg/kg/day in the male rats. No other changes in body weights were observed. There were no changes in food consumption or ophthalmic examinations. Decreases in forelimb strength was observed in males only at the 1000 mg/kg/day dose and were associated with changes in body weight. Increases in relative liver and kidney weights were observed at 1,000 mg/kg/day in males and females. Males also had increased relative brain and testes weights at the high dose. Absolute and relative pituitary weights were decreased in females at 62.5 and 125 mg/kg/day but not at other dose levels. In males, absolute pituitary weight was reduced at 62.5, 500, and 1,000 mg/kg/day, while relative pituitary weight was reduced at 125 mg/kg/day. There were no microscopic findings observed in any of the 1,000 mg/kg/day exposures. Gross observations were either not accompanied by microscopic findings or were considered incidental lesions. **Conclusions:** Since the changes in organ weights were not accompanied by consistent dose response trends or histopathological changes, these effects were deemed not adverse. The overall evaluation indicates that there were toxicological effects observed at the 1,000 mg/kg/day dose and the no observed adverse effect level for PFPeA is 500 mg/kg/day. (The views expressed in this abstract are those of the authors and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency)

PS 3907 Tire-Derived 6-PPDq Impairs Mitochondrial Function and Viability in Human Cardio-Pulmonary Cells.

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Background and Purpose: N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine quinone (6-PPDq), an ozone-derived transformation product of the tire antioxidant 6-PPD, has recently emerged as an airborne toxicant detected in particulate matter and aerosols. While human exposure likely occurs at nanogram-microgram levels, its impact on cardio-pulmonary health remains poorly defined. **Methods:** Lung tissue from individuals with chronic obstructive pulmonary disease (COPD) and healthy controls were analyzed for 6-PPDq using LC-MS/MS. Human lung epithelial (A549),

airway smooth muscle (ASM) and cardiomyocyte (AC16) cells were cultured as 2D monolayers and 3D spheroids using A549 cells. Cytotoxicity was quantified by MTT assay (1-1000 ng/ml). Mitochondrial function was assessed via extracellular flux analysis (Seahorse XF Cell Mito Stress Test), JC-10 dye assay for mitochondrial membrane potential. Cell injury and epithelial integrity were assessed by LDH release and transepithelial electrical resistance (TEER), respectively. Epithelial-mesenchymal transition (EMT) markers were analyzed by immunoblotting. **Results:** 6-PPDq accumulation was approximately twofold higher in COPD lung tissues compared to healthy samples (n=5). *In vitro*, 6-PPDq induced a concentration-dependent loss of viability in A549 and ASM cells after 48h ($p<0.001$) and in AC16 cells within 24h ($p<0.001$). Mitochondrial stress testing revealed impaired spare respiratory capacity and significant loss of membrane potential using after 12 hours ($p<0.05$), accompanied by elevated LDH release ($p<0.01$). TEER analysis demonstrated a -dependent decline in epithelial cell barrier integrity. In 3D epithelium spheroids, 6-PPDq exposure exhibited altered morphology and reduced viability, suggesting enhanced sensitivity of multicellular structures. EMT marker upregulation (N-Cadherin, Vimentin, and α -SMA) indicated a phenotypic shift towards mesenchymal characteristics. **Conclusions:** These data identify 6-PPDq as a cardiopulmonary toxicant that accumulates in human lung tissue, disrupts mitochondrial energy metabolism, impairs epithelial barrier function, and promotes EMT. The findings highlight a potential mechanistic basis for cardiopulmonary injury following environmental 6-PPDq exposure and underscore the need for further risk assessment.

PS 3908 Chemical-dependent Effects of Acute Wildfire Smoke Exposure on Shared Inflammatory Dysregulations in the Lung and Brain

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Background and Purpose: Health impacts of wildfire smoke have arisen as a public concern with the increase in global prevalence of wildfire. Epidemiology and animal studies have both shown negative respiratory health effects of wildfire smoke. More recently, the effects of wildfire smoke have been further assessed in its neurotoxic effects and have been associated with dementia and cognitive impairment. Wildfire smoke is a complex mixture of gases and particulate matter which vary in degree by the source of biomass, chemical composition, and combustion scale and phases. To untangle these complexities and investigate the effect of wildfire in the lung and brain, we exposed rats to woodsmoke extracts (WSE) reconstituted with different classes of chemicals commonly found in real-world wildfire. We hypothesize that chemicals reconstituted in WSE will differentially induce inflammatory molecular changes in similar ways in the lung and the brain, potentially acting through shared regulatory mechanisms. **Methods:** Male Sprague Dawley rats (n=7-10) were intranasally instilled with 50µg/kg of either ¹⁴C-palmitic acid (PA), ¹⁴C-benzo[a]pyrene (B[a]P), or ¹⁴C-catechol (CAT) reconstituted in 1mg/mL of smoldering eucalyptus WSE for 24h. Controls received equal volume of saline. The left-brain hemisphere and left lung lobe were fixed for histology. Paraffin sections were stained for cyclooxygenase-2 (COX-2), a biomarker of acute inflammation, COX-2 and heme oxygenase-1 (HO-1), an indicator of antioxidant response, using fluorescent immunohistochemistry (IHC). COX-2 and HO-1 were each co-stained with neuron (NeuN) on brain sections and macrophages (CD68) on lung sections. Quantitative IHC analysis was done for each biomarker using customized macros in Fiji. IHC image data were analyzed with linear mixed-effects regression for brain and simple linear regression for lung sections, followed by BH FDR correction, in R Studio. **Results:** In the lung, COX-2-positive cell density was significantly increased with B[a]P exposure, and interestingly, the same trend was observed in B[a]P-exposed hippocampal sections without reaching significance ($p_{adj}=0.109$). The number of HO-1-positive macrophages in the lung was reduced with PA exposure ($p_{adj}=0.068$). While a similar trend in HO-1-positive neuronal counts were observed in the hippocampus, changes were statistically insignificant with high variance. No notable changes were observed in astrocytes in the dentate gyrus brain region, while there was 4.2 fold increase in percent area of microglial expression in the B[a]P exposure group ($p_{adj}=0.056$). **Conclusions:** Our results indicate that WSE caused chemical-dependent inflammatory dysregulations in both the lung and brain. Similar patterns of chemical-induced changes in COX-2 and HO-1 expression in the lung and brain suggest either: (1) B[a]P and PA may act through shared molecular pathways upon entering lung and brain, or (2) that mediators originating in the lung circulate systemically to the brain and drive neuroinflammation. These findings warrant further mechanistic studies to delineate the temporal sequence of events to establish the lung-brain axis. Nevertheless, our results still indicate that the brain is affected by WSE-reconstituted chemicals in a manner similar to the lung. Moreover, these findings raise the importance of identifying main chemicals in wildfire to provide more tailored and effective medical care to exposed individuals. Supported by LLNL LDRD 23-ERD-020 and leveraged Fiji macro-driven automated IHC image analysis pipeline developed under NIH RF1 AG074709. This work performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344. LLNL-ABS-2013563.

PS 3909 Study of Neighborhood Air near Petroleum Sources (SNAPS): California's characterization of air quality and health risks in communities near oil and gas operations

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Background and Purpose: California's Office of Environmental Health Hazard Assessment (OEHHA) and the California Air Resources Board (CARB) developed the Study of Neighborhood Air near Petroleum Sources (SNAPS) program to characterize air quality and corresponding health risks in communities near oil and gas operations in California. SNAPS is a first-of-its-kind, community-driven monitoring effort focused on communities living near oil and gas operations. Lost Hills, CA (Kern County) is the first SNAPS location. It is a small community downwind of oil and gas operations. It is close to an interstate, trucking route and agricultural activities. Lost Hills is a disadvantaged community with a CalEnviroScreen score in the 86th percentile and pollution burden in the 91st percentile. **Methods:** OEHHA and CARB used public engagement to develop the SNAPS Lost Hills program. Input and lived expertise from communities informed monitoring locations, mobile monitoring timing, and communication of complex data to a broad audience. Staff attended and led in-person and virtual community meetings to provide updates and incorporate feedback. SNAPS measured pollutants in Lost Hills from May 2019 through April 2020 using a suite of instruments housed in a stationary trailer and a mobile platform. CARB conducted monitoring according to a community-specific air monitoring plan and a quality assurance project plan and processed data to provide 1-hour and 24-hour pollutant concentrations. Using the collected monitoring data, OEHHA performed human health risk assessments using OEHHA's Air Toxics Hot Spots methods. We used hazard quotient and hazard index to assess individual chemical hazard and cumulative hazards, respectively. We estimated cancer risk with cancer slope factors and estimated cumulative risk as the sum of individual risks. We used the single highest 24-hour or 1-hour concentration for acute assessment and the annual average concentration for chronic and cancer assessment. SNAPS communicated data and results at public meetings and via posting on the SNAPS website. The final report was posted in October 2025. **Results:** In Lost Hills, SNAPS monitored close to 200 compounds, including criteria pollutants and toxic air contaminants. Concentrations of particulate matter (PM_{2.5}), ozone, carbon monoxide, lead, and hydrogen sulfide met ambient air quality standards. Pollution levels were similar to other Central Valley locations, with the exception that acrolein concentrations were higher. Source apportionment showed that oil and gas-related sources contributed 39-55% of benzene, toluene, ethyl benzene and xylene (BTEX) concentrations. Similarly, oil and gas-related sources contributed over 80% of other volatile organic compounds (VOC) analyzed. When wind came from the direction of the Cahn 3 Gas Processing Plant, increased hydrocarbons and VOCs were observed in Lost Hills. Black carbon was primarily from mobile sources. Noncancer health hazards of concern (hazard index or hazard quotient >1) were identified for the respiratory system (acute HI=4.5, chronic HI=6.6), eyes (acute HI=2.1), and nervous system (chronic HI=1.1). Acrolein (acute HQ=2.2, chronic HQ=5.5), dimethyl disulfide (acute HQ=2.0), metals and benzene levels contributed to these hazards. Most carcinogens were detected at concentrations similar to other areas of California not directly associated with oil and gas production. Key contributors to cancer risk were diesel particulate matter, carbon tetrachloride, formaldehyde, and benzene, with cancer risk estimates of 460, 67, 66 and 36 excess cases per million, respectively. Cumulative cancer risk was 760 excess cases per million. No health value was available to assess cancer risk from acrolein. **Conclusions:** SNAPS is a novel air monitoring program that aims to improve the understanding of pollutants that residents living near oil and gas production may be exposed to. Results in Lost Hills showed potential hazards to eyes, the respiratory system and nervous system. Monitoring showed elevated levels of acrolein compared to other parts of the state. Acrolein is a carcinogen but has no health guidance value available to assess cancer risk. In response, OEHHA is developing a cancer potency factor for acrolein.

PS 3910 Impaired efferocytosis by MerTK shedding in macrophages attributes to pathogenesis of cadmium induced emphysema

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Background and Purpose: Cadmium (Cd) is a widely recognized toxic heavy metal that is commonly found as an occupational and environmental pollutant. Exposure to Cd accumulated in the lungs and other organs may inevitably affect intracellular signaling, resulting in chronic inflammation, COPD, and emphysema. In this study, we aimed to investigate the mechanisms underlying Cd-induced impairment of efferocytosis (phagocytic clearance of apoptotic cells), leading to chronic inflammation and emphysema. **Methods:** Mouse alveolar macrophage cells treated with Cd, and were assessed for their efferocytosis ability. The cell lysates were used for immunoblotting for the efferocytosis protein MerTK, pADAM17, and ADAM17. C57BL/6 mice (8-10 weeks old, both male and female), or MerTK^{-/-} were exposed to cadmium and evaluated for immunohistochemical analysis, lung functions, and lung injury at Day 21st. *Ex vivo* and *in situ* efferocytosis assays were performed to analyze efferocytosis function in alveolar macrophages of control and Cd-exposed mice. The effects of soluble Mer (sMer) treatments were analyzed in control and Cd-exposed mice. **Results:** Cadmium reduced the efferocytosis index in alveolar macrophages;

however, it upregulated MertK expression. Furthermore, we found that Cd treatment caused MerTK shedding through activation of ADAM17. Proteolytic cleavage of Mer produced an extracellular form of Mer (sMer), which acted as a decoy receptor by binding to Gas6 and inhibiting efferocytosis. *In vivo* studies showed that Cd increased sMer levels and ADAM17 activity and inhibited efferocytosis, leading to emphysema. Intratracheal administration of sMer was itself pathological, decreasing the efferocytosis index and increasing lung inflammation and emphysema. Co-treatment with sMer and Cd worsened inflammation and emphysema and further reduced the efferocytosis index. Additionally, we observed more severe emphysema in MerTK^{-/-} mice than in MerTK^{+/+} Cd-exposed mice. Interestingly, COPD plasma samples showed elevated sMer levels compared to control subjects. **Conclusions:** Cadmium increases levels of extracellular Mer and which then acts as decoy scavenger receptor, leading to defective efferocytosis. The anti-efferocytic activity of sMer perpetuates inflammation and causes emphysema.

PS 3911 Air Pollution Derived Fe-Oxide Inhalation Produces Persistent Sex-Specific Behavioral and Morphological Deficits in Mice

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Background and Purpose: Iron-overload, a hallmark and potential contributor to neurodegeneration, comprises a large portion of ultrafine particulate (UFP) matter of air pollution, with especially high concentrations in US subway systems. Previous research demonstrated that Fe-oxide nanoparticles can translocate along cranial nerves into the brain, form deposits within the brain, and associate with degenerating myelin. We hypothesize that the essential role Fe plays in myelination and the direct physical damage caused by Fe-oxide nanoparticle transport along axons can contribute to the sex-specific white matter dysregulation observed in neurodegeneration. **Methods:** To assess the impact of Fe-oxide nanoparticle inhalation and age-related increases in brain Fe content on white matter, two cohorts of 10-week-old C57BL/6 mice were randomly exposed to HEPA-filtered air or 1.5 ng/L iron-oxide UFPs for two hours daily for 20 days. An acute cohort was either acutely sacrificed followed by MRH or subjected to a behavioral battery. The other cohort aged undisturbed in their home cage until MRH, and a broad behavioral battery were performed at 12 and reassessed at 18 months. Micro-dissected corpus callosum from 18-month-old animals were sent for electron microscopy imaging. **Results:** Acutely, Fe-exposure produced sex-specific diffusivity differences in olfactory bulb, with Fe-exposed females exhibiting increases in diffusion indices and males decreases. At 12 months, significant increases, not decreases, in fractional anisotropy in both sexes were observed in caudal regions along the olfactory circuit. In Novel Object Recognition, Fe-exposed female mice had significantly lower recognition indices acutely and at 12 months indicating a persistent, female-specific memory deficit. No locomotor or motor changes were observed in either sex at 12 months of age, however, at 18 months, Fe-exposed male mice exhibited significant hypoactivity on a locomotor assay and significantly more footslips on a 2.5cm and 1.25cm balance beam on days 2 and 3, representing an emergent male-specific motor deficit after early adulthood Fe-oxide exposure. Ultrastructural 18-month corpus callosum myelin analyses demonstrated Fe-induced female-specific decreases in g-ratio and increases in axon, fiber, and myelin area indicating thicker myelin sheaths. However, Fe-exposed female mice were also found to have significantly more split myelin sheaths indicating significant myelin dysregulation in Fe-exposed females. Contrastingly, Fe exposure significantly increased total fiber and myelinated areas in males without concomitant decreases to g-ratio or increases in axonal area. Similar to Fe-exposure in females, Fe-exposed males also had significantly more split myelin area. However, when considering geometry of the axons, significant sex-specific changes to circularity index were observed with Fe exposure reducing circularity index in females but increasing the index in males. This change in axonal geometry could potentially underlie sex differences in diffusivity. **Conclusions:** Early adulthood Fe-oxide nanoparticle inhalation produces sex-specific behavioral and morphological differences that persist and even, emerge, with age. The observed behavioral differences indicate that Fe-exposures produces sex-specific behavioral alterations with females exhibiting persistent short-term memory deficits and males exhibiting emergent motor deficits at 18 months. Fe-exposure produced acute sex-specific differences in white matter in the olfactory bulb and these diffusivity differences moved caudally with age. At the ultrastructural level, Fe-exposure produced sex-specific changes in g-ratio and axonal geometry which could potentially underlie diffusion differences. These data suggest that Fe-oxide inhalation can cause sex-specific white matter dysregulation with concomitant behavioral deficits.

PS 3912 Simulated wildfire smoke particulate matter exposure elicits an inflammatory and oxidative response in RAW264.7 macrophage and C57BL6 mixed glia

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Background and Purpose: Wildfire smoke (WFS) is a major source of ambient particulate matter (PM) exposure and has been associated with adverse respiratory and neurological outcomes, including increased inflammation. However, the cellular mechanisms linking WFS exposure to inflammatory signaling within the lungs and periphery and how this in turn affects the blood-brain barrier and finally the brain itself remains unclear. **Methods:** To better understand the lung-brain axis we established a lung and brain exposure model to begin to investigate the effects of PM_{2.5} generated from a 50:50 mixture of eucalyptus:manzanita (E/M)—a common wildfire smoke source in California and Australia, on macrophage activation using the murine RAW264.7 cell line and on glial activation, using primary C57BL/6 mixed glia. Macrophages or glia were exposed to a concentration range of 0.01-10 ug/ml WFS PM_{2.5}, and outcomes include mitochondrial superoxide via Mitosox, cytokine gene profiling (IL-1 β , TNF- α , IL-6, C3) via qPCR, NLRP3 inflammasome expression and p65 phosphorylation through western blotting. Data were analyzed using one-way ANOVA with appropriate multiple-test corrections. **Results:** Exposure to E/M WFS PM_{2.5} induced a dose and time dependent increase in proinflammatory cytokine TNF- α , within macrophages; glia exhibit the most consistent and significant upregulation of IL-1 β , TNF- α , and C3. Following exposure, RAW264.7 cells also exhibited a dose and time dependent increase in mitochondrial superoxide, highlighting a pro-inflammatory and oxidative cellular response. Importantly, cell viability remained largely intact at relevant exposure concentrations, indicating that the inflammatory activation was not driven by overt cytotoxicity. **Conclusions:** These findings demonstrate that WFS PM_{2.5} robustly activates macrophage and glial inflammatory pathways. Establishing RAW264.7 macrophages and murine primary glia as a tractable model for WFS-induced inflammation, this work lays the foundation for future studies investigating downstream effects on the blood-brain barrier and neuroinflammatory signaling.

PS 3913 Effectsof Diesel Exhaust onLipid Remodeling and Endocrine Regulation in Obese Mice

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Background and Purpose: Obesity, whose incidence has tripled since 1975, is a major global health concern. Emerging evidence implicates air pollution, particularly ultrafine particles (UFP) and traffic-related air pollution (TRAP) as contributors to metabolic dysfunction. These pollutants can activate oxidative and inflammatory pathways, potentially influencing adipose tissue and endocrine regulation. We hypothesize that UFP/TRAP exposure alters lipid signaling through systemic oxidative stress and inflammation, impairing neuroendocrine regulation and metabolic homeostasis, particularly under high-fat diet (HFD) conditions. **Methods:** Adult male and female low-density lipoprotein receptor knockout (Ldlr KO) mice were exposed to UFP/TRAP emissions or filtered air while fed chow or HFD for 18 weeks. Exposure conditions mimicked ambient roadway air pollution using a diesel generator producing 250 μ g/m³ PM_{2.5} for 6 h/day, 5 days/week, while control mice received HEPA and carbon-filtered air. At sacrifice, blood was collected via orbital bleeding in heparinized tubes. After PBS perfusion, epididymal white adipose tissue was collected for untargeted lipidomics using liquid chromatography-ion mobility mass spectrometry. Lipid features were annotated against spectral libraries. Statistical analyses were conducted with LipidSigR package using two-group tests with FDR correction, and two-way ANOVA assessed exposure x diet interactions. Plasma thyroid markers (TSH, T3, T4) were quantified using multiplex assays. **Results:** Significant differences in lipid class abundance were identified between exposure groups using characteristic-level association analysis with FDR correction ($p < 0.05$). Diesel exhaust (DE) exposure strongly increased N-acyl ethanolamine (NAE) (log₂ FC 4.77), Lysophosphatidylglycerol (LPG) (2.89), Phosphatidylinositol-3,4,5-trisphosphate (PIP3) (2.21), and Glucosylceramide (GlcCer) (2.03), with rises in PIP, PG, TG, PI, MGDG, and DG. Pronounced reductions occurred in Hexosylceramide (HexCer) (-14.77), Ceramide (Cer) (-4.82), Lysophosphatidylcholine (LPC) (-3.70), Phosphatidylserine (PS) (-3.39), Phosphatidylcholine (PC) (-2.91), Digalactosyldiacylglycerol (DGDG) (-2.85), and Cardiolipin (CL) (-2.10), and smaller decreases in PE, PA, PIP2, LPE, and LPA. GATOM network highlighted arachidonoyl-CoA across multiple reactions. Chow vs. HFD comparison showed 11 classes with consistent directional changes under DE, including increases in PIP, PG, TG, PC and decreases in DGDG, PS, PI, Cer, DG, PE, PIP2. Four classes reduced under chow (LPE, LPC, CL, PA) increased under HFD, indicating diet-dependent regulation. Multiplex assay revealed elevated TSH in chow-fed Ldlr KO males under DE. HFD-fed mice had higher TSH overall with sex-dependent differences. Plasma T4 decreased in HFD-fed mice, while body weight rose with HFD but was not significant across exposures. **Conclusions:** DE exposure in chow-fed mice caused major lipid shifts, increasing signaling lipids (NAE, LPG, PIP3, GlcCer), and reducing sphingolipids and glycerophospholipids. Under HFD, similar patterns emerged, with some chow-decreased classes (LPE, LPC, CL, PA) increasing,

showing diet-dependent effects. Dysregulation of bioactive lipids tied to inflammation suggests DE could potentially trigger inflammatory pathways in adipose tissue. Disruption of TG, PC, PI, and structural lipids (PC, PE, PS, CL) could signify downstream impairments in cellular metabolism, systemic energy balance, and membrane structural integrity and function. Together, these lipidomic, network, and endocrine results indicate differences in metabolic and signaling pathways varying with exposure and diet.

PS 3914 Occupational Health Hazards: Aerosol toxicity and cardiopulmonary effects on a restaurant cohort

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Background and Purpose: Workers disproportionately exposed to air pollution have an increased risk for adverse health or cardiopulmonary outcomes. However, both toxicological and epidemiological data on cooking emissions exposure and their impacts on restaurant workers remains limited. **Methods:** We conducted a pre-post shift panel study among front-of-house (FoH) and back-of-house (BoH) for n = 39 non-smoking restaurant workers to assess acute changes in cardiopulmonary health associated with cooking-related PM_{2.5} exposure. Data were stratified by reported amount of time spent in kitchen ($\geq$ or $<$ 50%). Average PM_{2.5} concentrations were estimated using low cost-sensors. Clinical measurements including spirometry and oscillometry were conducted pre-post shift, along with blood pressure, O₂ saturation, and short-term health survey data (REDcap). Urine was collected for lipid peroxidation (TBARS) examination. Dried blood spots (DBS) and nasal epithelial lining fluid (NELF) were collected for pro-inflammatory cytokine (IL-1 β , IL-6, IL-8, and TNF- α) quantification via Meso Scale Discovery (MSD) plates. **Results:** Participants (n = 39) were 74.4% male and 25.6% female, with an overwhelming majority being Hispanic or Latino/a (66.7%) while 10.3% were Asian or Asian American, 15.4% Black or African American, and 12.8% White or European. Higher percentages of back pain and shortness of breath amongst workers were reported post-shift. Statistically significant baseline (pre-shift) blood pressure or O₂ saturation differences were not detected between BoH and FoH groups, particularly when controlling for average PM_{2.5} concentrations; however, post-shift systolic blood pressure was higher amongst BoH workers compared to their pre-shift values ($p \leq 0.05$). There were no significant differences for pre- and post-shift FEV₁ ($p = 0.071$), FEV₆ ($p = 0.49$), or FVC ($p = 0.34$) for all workers with acceptable spirometry (n = 20); additionally, neither BoH nor FoH showed significant short-term differences. For all acceptable pre-shift pulmonary function tests, 34.6% (9/26) indicated moderate obstructions or possible restrictive spirometry's. **Conclusions:** Restaurant employees are an understudied population, often facing 50-to-70-hour workweeks, high heat, stress, noise, and air pollution. These preliminary clinical findings may suggest adverse short-term health effects for BoH workers correlated with air pollution exposure, but further examination of a larger population is necessary.

PS 3915 Spatial single-cell transcriptomic analysis of benzene-exposed atherosclerotic plaque

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Background and Purpose: Chronic exposure to airborne pollutants, including benzene, has been linked to an increased risk of myocardial infarction, heart failure, and cerebrovascular events. Benzene is a pervasive environmental contaminant, ranking sixth on the Agency for Toxic Substances and Disease Registry's priority list. It ranks among the top 20 chemicals produced by industrial activities in the United States and constitutes a principal toxic component in vehicular emissions and cigarette smoke. Our recent studies have revealed that benzene exposure is sufficient to induce endothelial dysfunction, toxicity, and heart failure. Extensive research has examined the atherogenic potential of various environmental pollutants, including particulate matter, ozone, acrolein, arsenic, and cadmium. Nevertheless, the impact of benzene on vascular inflammation and the stability of atherosclerotic plaques remains poorly understood. The primary objective of this investigation is to delineate the molecular mechanisms underlying benzene-mediated plaque inflammation and attenuation of fibrous cap integrity. **Methods:** Atherosclerosis-prone low-density lipoprotein receptor knockout (LDLr-KO) mice were maintained on a Western Diet for 10 weeks to establish atherosclerotic lesions. Mice were then randomized to receive whole-body inhalation exposure to either 1ppm benzene or HEPA-filtered air (control) for 6 hours/day, 5 days/week, for 4 weeks, while remaining on Western Diet. Following the 4 weeks of exposure, innominate arteries were harvested, cleaned, sectioned, and analyzed using the Nanostring CoxMx™ Spatial Molecular Imager. A 1,000-plex gene panel was used to assess single-cell gene expression profiles *in situ*. High-resolution imaging datasets facilitated the delineation of three regions of interest (ROIs): the vessel wall, plaque core, and fibrous cap. Visualization and spatial segmentation of RNA transcripts were conducted utilizing the Napari software. Expression matrices, associated metadata, and field-of-view (FOV) files were imported and processed using the Seurat package in R. Cells exhibiting aberrant

gene counts (either excessively high or low) were excluded from further analysis. Inter-cellular communication was evaluated through receptor-ligand interactions using databases integrated into the NicheNet R package. **Results:** Using the Leiden clustering approach, six cell clusters were identified: Activated Endothelial Cells, Proliferating Endothelial Cells, Fibroblasts, Macrophages, and two distinct Vascular Smooth Muscle Cell populations. Significant downregulation of the major structural Col3a1 gene was identified in the fibroblasts residing in the fibrous cap and plaque areas of benzene-exposed animals compared to air-exposed controls ($p < 0.001$). Conversely, significant upregulation of atherogenic and inflammatory Psap and ApoE genes in macrophages localized to the same regions was observed ($p < 0.001$). In addition, a reduction in the number of fibroblasts was detected in benzene vs air fibrous cap areas. These data suggest that benzene and its metabolites compromised the integrity of the protective fibrous cap in the plaque. Next, we explored cell-cell interactions, focusing on endothelial cells as signal senders and macrophages as signal recipients. Several ligands with high target gene prediction ability were identified in endothelial cells (Csf1, Tgfb1, and Fgf7), which were expressed at higher levels in activated EC and were differentially regulated by benzene exposure. These ligands have a high probability of interacting with target genes, including Apoe, Ctsd, and Tgfb1, which are expressed in macrophages. **Conclusions:** Our data suggest that short-term benzene exposure alters the stability of pre-established atherosclerotic plaques by affecting lesion cellularity and inflammation, and fibrous cap integrity.

PS 3916 Function-Barrier changes in the lungs of Apoe^{-/-} mice following repeated exposure to flaming eucalyptus-manzanita smoke.

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Background and Purpose: Public and occupational health concerns regarding wild-fire smoke (WFS) inhalation have increased markedly due to the rising intensity and frequency of wildland fires. Wildland fires generate both flaming and smoldering smoke, however little is known about how flaming smoke affects lung function and barrier integrity. Flames generate smaller and more toxic particles than smoldering smoke, with significant lung toxicity potential (PMID: 29369516). WFS particles are hypothesized to impact extra-pulmonary organs by bypassing the lung barrier and translocating into the systemic circulation (PMID: 38662865). Here, we tested the consequences of flaming exposure on lung function and proposed a novel method for quantifying whole-lung permeability. **Methods:** Female Apoe^{-/-} mice (9 $\pm$ 1 weeks, N=20) were assigned to flaming WFS (50:50 eucalyptus/manzanita), filtered lab-air controls, or LPS- or PBS-treated controls for inflammation-driven comparisons. Flaming and air-control mice were exposed in a whole-body chamber for 2 hours daily over 7 days to smoke or lab air, respectively. LPS mice received a single intratracheal dose of 2 mg/kg b.w. LPS. Flaming parameters were 16 g fuel mass and 600 °C furnace temperature. After the 7th exposure day, or 2 days after LPS/PBS administration, lung function was assessed using derecruitability tests on the flexiVent (SCIREQ). Solution of the constant-phase model yielded coefficients of tissue elastance (H), tissue damping (G), and airway resistance (Rn). Barrier integrity was then evaluated using ventilator-induced lung injury (VILI) and ventilator-assisted aerosol delivery (VAAD) of 4 kDa FITC-dextran. For the VILI protocol, mice underwent 5 minutes of injurious ventilation (50 ml/kg tidal volume, 37.5 cmH₂O plateau pressure, zero PEEP, 50 breaths/min) to determine VILI onset, defined as the time when H doubled. For the VAAD approach, an Aerogen vibrating mesh nebulizer (2.5-4 μ m droplets) integrated with the flexiVent delivered 3.84 mg of FITC-dextran (32 mg/ml) under 120 RR, 2:1 I:E, and 20 ml/kg b.w. tidal volume. This method was validated in LPS mice, where one-hour post-nebulization, whole blood and bronchoalveolar lavage fluid (BALF) were collected. Concentration of FITC-dextran was measured in plasma, and mass in BALF (μ g) was calculated by multiplying the measured concentration by the fluid volume recovered. The permeability index (%) was defined as the ratio of fluorescence intensity plasma/BALF. **Results:** Flaming smoke contained 328 ppm CO and 4 mg/m³ PM. Flaming WFS-exposed mice showed significantly elevated H at intermediate PEEP levels (9, 6, 3 cmH₂O), similar to LPS mice, with no changes at 12 or 0 cmH₂O. Rn increased at all PEEP levels except zero, while G remained unchanged. These PEEP-dependent changes produced significantly lower hysteresivity ($\eta = G/H$) at all PEEP levels except ZEEP. VILI onset occurred earlier in flaming exposed mice than air controls. Nebulized Evans Blue dye showed a lung deposition pattern comparable to inhaled flaming smoke lungs under the VAAD configuration. The lungs of LPS-treated mice were more permeable than those of PBS-treated controls. This permeability assay will be leveraged in our smoke exposure studies to assess lung permeability following functional test. **Conclusions:** Repeated flaming WFS inhalation significantly impairs lung function and may underlie inflammatory responses associated with barrier compromise. Future work will incorporate smoldering WFS inhalation to delineate the differential impact of combustion mode on the lung function-structure relationship and barrier integrity. We advocate that tailored protective measures and health guidelines be implemented for individuals facing repeated smoke exposure.

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Background and Purpose: Traffic-related air pollution (TRAP) is a heterogeneous mixture of particulate matter (PM) and gaseous pollutants. TRAP is implicated in both respiratory and Alzheimer's disease (AD). However, it is unknown which components in TRAP drive adverse health effects and whether the respiratory and neurologic effects are linked. Here we addressed the hypothesis that TRAP triggers pulmonary inflammation, specifically, increased TGFβ. Increased pulmonary TGFβ can subsequently release TGFβ into the systemic circulation, which can impact the blood brain barrier and promote AD relevant phenotypes. We also examined the relative contribution of TRAP components on lung TGFβ levels. **Methods:** Male and female TgF344-AD rats, which express two human AD risk genes, were chronically exposed to ambient whole TRAP (containing both particles and gases), TRAP derived PM only or TRAP derived gases only collected from the emissions of light-duty vehicles (LDV) vs. light duty and heavy-duty vehicles (LDV+HDV). Age and sex matched controls housed at the same facility were exposed to filtered air (FA). Exposures began when animals were 1 month old with samples collected at 4, 9, 12, and 15 months (mos) of age. Bronchoalveolar lavage fluid (BALF) was collected to assess total protein leak and lung tissue was collected to measure protein levels of three TGFβ isoforms. **Results:** Protein leak in response to TRAP was minimal at 4 mos but a notable increase in total protein concentration in BALF supernatant was observed at 12 and 15 mos. At 12 mos, male rats exposed to PM + Gas (LDV + HDV) demonstrated a significant increase in BALF protein levels (p_{adj} 0.0116) and although 12 mos female rats exposed to PM (LDV + HDV) reached the highest mean BALF protein levels (>3000 µg/mL), this increase was not statistically significant compared to FA controls. At 15 mos, female BALF protein concentrations in all exposure groups diminished to levels comparable to FA controls but males continued to display elevated protein concentrations across all TRAP groups. TGFβ protein measurement in homogenized caudal lobes showed varying changes in TGFβ isoforms across timepoints, most prominently in males. While there were no clear or consistent changes across all timepoints, every PM containing TRAP exposure group had significantly increased ($p_{adj}<0.1$) pulmonary TGFβ-2 levels at 4 and 12 mos in males, but the same was not observed in females. Additionally, a few female TRAP groups had significantly reduced TGFβ-1 levels at 4 mos, but the same was not seen in males. All TRAP-induced changes in TGFβ-1 and 2 returned to near baseline (compared to FA) at 15 mos in females. **Conclusions:** Across all timepoints and TRAP exposures there appeared to be sex-dependent variability in TRAP-induced increases in BALF protein, indicating cellular injury in the lung, most notably in the male lung. There were progressive, sex-dependent increases in BALF protein concentrations over time. Changes in TGFβ protein, which play important roles in pulmonary fibrosis, were most prominently observed in the male lung. Together, these results suggest sex-dependent pulmonary vulnerability to chronic TRAP exposure, which merits further investigation into the sequence of pathologic events triggered by TRAP in the lung, the systemic circulation, and the brain. This study was supported by NIH grants RF1 AG074709, T32 HL007013, P30 ES023513, and T34GM136469.

PS 3918 Benzene-Induced Endothelial Activation: Mitigation by Heat Shock Proteins

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Background and Purpose: Benzene is a widespread environmental pollutant found at superfund sites and is abundant in petroleum products, tobacco smoke, and various household goods like cleaners, paints, and adhesives. It is a well-known human carcinogen with well-documented hematopoietic and immunotoxic effects. Epidemiological evidence also links benzene exposure to an increased risk of all-cause mortality, cardiovascular mortality, and stroke, though the biological mechanisms underlying these associations remain unclear. Endothelial activation plays a pivotal role in vascular inflammation and atherosclerosis, the underlying cause of myocardial ischemia and stroke. This study examines the impact of benzene exposure on endothelial cell activation and the underlying mechanisms. **Methods:** To understand the potential functional changes to the endothelium from benzene exposure, we used multiphoton-intravital microscopy (IVM). IVM allowed us to visualize real-time interactions between leukocytes and the endothelium in the mouse ear microvasculature. For all IVM experiments, Fluorescein-dextran (70 kDa; 1.5 µL/g in saline, intravenous) was used to label vasculature, and Rhodamine 6G (0.2% v/v in saline, intraperitoneal) to label leukocytes. For each mouse, two separate two-minute videos were taken of a blood vessel within the ear. For analysis, leukocyte dynamics (free-flowing, rolling, adhering) were quantified manually and the counts from both videos were averaged. First, male and female C57BL/6 mice (n=10/group) were exposed to benzene (1ppm, 6h/d 5d/wk, 4 weeks) or HEPA-filtered air and leukocyte rolling and adhesion was examined by IVM. *In vitro* we exposed human aortic endothelial cells (HAECs) to a benzene metabolite, t,t-muconaldehyde (MA; 10 µM) and assessed adhesion and transmigration of a leukocytic cell line (dTHP-1 cells). Our previous studies revealed that heat shock proteins, especially HSPA1B, protect against benzene-induced endothelial toxicity. To further assess the role of heat shock

proteins in benzene-induced endothelial activation, we exposed transgenic mice that selectively overexpress HSPA1B in the endothelium (EC-HSPA1B-TG) to either benzene (1ppm) or HEPA-filtered air and assessed leukocyte rolling and adhesion by IVM. **Results:** Chronic inhaled benzene exposure induced a significant increase in leukocyte rolling and adhesion, demonstrating a clear sex difference in response. While male mice showed increases in leukocyte rolling (16-fold, $p=0.0009$) and adhesion (11-fold, $p<0.0001$), female mice exhibited a significantly greater response (rolling: 20-fold, $p=0.001$; adhesion: 43-fold, $p<0.0001$). These sex differences were statistically significant for leukocyte rolling ($p=0.0029$) and adhesion ($p=0.0001$). *In vitro*, MA exposure enhanced leukocyte adhesion and transmigration and phosphorylation of eIF2α (reflecting endoplasmic reticulum (ER) stress and unfolded protein response). The endothelial-specific overexpression of HSPA1B reduced benzene-induced leukocyte rolling (5.4-fold decrease compared to WT, $p=0.0002$) and leukocyte adhesion (2-fold decrease compared to WT, $p=0.0026$). **Conclusions:** These findings suggest that benzene contributes to cardiovascular risk by promoting endothelial activation which could be prevented by endothelial cell specific overexpression of HSPA1B.

PS 3919 Inhaled Copper Nanoparticles Induce Pulmonary Inflammation and Blood-Brain Barrier Dysfunction in a Late-Onset Alzheimer's Disease Mouse Model

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Background and Purpose: Inhalation of nanosized particles (<100 nm) is increasingly recognized as a contributor to neurotoxicity, yet the mechanistic link between pulmonary exposure and brain outcomes remains unclear. Recent findings indicate that systemic inflammation and blood-brain barrier (BBB) disruption may mediate the connection between lung injury and neural damage. Thus, this study investigated the pulmonary and neurotoxic effects of copper oxide nanoparticles (CuO NPs) in a late-onset Alzheimer's disease (LOAD2) mouse model carrying genetic risk factors such as *APOEε4*, *h-APP*, and *Trem2*^{R47H} mutations. We aimed to evaluate whether inhaled CuO NPs exacerbate systemic inflammation, impair BBB integrity, and contribute to neuroinflammatory signaling in brain regions associated with cognition. **Methods:** Wild-type and LOAD2 mice were exposed to CuO NP aerosols (3.5 mg/m³, 50 nm) using a dynamic whole-body inhalation exposure system (4 h/day, 5 days/week, for 4 weeks). Necropsies were performed 5 days post-exposure. Pulmonary responses were assessed by bronchoalveolar lavage fluid (BALF) analyses, including inflammatory cell counts, total protein, and lactate dehydrogenase (LDH) levels. Systemic responses were characterized by serum matrix metalloproteinase-9 (MMP-9) and S100B, a marker of BBB disruption. Tissue Cu accumulation was quantified using inductively coupled plasma-mass spectrometry (ICP-MS). Neuroinflammatory responses are currently being assessed in neocortical tissue at the protein and mRNA level by measuring cytokine levels, immune markers (CD68), and astrocytic activation markers (GFAP). **Results:** CuO NP exposure induced pronounced pulmonary inflammation, with elevated total and differential BALF macrophages, neutrophils, LDH activity, and total protein. These responses were observed in both wild type and LOAD2 mice, indicating a robust inflammatory response to CuO NP inhalation regardless of genotype. Systemically, CuO NP exposure elevated serum MMP-9 and S100B levels, consistent with tight-junction degradation and BBB compromise. S100B levels tended to be higher in LOAD2 mice, suggesting increased BBB susceptibility in the Alzheimer's disease genotype. ICP-MS analysis confirmed Cu accumulation in the lungs and brain, supporting hematogenous transport of NPs or dissolved Cu ions into the brain. Preliminary Western blot analysis of neocortical tissue revealed a trending increase in CD68 expression in CuO-exposed LOAD2 mice, suggesting potential microglial/macrophage activation. GFAP levels showed slight, non-significant elevation in LOAD2 mice, but the pattern was inconsistent across groups. Additional analyses are ongoing to define astrocytic responses and BBB-related changes. To further explore these findings, ongoing qPCR and RNA sequencing will further define the molecular pathways involved in CuO NP-induced neurotoxicity. **Conclusions:** Our data support the hypothesis that inhaled CuO NPs induce a cascade of pulmonary and systemic inflammatory responses that contribute to BBB leakage and neuroinflammation priming in a mouse model of Alzheimer's disease. Ongoing molecular analyses, including RNA sequencing, aim to elucidate pathway-specific mechanisms that link NP exposure to neurodegenerative vulnerability. These results provide mechanistic insight into the lung-brain axis in NP-induced neurotoxicity and underscores the importance of considering metal-based NPs as environmental risk factors of Alzheimer's disease progression.

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Background and Purpose: California's Office of Environmental Health Hazard Assessment (OEHHA) conducts health risk assessments under the Air Toxics Hot Spots Program. Reference Exposure Levels (RELs) are airborne concentrations of a chemical that are not anticipated to result in adverse noncancer health effects for specified exposure durations in the general population and in sensitive subpopulations (e.g., children). RELs are based on the most sensitive and relevant noncancer health effects reported in the medical and toxicological literature. The Acute REL is intended to protect against infrequent 1-hour exposures; the 8-hour REL is for exposures that occur on a recurrent basis for a portion of each day; and the chronic value represents continuous exposure for a significant fraction of a lifetime. The California Air Resources Board then incorporates the RELs into the Hot Spots program to be used by stationary facilities and air districts in risk assessments. As such, RELs are a valuable tool for communities and regulators to use in evaluating the health impacts from air toxics exposures. OEHHA evaluated Trimethylbenzenes (TMBs), a group of volatile aromatic hydrocarbons (VOCs). TMBs exist as three isomeric forms: 1,2,3-, 1,2,4-, and 1,3,5-TMB. These VOCs are common components of gasoline, and emission sources include steel-making facilities, coal-fired plants, construction, cement, paving mixtures, asphalt, and metal coatings, amongst others. TMBs are found in printing inks, paint solvents, hydraulic fracturing fluids, and biogas. **Methods:** Searches were conducted in SCOPUS, PUBMED, and Google Scholar for relevant medical and toxicological literature, with an emphasis on human and animal inhalation toxicity studies. The most sensitive health effect was chosen to develop the RELs. Dose-response data from human and animal studies were used to develop acute, 8-hour, and chronic RELs using US EPA's Benchmark Dose Software. The resulting Benchmark Concentrations (BMCs) were adjusted for exposure duration. A Human Equivalent Concentration was calculated and Uncertainty Factors (UFs) were applied to the BMC in accordance with OEHHA's Technical Support Document methodology. **Results:** TMBs are readily absorbed via inhalation and accumulate in adipose tissue. Both acute and chronic exposures to TMBs in animal and human studies showed primarily respiratory and neurotoxic effects. Adverse effects on hematology, clinical chemistry, development, and several other organ systems were also seen. For all three REL values, neurotoxicity was the most sensitive endpoint. The Acute TMB REL is based on a neurobehavioral study in rats in which significant latencies were seen in a number of neurobehavioral tests following exposure to the 1,2,4-TMB isomer for up to 8 hours. The 8-hour and chronic RELs are based on a subchronic neurotoxicity study in rats in which exposure to 1,2,3- or 1,2,4-TMB for 3 months resulted in concentration-dependent disturbances in pain sensitivity and neuromuscular function. Inter- and intra-species UFs were applied and, for all TMB REL values, the intraspecies toxicodynamic component of the UF was increased to 10 from the default factor of 3 because TMBs are neurotoxicants, and likely to impact infants and children disproportionately. An additional subchronic to chronic UF was applied to the chronic and 8-hour REL values since the critical study was only 13 weeks in duration. This yielded an acute TMB REL of 2400 µg/m³ (490 ppb), an 8-hour TMB REL of 8 µg/m³ (2 ppb), and a chronic TMB REL of 4 µg/m³ (1 ppb). **Conclusions:** Because the three TMB isomers share multiple similarities regarding their chemical, toxicokinetic, and toxicological properties, it is expected that the REL values based on one isomer would be adequately health protective for the other isomers. US EPA has adopted a similar approach in the development of their Reference Concentrations for the TMB isomers (USEPA, 2012). Following approval by California's Scientific Review Panel on Toxic Air Contaminants in 2023, the TMB RELs were adopted by OEHHA for use in the Hot Spots Program to assess human health risks associated with emissions of TMBs state-wide. The TMB RELs will be used to identify facilities having localized impacts on nearby communities. If concentrations exceed certain exposure levels, then nearby residents will be informed of potential health risks, and strategies for reducing exposure to acceptable levels can be implemented.

PS 3921 Estrogen Signaling As a Determinant of Immune Vulnerability to Wildfire Smoke Exposure

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Background and Purpose: Wildfires are an escalating public health concern that contribute not only to acute respiratory injury but also to long-term impacts on cardiovascular, metabolic, cancer-related and immune-mediated disease. Wood smoke (WS), a major component of wildfire emissions, contains a complex mixture of particulate matter (PM), gases, and volatile organic compounds that can impair host defense and alter systemic immune regulation. Epidemiologic studies have reported associations between air pollution exposure and earlier menopause, as well as links between aging and increased immunological susceptibility following inhaled exposures. Despite these large-scale observations, there remains a significant gap in knowledge regarding immunotoxicological susceptibility during the menopausal transition and the mechanistic role of estradiol, post-WS exposure. **Methods:** In this study we subjected mice (n=10/group) to an acute wood smoke exposure paradigm (0.5mg/m³ 4h/d for 2d) or filtered air (FA, controls) in 1) sham vs. ovariectomized (OVX) mice 2) a mouse model of accelerated ovarian failure (AOF) and 3) wildtype (WT) vs. g-coupled protein estrogen receptor deficient (GPER-/-) mice. **Results:** Bone marrow immune cell profiling indicated significant increase (p<0.05) in CD11c+

dendritic cells and CD19+ B cells, and a decrease in Gr1+ neutrophils and CD11b+ monocyte/macrophage post WS-exposure in OVX mice, but not in Sham mice, as determined by a 2x2 factorial ANOVA. Single cell bone marrow transcriptomics demonstrated statistically significant differential expression between FA and WS in the OVX mice (p<0.05). A moderate AOF mouse model demonstrated diminished CD11b+ cells that was rescued with estradiol supplementation (0.2mg/kg for 60d). M-CSF differentiated macrophages from OVX mice demonstrated metabolic hypersensitivity, as shown by the Seahorse Mito Stress and glycolysis tests. However, this effect has not mediated by GPER, indicating some other estrogen receptor (ERα, ERβ), as a likely mechanistic target. **Conclusions:** Our exciting preliminary studies demonstrate that WS-induced immunotoxicity is dependent on estrogen signaling, highlighting the need to investigate endocrine-immune interactions in environmental susceptibility.

PS 3922 Traffic-related air pollution exposure across the lifespan accelerates age-related renal injury in rats

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Background and Purpose: Traffic-related air pollution (TRAP) comprises a complex mixture of exhaust and non-exhaust emissions, including gases, volatile organic compounds, and particulate matter enriched with metals. Epidemiological studies increasingly link TRAP exposure to heightened chronic kidney disease (CKD) risk, yet causal evidence remains limited because TRAP composition varies by location, time, and meteorological conditions, making it difficult to model true human exposures experimentally. To address this gap, we assessed real-world TRAP exposure by housing rats adjacent to a major freeway and comparing kidneys from animals exposed to either ambient TRAP or filtered air (FA). **Hypothesis:** Exposure to TRAP accelerates age-related renal injury that leads to CKD onset. **Aim:** To assess the effects of TRAP exposure across the lifespan on renal tubule morphology and CKD progression. **Methods:** Thirty-six transgenic Fischer 344 rats were transferred at 1 month of age to a vivarium situated beside a major freeway tunnel system in California. Ambient tunnel air (TRAP; n=18) was delivered unaltered and in real time through supply lines to the exposure chambers for 14 months. As a control, the same ambient air was routed through a series of emissions-control technologies to remove airborne pollutants before entering the chambers to provide clean filtered-air (FA; n=18). All rats were euthanized at 15 months of age, and the kidneys were dissected to assess renal injury and disease. The left kidney was fixed in 4% PFA, paraffin-embedded, sectioned, and stained using histological dyes and immunofluorescence. Whole-kidney brightfield and fluorescence images were acquired using the Zeiss AxioScan. The right kidney was snap-frozen for single-particle time-of-flight ICP-MS to quantify metallic nanoparticles in FA and TRAP kidneys. **Results:** Kidney weights did not differ significantly between TRAP- and FA-exposed animals (mean TRAP = 1.14 g; mean FA = 1.15 g, n = 18/group), including when normalised to body weight. Periodic acid-Schiff staining revealed abundant intratubular renal casts - a hallmark of tubular disease of the kidney - distributed throughout the renal cortex, medulla, and pelvis. Whole-kidney quantification showed a 2.5-fold increase in cast burden in TRAP kidneys (mean 711 per kidney) compared with FA controls (mean 286 per kidney) (n = 5/group). Immunofluorescent staining for kidney injury molecule-1 (KIM-1), a proximal tubule protein that is a biomarker of acute and chronic tubular injury, showed a marked increase in KIM-1-positive tubule cross-sections in TRAP kidneys (mean 104 per kidney) compared with FA kidneys (mean 19 per kidney), representing a 5.5-fold increase (n=3 kidney sections/group). Single-particle time-of-flight ICP-MS identified multiple metallic nanoparticles in TRAP-exposed filters, including Ti, Cr, Mn, Cu, Ag, Sn, Sb, Hf, Pb, and Bi (n=3). Among the elements detected in kidney tissue, Sn was the most enriched, with TRAP-exposed animals exhibiting a six-fold increase in Sn particles compared with FA controls (n=3/group). **Conclusions:** Lifetime TRAP exposure in rats increased the burden of intratubular renal casts, proteinaceous obstructive structures indicative of tubular injury, and markedly elevated KIM-1 expression, suggesting accelerated renal aging and heightened CKD risk. Nanoparticle profiling identified Sn as the most enriched metallic species in TRAP-exposed kidneys, indicating tissue accumulation and highlighting the need to determine the mechanistic contribution of Sn-containing particles to kidney injury and disease.

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Background and Purpose: Diesel exhaust (DE), an important source of particulate matter and other toxic compounds within traffic-related air pollution, generates reactive oxygen species (ROS), often leading to oxidative stress and inflammation in the body. The combination of particulate matter and a high-fat diet (HFD) has been linked to glucose intolerance, as well as an increase in inflammation and oxidative stress. Oxidative stress and inflammation can also lead to dysfunctional high-density lipoproteins (HDL), which can further increase oxidative stress and inflammation. The purpose of this research was to assess the impact of chronic DE exposure and a HFD on oxidative stress and inflammation in plasma, as well as HDL function in mice. An additional aim was to highlight how effects differ based on sex. **Methods:** Two different experimental groups of low-density lipoprotein receptor (*Ldlr*) knockout mice were used: chronic exposure mice (Group 1; male and female) and chronic exposure followed by diet recovery mice (Group 2; male only). Group 1 mice were exposed to filtered air (FA) or DE for 18 weeks, while fed either a high-fat diet (HFD) or Chow. Group 2 mice were exposed to FA or DE and fed HFD for 18 weeks, then fed Chow and exposed to DE or FA for 2 or 8 additional weeks. At the end of exposures, mice were sacrificed, and blood was collected via orbital bleeding and placed in heparinized tubes. Plasma was separated and kept at -80°C until needed for assays. Mouse paraoxonase-1 (Pon1) was measured in plasma using an enzymatic assay with diazoxon as a substrate, as a biomarker of oxidative stress, and serum amyloid A (SAA) was measured in plasma using a sandwich ELISA assay as a biomarker of inflammation. The SAA/Pon1 ratio was calculated and used to assess HDL function. **Results:** Group 1 DE HFD male mice had significantly higher SAA levels than DE HFD female mice. SAA levels in group 1 HFD male mice increased with both FA and DE compared to Chow. Group 1 DE HFD male mice had significantly higher SAA/Pon1 ratios compared to DE Chow male mice and DE HFD female mice. In Group 2 mice, the SAA/Pon1 ratios progressively declined with increasing weeks of diet recovery compared to the ratios observed in Group 1 DE HFD mice. The mice with 8 weeks of diet recovery showed the lowest SAA/Pon1 ratios, although levels remained higher in DE males than in FA males. **Conclusions:** Overall, exposure to DE and HFD in male mice leads to increased inflammation, decreased antioxidant capacity, and ultimately dysfunctional HDL, illustrated by the increased SAA/Pon1 ratios. Sex had a significant effect on SAA levels and SAA/Pon1 ratios in mice exposed to DE and fed HFD, suggesting male mice are more sensitive to the combination of DE and HFD compared to female mice. When HFD male mice exposed to DE for 18 weeks were switched to Chow diet for 8 weeks while exposed to DE, the SAA/Pon1 ratios decreased, indicating improved HDL functionality.

PS 3924 Genetic and Chemical Modulation of Heme Oxygenase-1 Regulates Ultrafine Particle-Induced Inflammation in Macrophages

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Background and Purpose: Ambient air pollution is a well-recognized, major risk factor for atherosclerosis, a chronic inflammatory disorder of the vasculature. Epidemiological, *in vivo*, and *in vitro* studies have supported a mechanism of inflammation and atherogenesis linked to the exposure of fine particles (or particulate matter (PM) with a diameter $\leq 2.5\mu\text{m}$). However, ultrafine particles (UFP; PM with a diameter $\leq 0.1\mu\text{m}$) are likely to pose a greater health risk due to their smaller size and greater adsorbed surface material, but the mechanisms are still unclear. It has been shown that upregulation of systemic Heme oxygenase-1 (HO-1) offers cytoprotection against vascular atherogenesis due to its important antioxidant and anti-inflammatory properties. With macrophages playing a central role in inflammation, atherogenesis, and PM clearance in the most distal regions of the lungs, it is pertinent to understand how PM, and more importantly, UFP, can affect inflammation. We hypothesize that UFP activate inflammatory processes via the upregulation of cytokines and the activation of the inflammasome in macrophages, all of which can be modulated by HO-1 expression. **Methods:** RAW 264.7 macrophages were seeded at 300,000 cells/well at passage 12 and grown overnight. Macrophages were pretreated with cobalt protoporphyrin (CoPPiX) (10 μM), tin protoporphyrin (SnPPiX) (10 μM), UFP (25 $\mu\text{g}/\text{mL}$) or a combination of chemical treatment and UFP for 24 hours. After the exposure duration, RNA was isolated from the cells and cDNA was synthesized. Bone marrow-derived macrophages (BMDMs) were harvested from the femurs of myeloid-specific Heme oxygenase-1 knockout mice (mHO-1-KO) and transgenic overexpressing Heme oxygenase-1 mice (mHO-1-Tg). For controls, BMDMs were harvested from their respective floxed counterparts. Briefly, marrow was extracted from the femurs of multiple mice and pooled (n=6-8) using complete RPMI media and frozen in 90% FBS and 10% DMSO until needed for experiments. BMDMs were cultured and differentiated in RPMI supplemented with M-CSF from L929 conditioned media. BMDMs were seeded at 500,000 cells/well and grown overnight. BMDMs were treated with UFP (25 $\mu\text{g}/\text{mL}$) for 24 hours. After the treatment duration,

RNA was isolated from the cells, and cDNA was synthesized. The expression of cytokines and inflammasome were assessed by quantitative PCR (Roche LightCycler 480) using Taqman probes on 384-well plates. **Results:** Our data show that exposure to UFP induced robust cytokine and inflammasome activation in RAW 264.7 macrophages (p<0.05). The data supports that chemical induction and inhibition of HO-1 modulates cytokine and inflammasome components expression in macrophages, as evidenced by the significant reduction or exacerbation in the expression of specific cytokines and NLRP3 (p<0.05). In BMDMs from control floxed mice, UFP treatment significantly increased cytokine expression. In BMDMs harvested from mHO-1-KO mice, there is an exacerbation of these respective cytokines (p<0.05) and NLRP3. On the contrary, BMDMs from mHO-1-Tg mice had a significant reduction in inflammatory cytokines (p<0.05) as well as decreased expression of NLRP3. **Conclusions:** These data warrant further investigation into the precise molecular mechanisms by which HO-1 exerts its regulatory effects. The observed modulatory capacity suggests that targeted manipulation of HO-1 expression levels could represent a viable therapeutic strategy for mitigating chronic inflammatory diseases, such as atherosclerosis, where environmental particle exposure contributes significantly to pathology.

PS 3925 CO₂ Mediated Regulation of Neutrophil NADPH Oxidase and Interaction with Reactive Nitrogen Species

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Background and Purpose: Carbon dioxide (CO₂) can modulate inflammatory and redox signaling pathways. Previously it's been shown that *in vivo* exposure of mice and *in vitro* exposure of isolated human PMNs to elevated CO₂ levels (10%) stimulated reactive oxygen species (ROS) production, leading to microparticle release and activation of NLRP3 inflammasome. These findings suggest that CO₂ can modify neutrophil function through redox-dependent mechanisms. Literature shows that CO₂ reacts with peroxynitrite (ONOO⁻) to form caged radical nitrosoperoxycarbonate (ONOCO₂⁻), redirecting oxidative chemistry toward nitration rather than direct oxidation. Extending these concepts to human exposures we reported that 12 healthy participants exposed to 2,500 ppm CO₂ exhibited altered oxidative-burst kinetics in circulating PMNs compared with pre-exposure values, suggesting that environmentally relevant CO₂ can modulate innate immune activation. To determine whether CO₂ directly alters neutrophil function, we used differentiated HL60 (dHL60) cells to evaluate bicarbonate-dependent modulation of NADPH oxidase and its interaction with nitric oxide-derived species. We also specifically compared three NO donors—NOC-5, GSNO, and SIN-1, representing distinct redox species. SIN-1 releases both NO and superoxide, forming peroxynitrite, which in the presence of CO₂ favors nitration. GSNO, an S-nitrosothiol donor, causing transnitrosylation of protein thiols, can modulate NOX activity, while NOC-5 releases 2NO leading to nitrosative signaling. **Methods:** HL60 cells were differentiated for 5 days with 1 μM all-trans retinoic acid (RA). Seahorse XFe96 assays were performed with 40,000 cells /well. Sequential injections were as follows with final concentrations: 1) NaHCO₃ (0, 12.5, 25, 50, 100 mM, pH adjusted to 7.4), 2) Rotenone/Antimycin A (0.5 μM), 3) PMA (100 ng/mL) to activate NADPH oxidase, and 4) DPI (10 μM) to inhibit it. The Oxidative Burst (OB) assay was used to study NADPH oxidase activity by measuring real-time oxygen consumption rate (OCR) upon PMA stimulation, which directly reflects NOX2-dependent superoxide generation. Because glycolytic and mitochondrial parameters were unchanged across bicarbonate concentrations, consistent with human data, subsequent experiments focused on OB assays. To test the influence of nitric oxide-related and peroxynitrite signaling, cells were also exposed to NO donors (NOC-5 [150 μM], SIN-1 [1 mM], or GSNO [100 μM]) after Rotenone/Antimycin A and before PMA. **Results:** Increasing NaHCO₃ concentrations produced a dose-dependent reduction in PMA-stimulated oxygen consumption [3412.8 $\pm$ 1391.5 pmole (0 mM NaHCO₃) vs 2691.8 $\pm$ 1042.2 pmole (12.5 mM NaHCO₃), p = 0.046] and [AG1] 3373.7 $\pm$ 1391.5 pmole (0 mM NaHCO₃) vs 1634.1 $\pm$ 1133.5 pmole (25 mM NaHCO₃), p < 0.001] (n=5), indicating suppression of NADPH oxidase activity[AG2]. When SIN-1 (1 mM) was added, AUC (pmole) decreased at low bicarbonate (1924 at 0 mM NaHCO₃ vs 3569 without SIN-1) and at 12.5 mM NaHCO₃ (2197 vs 3473 without SIN-1), but the inhibitory effect plateaued at higher bicarbonate (25 mM = 1732 vs 1747 without SIN-1), suggesting that increasing NaHCO₃ concentration blunted SIN-1's effect. Across replicate runs, consistent dose-dependent inhibition patterns were observed with different NO donors. NOC-5 significantly reduced AUC values (pmole) (0 mM: 4889 $\rightarrow$ 1491; 12.5 mM: 3505 $\rightarrow$ 1672; 25 mM: 2711 $\rightarrow$ 460.6; 50 mM: 202.5 $\rightarrow$ 39.15), while GSNO didn't effect OB (0 mM: 4608 $\rightarrow$ 4025; 12.5 mM: 3348 $\rightarrow$ 3302; 25 mM: 2799 $\rightarrow$ 2594; 50 mM: 470.8 $\rightarrow$ 173.4). **Conclusions:** These data collectively indicate that while bicarbonate alone suppresses NADPH oxidase activity, RNS donors, especially NOC-5 reduces the NOX activity irrespective of bicarbonate and GSNO didn't change PMA induced OB or the effects of bicarbonate. SIN-1 reduces the PMA activity but with bicarbonate the NOX activity loss is restored. We propose that nitrosylation, nitrosation, and nitration are all means by which NOX activity can be regulated and that the presence of CO₂ can alter these reactions. In future studies we will examine how CO₂ and RNS cooperatively regulate NOX and contribute to systemic inflammatory and vascular changes associated with CO₂ exposure. Together, these results support a model in which CO₂ acts not as an inert gas but as a signaling molecule capable of altering neutrophil redox responses and immune activation.

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Background and Purpose: Ethylene oxide (EO) is a small, reactive gas widely used as a chemical intermediate and as a sterilizing agent for approximately half of all medical devices - many of which cannot be sterilized safely using alternative methods. In 2016, the U.S. Environmental Protection Agency (EPA) released an updated IRIS assessment that classified EO as carcinogenic to humans and derived new inhalation unit risk (IUR) estimates based on dose-response associations in a single sterilization-worker cohort. This revision increased EO potency by roughly 50-fold and introduced substantial regulatory and public health communication challenges, as elevated lifetime excess cancer risks were identified near EO-emitting facilities nationwide, including hospitals previously below reporting thresholds. Subsequent federal and state air monitoring campaigns revealed that EO is ubiquitous in ambient air at concentrations exceeding the 1-in-1-million excess cancer risk benchmark. Characterizing EO in background air is therefore essential to effectively evaluating, managing, and communicating public health risks from a specific EO source. The aim of this study was to compile and summarize federal and state air sampling data across the U.S. to support these data needs. **Methods:** We compiled publicly available US EO monitoring data from federal and state agencies and then conducted a descriptive analysis of the body of existing data, including trends by year, state, and monitoring location. **Results:** EO ambient air sampling data were sparse prior to 2018. Most measurements were first reported in 2018 following EPA's 2014 National Air Toxics Assessment, which incorporated the updated IUR. Between 2018 and 2024, EO monitoring data were available for 23 states and 85 monitoring locations. Monitoring density varied by state, with New York and Washington having the most stations, and years of available data differed across sites. Seven-year background EO concentrations across the 85 stations ranged from 0.013 ppb (10th percentile) to 0.515 ppb (98th percentile), with an overall average of 0.11 ppb. The highest single concentration (20 ppb) was measured in South Carolina in 2022; Virginia reported the highest average concentration. States with the lowest average and maximum levels included Florida, New York, Oregon, California, and Vermont. Overall, most average background concentrations fell between 0.038 and 0.512 ppb. Temporal trends were also investigated due to evolving laboratory detection methods. Colorado, for example, had the highest average EO concentrations in 2018 and lowest in 2024. **Conclusions:** EO is ubiquitous in ambient air across the U.S., and average concentrations are generally consistent across regions. Because current analytical methods cannot reliably detect EO below approximately 0.01 ppb, the concentration corresponding to a 100-in-1-million excess cancer risk, all detectable background levels exceed this regulatory benchmark. Exceedances of the 1- and 100-in-1-million excess cancer risk thresholds are therefore common, even in background air. As a result, quantifying EO in background environments is essential for site-specific risk assessments and for public health assessments associated with EO emissions.

PS 3927 CHEMR23 Signaling Protects Against Ozone-Induced Airspace Inflammation through RvE1 Signaling.

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Background and Purpose: Ground-level ozone (O₃) is a criteria air pollutant known to trigger exacerbations of chronic respiratory diseases. Inhaled O₃ induces an acute pulmonary inflammatory response involving leukocyte recruitment, proinflammatory cytokine release, and proinflammatory lipid mediator production via n-6 polyunsaturated fatty acids (PUFAs). Specialized pro-resolving mediators (SPMs) are bioactive lipids that are produced during inflammation to promote resolution and restore tissue homeostasis, in part by binding to their corresponding G-protein coupled receptors. Most SPMs are derived from n-3 PUFAs, including Eicosapentaenoic Acid which can be metabolized into Resolvin E1 (RvE1). Prior research from our lab showed that O₃ exposure reduces the pulmonary expression of the RvE1 receptor CHEMR23. CHEMR23 is predominately expressed on macrophages, which play a role in initiating and resolving inflammation. Therefore, we hypothesize that CHEMR23 expression on pulmonary macrophages alters O₃-induced lung inflammation. **Methods:** Male C57BL/6J wild-type (WT) and CHEMR23-knockout (KO) mice were exposed to filtered air or 1 part per million O₃ for 3 hours. At 24 hours post-exposure, bronchoalveolar lavage fluid (BAL) and lung tissue were collected to assess injury and inflammation as well as immune cell quantification via flow cytometry. To evaluate macrophage specific functions of CHEMR23, bone marrow was collected from WT and KO mice and differentiated into bone marrow derived macrophages (BMDMs). BMDMs were treated with lipopolysaccharide (LPS, 100 ng/mL) or PBS followed by RvE1 (100 nM) or vehicle control. After a 4-hour incubation, cell supernatants and lysates were collected to quantify production of pro-inflammatory cytokines. **Results:** Following O₃ exposure, KO mice had increased airspace inflammation compared to WT mice with elevated airspace neutrophilia and proinflammatory cytokines. Furthermore, KO mice exhibited increases in pulmonary lymphocytes after O₃ exposure. RvE1 levels decreased post O₃ exposure in the airspace of WT mice which were further reduced

in KO mice. Using BMDMs collected from WT and KO mice, RvE1 treatment following LPS stimulation reduced cytokine expression in WT BMDMs. However, there was no change in LPS induction of cytokines in KO BMDMs following RvE1 treatment. **Conclusions:** Taken together, our results indicate that the absence of the CHEMR23 receptor augments O₃-induced airspace inflammation. LPS-induced inflammation was reduced with RvE1 treatment in WT BMDMs, but not in KO BMDMs, suggesting RvE1's anti-inflammatory treatment is mediated through CHEMR23 signaling. Future studies will analyze primary macrophages in the lung after O₃ exposure to determine if CHEMR23 signaling drives resolution responses.

PS 3928 Exploring the Role of Retinoids in Protecting the Lungs Against Inhaled Ozone

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Background and Purpose: Ozone (O₃) is a criteria air pollutant and a powerful oxidant that can damage cell membranes in the lungs, leading to the activation of both proinflammatory and anti-inflammatory macrophages. The extent of lung inflammatory responses to O₃ exposure is defined by the alveolar microenvironment, specifically by the ability of macrophages residing in the alveolar-epithelial region to tolerate the direct cytotoxic actions of O₃ and to maintain effective intercellular signaling aimed at repairing injured tissue. Retinoids (vitamin A and its metabolites) are vital for maintaining adult lung health and function, especially during lung injury. These effects are predominantly mediated via the actions of all-trans-retinoic acid (ATRA), the most active retinoid metabolite, which serves as a transcriptional regulator of cell proliferation, differentiation, and intercellular signaling within the alveolar niche. The adult lungs accumulate retinoids due to the action of lecithin:retinol acyltransferase (Lrat), which catalyzes the esterification of dietary retinol (ROH) and allows for the accumulation of retinyl esters (RE). Locally stored REs have been shown to protect the lung against acute injury. These studies aimed to explore the role of retinoids in O₃ toxicity and their underlying mechanisms of action. **Methods:** Groups of male and female mice (10-12 wk, n=5-10/group) were exposed to ultrapure air or O₃ (0.8 ppm) for 3 h. To explore the actions of retinoids, groups of animals with different lung retinoid levels were employed, including: 1) *Lrat*^{-/-} mice (a genetic animal model deficient in tissue REs), 2) *Lrat*^{+/+} (wild type) mice (a control group kept on a chow diet with 15 IU/g of vitamin A) and 3) *Lrat*^{+/+} + VA mice (a high vitamin A group fed a chow diet with 200 IU/g of vitamin A for 6 wk prior to air or ozone exposure). Mice were euthanized 24, 48, and 72 h after exposure. Bronchoalveolar lavage (BAL) fluid, macrophages, plasma, and lung tissues were collected. Lung and plasma retinoids were analyzed using HPLC. Total RNAs were isolated from lung tissues and AMS, followed by cDNA synthesis using 1 µg of total RNA, and assessing gene expression by qRT-PCR. Expression levels of target genes were normalized to the reference gene 18S. Statistical differences between air- and O₃-treated groups, as well as between groups with different lung retinoid concentrations, were analyzed by ANOVA, p<0.05 was considered significant. **Results:** Exposure of wild-type mice to O₃ resulted in pulmonary inflammation, characterized by upregulation of lung proinflammatory genes (*Tnfa*, *Il-1β*, *Ptges*) as early as 48 h postexposure, followed by upregulated expression of reparative genes (*Tgfb*, *Col1a1*, *Acta2*) at 72 h postexposure. This was associated with a progressive decline in retinoid concentrations in lung tissue in both males and females as early as 24 h post-exposure. A significant elevation of canonical ATRA-responsive genes (*Rarg*, *Stra6*, *Cyp26b1*) was also noted at this time, suggesting activation of local lung retinoid mobilization required to assure upregulated ATRA-mediated signaling, a protective response to O₃. *Lrat*^{-/-} animals exhibited earlier (24 h post exposure) and significantly greater upregulation of proinflammatory gene expression, suggesting a more pronounced inflammatory response to O₃ by lungs lacking locally stored REs. Conversely, lung expression of proinflammatory genes in animals supplemented with dietary vitamin A prior to O₃ exposure was significantly lower when compared to wild-type and *Lrat*^{-/-} mice and was not different from that of air-treated animals, even 72 h post-exposure, suggesting that inflammation was mitigated in lungs with high RE stores. **Conclusions:** Lung retinoid metabolism and signaling are responsive to cellular damage induced by acute O₃ exposure. A lack of tissue retinoids contributes to an amplified inflammatory response. Dietary supplementation with vitamin A, resulting in higher lung retinoid concentrations, has a protective effect on the lungs against O₃ exposure. **Support:** NIH Grants ES005022, ES004738, HL171112.

PS 3929 Targeting All-trans-Retinoic Acid Signaling to Mitigate Ozone Respiratory Toxicity

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Background and Purpose: Environmental exposure to air pollutants is the leading cause of lung functional impairments and respiratory diseases worldwide. According to the WHO, more than 7 million premature deaths occur annually due to polluted air and air pollution-related illnesses. It is essential to identify effective and impactful strategies, including nutritional and pharmacological interventions, aimed at alleviating lung injuries resulting from environmental exposure to polluted air and preventing long-term lung pathologies. Retinoids (vitamin A and its metabolites) are essential for pre- and post-natal lung development and lung health. All-trans-retinoic

acid (ATRA), a transcriptionally active retinoid species, and its three distinct cognate nuclear hormone receptors, retinoic acid receptors (RAR α , - β , and - γ), mediate these processes by regulating lung cell proliferation, differentiation, and communication among lung macrophages, alveolar fibroblasts, epithelial, and endothelial cells. The physiological ramifications of ATRA-RAR signaling in the context of respiratory toxicology, as well as its cell-specific targeting to mitigate polluted air-borne lung injuries, have not been systematically explored. Our studies focus on understanding the mechanisms behind the protective actions of ATRA-RAR signaling in mitigating respiratory toxicity and associated lung disease resulting from exposure to air pollutants using ozone (O₃) as a criteria air pollutant. **Methods:** To test the hypotheses that stimulation of lung cell-specific ATRA-RAR signaling enhances lung resilience and function after exposure to O₃, mouse models of chronic and acute O₃ exposure are utilized, coupled with dietary (vitamin A-containing diets), pharmacological (RAR isoform-specific agonists and antagonists), and genetic (animal models lacking tissue retinoid stores or ablated RAR signaling) manipulations. Single-cell RNA-sequencing (scRNA-seq) is employed to assess lung cell-specific responses to O₃ exposure, followed by validation of transcriptional responses (qRT-PCR), protein expression (immunoblot and immunofluorescent staining), and retinoid concentrations (HPLC) in either whole lung tissue or primary lung cell populations (macrophages, fibroblasts, epithelial, and endothelial cells) isolated postexposure from animals with different vitamin A supplementation or altered ATRA-RAR signaling. **Results:** Exposure of mice to O₃ results in pulmonary inflammation, characterized by upregulation of lung proinflammatory genes (*Tnfa*, *Il-1 β* , *Ptges*) in transcriptionally diverse macrophages, followed by upregulation of reparative gene (*Tgfb β* , *Col1a1*, *Acta2*) expression by functionally heterogeneous activated lung fibroblast populations. This response is associated with a progressive decline in retinoid (retinol and retinyl esters) concentrations in lung tissue and a significant elevation of canonical ATRA-responsive genes driven by RAR α in lung structural cells and RAR γ in immune cells. Taken together, the data suggest activation of local lung retinoid mobilization for enhanced ATRA synthesis and upregulated cell-specific ATRA-RAR signaling, a protective response to O₃. Moreover, a lack of sufficient retinoid stores, as well as compromised RAR signaling, makes the lung highly susceptible to the damaging actions of O₃, resulting in enhanced lung inflammation and functional impairments. Dietary interventions employing vitamin A-supplemented diets or pharmacological manipulations involving RAR α agonists and RAR γ antagonists rescue the altered lung phenotype by reducing O₃-induced macrophage-associated inflammation and improving functional activities of lung epithelial and endothelial cells. **Conclusions:** Lung ATRA-RAR signaling is responsive to cellular damage induced by O₃ exposure in a cell-specific manner. Targeted stimulation of ATRA-RAR signaling with dietary supplementation with vitamin A, resulting in higher lung retinoid concentrations, has a protective effect on the lungs against O₃ exposure. Our studies can have a meaningful impact on public health by providing new mechanistic insights into how vitamin A can protect the lungs from O₃ and how individual nutritional interventions with vitamin A can minimize the risk of lung injury and optimize lung health during inhaled exposures. **Support:** NIH Grants ES005022, ES004738, HL171112.

PS 3930 The Interactions between Chromosomal and Gonadal Sex Affect Lung Function After Sub-Chronic Ozone Exposure

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Background and Purpose: Epidemiological studies have shown that when exposed to ambient ozone, women typically have worse lung health outcomes such as higher mortality and decreased lung function compared to men. Animal models of acute ozone exposure have recapitulated sex differences in pulmonary responses; however, data remain conflictive. Previously, our lab found that hormone status modulates lung responses to acute ozone exposure. The contributions of sex hormones and the sex-specific responses to chronic or sub-chronic exposures remain understudied. To address this gap, we are using the Four-Core Genotype (FCG) mice, a mouse model where gonadal sex and chromosomal sex are independent of each other, to understand how same-sex and opposite-sex hormones alter the response to sub-chronic ozone. **Methods:** We exposed adult FCG mice to 1.5 ppm of ozone for 2 hours, twice a week, for 6 weeks (4-7 mice per experimental group). At 24 hours after the last exposure, mice were anesthetized, administered a paralytic, and attached to a rodent ventilator (flexiVent, Scireq). Baseline lung function data was recorded, including PV loops, inspiratory capacity, airway resistance, elastance, and compliance. Three-way ANOVAs with Tukey tests were used for statistical analyses with significance at p-value < 0.05. **Results:** Our preliminary results indicate that PV loops for the FCG mouse model varied after exposure to ozone depending on their combined chromosome and gonad composition. Mice with the XY sex-chromosome complement had no significant response to the ozone exposure, however mice with the XX sex-chromosome complement had changes to their PV loops. XX mice with testes had a reduction in volume for the given pressures, and XX mice with ovaries had an increase in volume for the given pressures. The XX mice with ovaries also showed a significant decrease in elastance (p < 0.0001), a significant increase in inspiratory capacity (p < 0.01), and a significant decrease in compliance (p < 0.001). **Conclusions:** After exposure to ozone, the increase in volumes for given pressures on the PV loop for the XX mice with ovaries suggests a shift towards emphysema, while the decrease in volumes for given pressures in the XX mice with testes suggests a shift towards fibrosis. Furthermore, the emphysema phenotype for the XX mice with ovaries is further evident by the significant increase in inspiratory capacity and decrease in elastance. In contrast, the XX mice with testes did not display the

typical increase in elastance expected of a fibrosis phenotype. In conclusion, our data show that the combination of gonads and hormones affect lung function after sub-chronic exposure to ozone. Future histological analysis will confirm these lung disease progressions in the response to ozone.

PS 3931 Effects of Sub-Chronic Ozone Exposure on Ovarian Follicular Development and Gene Expression in Adult Female Mice

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Background and Purpose: Ozone is a highly reactive gas known to trigger lung inflammation, oxidative stress, and other deleterious health effects when inhaled. Previous work in our lab has also shown that these systemic inflammatory responses may affect the hypothalamic-pituitary-gonadal (HPG) axis. The HPG axis is responsible for steroidogenesis for reproductive processes. These hormones contribute to the development and maturation of ovarian follicles, which are eventually released during ovulation. This study aimed to quantify changes in hormone production and follicle development following a sub-chronic ozone exposure in mice. Based on prior studies on acute exposures, we hypothesized that sub-chronic ozone exposure would result in stunted follicular development and altered hormone gene expression in the HPG axis. **Methods:** To test this hypothesis, we exposed adult female mice to 1.5 ppm ozone for 2 hours, twice a week for 6 weeks (n=5/group), or filtered air (control). At 24 hours after the last exposure, mice were euthanized, and blood, brains, and ovaries were collected. One ovary was used for histological analysis, where ovarian follicles were counted at varying stages of development. RNA was extracted from the other ovary, the pituitary gland, and the hypothalamus. Real-time qPCR with Taqman assays was used to determine the expression of gonadotropin-releasing hormone (GnRH) in the hypothalamus, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and GnRH receptor (GnRHR) in the pituitary gland, FSH receptor (FSHR), progesterone receptor (PGR), and LH receptor (LHCGR) in the ovaries, and 18S RNA as the endogenous control. Two-sample t-tests compared gene expression between ozone-exposed mice and controls in all tissues, with p-values < 0.05 considered significant. **Results:** Our preliminary results showed no significant difference in gene expression for any of the hormones or receptors measured. However, ozone-exposed mice showed a large increase in pituitary FSH expression, with a relative fold change 4 times higher than mice exposed to filtered air. The ozone group also showed significantly lower corpora lutea counts in ovarian histology (p<0.05), with means of 5.25 $\pm$ 0.24 in filtered air and 2.83 $\pm$ 0.07 in ozone. There were no significant changes in all other follicle stages. **Conclusions:** In conclusion, a lower corpus luteum count after sub-chronic ozone exposure suggests less frequent ovulation success. The potentially higher FSH expression could indicate the staggered peaking of FSH and LH to stimulate ovulation. Because LH and FSH together control the ovulation process, a relative difference in levels of the two may decrease ovulation success, which may contribute to the differential corpus lutea count. Future directions include increasing sample size to reduce variability, measuring hormones, and investigating intermediates induced by FSH prior to ovulation.

PS 3932 Ozone-Induced Lung Injury Increases Susceptibility to Lower Respiratory Infections by Elevating Airspace Iron Concentrations

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Background and Purpose: Epidemiological studies reported that increases in ambient air pollution associate with increased risk for hospitalization and/or mortality from lower respiratory tract infections. Acute elevations in ground level ozone (O₃), a criteria air pollutant produced by heat and sunlight reacting with volatile organic compounds and nitric oxides, increase hospitalization due to respiratory infections such as community acquired pneumonia. Despite this apparent susceptibility to infection, acute O₃ exposure stimulates the pulmonary immune response, and induces pulmonary inflammation through disruption of iron homeostasis. Prior research in our lab and others identified that acute O₃ exposure increases airspace iron and iron associated proteins. However, if this contributes to increased lung infection susceptibility is not fully understood. To uncover these specific mechanisms, we utilized a murine model of O₃-exposure followed by a pulmonary infection to test the hypothesis that O₃ increases susceptibility to pneumonia by promoting an airway microenvironment that favors pathogen growth. **Methods:** Male C57Bl/6J (WT) mice were exposed to filtered air (FA) or 1 ppm O₃ for 3h, and then 24h after exposure, mice were infected by oropharyngeal aspiration with 2000 colony forming units of the gram-negative bacteria *Klebsiella pneumoniae* (Kp). Mice were necropsied 24h post-infection and lung tissue and blood were obtained to quantify bacterial burden. Bronchoalveolar lavage fluid (BAL) was evaluated to define the pulmonary immune response. BAL protein levels were assessed to quantify lung injury, whereas lung inflammation was assessed by BAL leukocyte differentials and histopathology. Inductively coupled plasma-mass spectrometry (ICP-MS) was used to measure iron levels within the BAL and lung tissue was used for bulk RNA-sequencing. To

determine if factors within O₃-exposed lung directly promote bacterial pathogenesis, BAL from WT mice 24h post FA or 1 ppm O₃ exposure was incubated with *Kp*, and bacterial proliferation was assessed. Lastly, to understand how iron impacts bacterial growth following O₃ exposure, we utilized *Kp* mutants deficient in siderophore production, a key bacterial mechanism of iron acquisition, to assess bacterial proliferation and markers of lung injury and inflammation. **Results:** 24h following *Kp* infection, O₃-exposed mice, when compared to FA controls, had a significant increase in bacterial burden in lung tissue and blood. O₃-exposed *Kp*-infected mice also had a significant increase in airspace neutrophilia and total BAL protein. Transcriptional analysis of lung tissue by RNA-sequencing indicated that O₃-exposed mice infected with *Kp* had a significant increase in expression of genes related to immune pathways. Assessment of BAL iron concentration indicated airspace iron levels were significantly increased 24h following O₃-exposure when compared to FA. Based on the evidence of increased iron and increased infection risk and severity, we tested the direct potential relationship to airspace iron. First, we determined if cell free BAL increased *Kp* growth, observing an increase with co-incubation of *Kp* with BAL from O₃ exposed mice. To remove the potential for BAL proteins as causal factors, we heat inactivated the BAL. Consistent with the suggestion of iron as the driving reason for increased *Kp* growth, heat-inactivated BAL from O₃ exposed mice continued to increase *Kp* growth. Finally, we assess if this was a direct effect by using BAL from O₃ exposed mice with a *Kp* that lacks siderophores that would allow it to take up iron. In this setting, the pro-growth support of BAL from O₃ exposed mice was lost. **Conclusions:** Taken together, these data suggest that O₃-induced increases in airspace iron promote a microenvironment conducive to bacterial infection, ultimately increasing disease severity. Ongoing studies will identify how iron supplementation or depletion, through use of iron chelators, affects airspace *Kp* proliferation. Additionally, investigations as to how iron deposits in the lung following O₃ exposure to assess the mechanism or cell type responsible for elevations in airspace iron following O₃ exposure.

PS 3933 Prior Viral Lung Injury Enhances Lung Inflammation to Subsequent Ozone Exposure

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Background and Purpose: Exposure to ozone (O₃) increases the risk of significant cardiopulmonary events, in part due to increased and persistent lung inflammation. One question is if prior lung injury events sensitize the lung to enhanced or more persistent O₃-induced lung injury. In the present study, we define if O₃-induced lung injury is enhanced in mice that recover from prior influenza infection. **Methods:** We inoculated mice with 25 PFUs of H1N1 (WSN33) or heat-inactivated virus suspended in PBS via intratracheal administration and monitored weights for 21 days post-infection. At 21 days, when the mice had recovered from infection, they were exposed to either filtered air or O₃ (2 ppm x 3 hours) in a whole rodent exposure chamber. 24 hours post-exposure, mice were euthanized with ketamine/xylazine and bronchoalveolar lavage fluid (BALF) was collected to analyze for total protein, cell counts, and cell differentials. One-way ANOVA with Tukey's post-hoc analyses evaluated relationships among treatment conditions and endpoints. **Results:** Mice exposed to influenza lost on average 18% of total body weight before returning to their original starting weight over a three week period, suggesting adequate infection and recovery responses. Maximum weight loss was observed on day 11 with mice returning to starting weight by day 15. No mice experienced mortality as a sublethal infection model was desired. Influenza/ozone-exposed mice had higher total protein in their BALF than influenza/filtered air and heat-inactivated/ozone mice, suggesting enhanced lung injury. Additionally, influenza/ozone-exposed mice had more BALF macrophages than heat inactivated/ozone mice and more BALF neutrophils than influenza/filtered air and heat-inactivated/ozone mice, suggesting enhanced lung inflammation. **Conclusions:** Our data suggest that following recovery from a viral infection, mice have enhanced lung inflammation and injury to subsequent O₃ exposure. This is evidenced by increases in total protein and BALF macrophages and neutrophils. This suggests that prior injury responses may prime the lung to enhanced lung injury to subsequent air pollution exposure and could be a reason for persistent respiratory symptoms following infection. Future work will define how changes in immune cell composition following influenza infection could drive augmented O₃-induced lung injury.

PS 3934 Exaggerated defects in pulmonary mechanics following ozone exposure in mice lacking GCN2

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Background and Purpose: Ozone is a criteria air pollutant known to cause injury, oxidative stress and inflammation in the lungs and to exacerbate chronic diseases such as asthma. This is associated with alterations in pulmonary function including airway hyperresponsiveness in both humans and rodents. We previously demonstrated increased airway resistance and decreased tissue elastance and lung volume

following acute ozone exposure in mice. Moreover, ozone causes a reduction in surfactant protein B (SP-B), a component of pulmonary surfactants, which are critical for proper lung functioning. Biochemical pathways underlying the effects of ozone are not fully understood. The integrated stress response (ISR) plays a key role in regulating anti-inflammatory and antioxidant responses. We discovered that ISR signaling through the activating kinase, GCN2, is important in protecting against ozone-induced lung injury and inflammation. Thus, loss of GCN2 exacerbates alveolar epithelial dysfunction leading to increased neutrophil accumulation in the lungs following ozone exposure. The effects of loss of GCN2 on pulmonary mechanics are unknown and were evaluated herein. We hypothesized that lack of GCN2 will lead to more pronounced lung dysfunction following acute ozone exposure. **Methods:** Male and female C57BL/6 wild-type (WT) and whole-body GCN2 knockout (GCN2^{-/-}) mice (11-14 wk) were exposed to filtered air or ozone (0.8 ppm) in a whole-body Plexiglass chamber for 3 hr. Mice were anesthetized 3 or 24 hr later and respiratory mechanics measured using a SCIREQ flexiVent small animal ventilator. A one-phase decay nonlinear regression line was fit to the real and imaginary values from forced oscillometry to analyze resistance and reactance respectively; Y0 and plateau values were compared to evaluate differences in the lower and upper respiratory tract. At 3 hr post-exposure, pressure-volume (PV) loops were generated and the area between inspiratory and expiratory curves calculated at positive end expiratory pressures (PEEP) of 1, 3, 6, and 9 cm H₂O. At 24 hr post-exposure, PV loops and PV loop area were generated at a PEEP of 3 cm H₂O; evaluation of airway resistance following methacholine challenge at 0, 6, 12, 48, 96 mg/mL was assessed. Lung expression of SP-B was analyzed by western blotting. **Results:** At 3 hr post ozone exposure, a significant decrease in PV loop area was noted in GCN2^{-/-} mice, but not WT mice at PEEPs 1 and 3 cm H₂O; this decrease persisted for at least 24 hr at PEEP 3 cm H₂O. Analysis of forced oscillometry revealed a frequency-dependent increase in airway resistance in GCN2^{-/-} mice compared to WT mice at PEEP 3 cm H₂O, 3 and 24 hr following ozone exposure, consistent with bronchoconstriction. Methacholine challenge at 24 hr resulted in a dose dependent increase in resistance in WT mice, with no difference between air and ozone exposures. GCN2^{-/-} mice were unresponsive to methacholine, a response independent of air or ozone exposure. No significant changes in expression of SP-B were observed following ozone exposure in either genotype. **Conclusions:** Loss of GCN2 leads to persistent functional alterations in the airways, but not in parenchymal tissue, as evidenced by differences in resistance at both 3 and 24 hr after exposure to ozone, with no change in reactance. This is supported by findings that ozone had no effect on expression of SP-B in GCN2^{-/-} mice and that air-exposed GCN2^{-/-} mice were unresponsive to methacholine. Alterations in airway function could also explain the significant reduction in PV loop area observed in GCN2^{-/-} mice following ozone exposure at lower PEEPs (1 and 3 cm H₂O). Moreover, because these effects are observed at 3 h post-exposure, it is likely that direct oxidative effects of ozone on the airways drive these changes. **Support:** NIH grants ES004738 and ES005022, and the Air Pollution Educational and Research Grant (APERG) Scholarship Program.

PS 3935 Prolonged Ozone Exposure Induces Sex-Specific Changes in the Gut Microbiota and Production of Short Chain Fatty Acids in Mice

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Background and Purpose: Prolonged exposure to ozone is known to induce pulmonary injury, oxidative stress and altered lung functioning; it has been linked to exacerbation of chronic respiratory diseases including asthma, bronchitis, and emphysema. Evidence suggests that the onset of these respiratory diseases is accompanied by changes in gastrointestinal health, the gut microbiota, and gut-derived metabolites such as short chain fatty acids (SCFAs). The effects of prolonged ozone exposure on the gut microbiota and cecal SCFAs are unknown and were assessed in the present studies. **Methods:** C57BL/6 mice (11 wk, male and female) were exposed to filtered air or 1.5 ppm ozone for 2h, 2x/wk for 6wk; mice were euthanized 24 hr after the final exposure. Fecal samples were collected from the distal colon for gut microbiota analysis. Genomic DNA was extracted using QIAamp PowerFecal Pro DNA kit and the V4 hypervariable region of the 16S rRNA gene was amplified using fusion primers, ligated to adapters, and then barcoded. Bacterial DNA sequencing was performed using the Ion GeneStudio S5, and amplicon sequence variants (ASVs) identified and matched. Alpha diversity (Shannon index, observed ASVs, and Faith's Phylogenetic Diversity (PD)) and relative abundance of bacteria were analyzed using QIIME2 and the phyloseq package in R. Statistical comparison was performed using a Kruskal Wallis test paired with Dunn's post hoc and p<0.05. SCFAs were assayed using gas chromatography-mass spectrometry from snap frozen cecal samples. Beta diversity (Bray-Curtis) was performed in the vegan R package and statistical comparisons of community dissimilarity between groups were analyzed using permutational multivariate analysis of variance paired with a Bonferroni post-hoc (p<0.05). Colon tissue mRNA expression of free fatty acid receptors (Ffar) 2 and 3, Olfr78, and Hcar2, which bind to and transport the SCFAs acetate, propionate, and butyrate, were analyzed using a two-way ANOVA paired with Tukey's posthoc and p<0.05. **Results:** Alpha diversity (gut microbiota differences within a community) identified a significant increase in observed ASVs within female, but not male, mice exposed to ozone when compared to air. No differences were observed in the Shannon Index, which examines species richness and diversity. Beta diversity (gut microbiota differences between communities) identified significant differences

between mice exposed to air compared to ozone in both sexes. At the phyla level, a significant increase in the relative abundance of Bacteroidota and a significant decrease in Bacillota and Proteobacteria was noted in both sexes. Following ozone exposure, there were significant decreases in the SCFA-producing genera *Alistipes* in female mice and *Faecalibaculum* in male mice compared to air-exposed mice. Additionally, an increased abundance of *Clostridia* UCG-014, a genus associated with inflammation, was observed in male mice exposed to ozone. Whereas decreases in acetate and propionate were evident in response to ozone in male and female mice, respectively, butyrate was increased in both sexes. Expression of *Ffar2* was significantly reduced in males, while *Hcar2* expression was significantly increased in female mice after ozone exposure. **Conclusions:** Prolonged ozone exposure alters bacterial communities in both male and female mice; notably, ozone causes a decrease in abundance of SCFA-producing bacteria and an increase in inflammatory gut microbes, responses that can influence cecal levels of acetate and propionate. Increases in butyrate likely reflect compensatory production following ozone exposure. mRNA expression of SCFA receptors is also altered by ozone in a sex-related manner, which suggests that there are distinct responses to ozone exposure in the colon of males and females.

PS 3936 Sex Differences in Airway Inflammation and Remodeling after Repeated Ozone Exposure in CC002 Mice

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Background and Purpose: An increased frequency of extreme weather events, including wildfires and heat waves, enhances the formation of ozone and results in more consecutive ozone action days. This is a significant problem as ozone is a highly reactive and oxidizing gas that increases the development of new cases and worsens existing cases of non-allergic asthma. In this regard, studies using the genetically diverse Collaborative Cross strain CC002 have reported increased pathological hallmarks of non-allergic asthma, including airway eosinophilia and collagen deposition, after repeated exposure to ozone. Epidemiological studies report females exhibit increased severity and prevalence of non-allergic asthma relative to males, but whether this is influenced by ozone exposure is not known. We hypothesized that repeated exposure to ozone would result in increased eosinophilic inflammation and airway remodeling in female CC002 mice compared to males. **Methods:** Adult male and female CC002 mice were exposed to filtered air or ozone (0.8 ppm, 4 hr/day for 9 days) and euthanized 24 hr after the last exposure. Total protein content, IgM, and cytokine levels were analyzed in bronchoalveolar lavage (BAL) fluid by BCA, ELISA, and MILLIPLEX Multiplex assays, respectively. Eosinophils were enumerated in differentially stained BAL cytosins. Collagen content around small airways was quantified in Masson's Trichrome stained lung histological sections using QuPath image analysis software. mRNA expression was analyzed in lung tissue by qPCR. Data were analyzed by two-way ANOVA followed by Šidák's multiple comparisons test. **Results:** Repeated exposure to ozone significantly increased BAL levels of biomarkers of lung injury (total protein and IgM). No sex differences were observed, indicating that anatomical variation between males and females did not affect ozone deposition and degree of injury. Interestingly, female mice exhibited a significant increase in the number of eosinophils and levels of the eosinophil-derived cytokine, CXCL1, in BAL, and CXCL1 mRNA expression in lung tissue after repeated ozone exposure compared to males. This was associated with significantly enhanced collagen deposition around the small airways in ozone-exposed females relative to males. **Conclusions:** These results indicate that repeated exposure to ozone results in a more pronounced eosinophilic inflammation and airway remodeling response in female mice compared to males. We speculate that this is due to immune-potentiating actions of female gonadal steroid hormones, as the degree of lung injury was similar in males and females. Importantly, these sex differences mirror those observed in humans; thus, this model represents an ideal tool to investigate mechanisms underlying sex differences in non-allergic asthma.

PS 3937 Impact of Ozone Exposure Timing on Lung Injury and Inflammation in a Model of SurfactantProtein-C-Driven Inflammatory Exacerbations of Pulmonary Fibrosis

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Background and Purpose: Pulmonary fibrosis (PF) is a form of interstitial lung disease characterized by progressive scarring of the alveolar compartment, leading to pathogenic loss of pulmonary function and mortality within 5 years of diagnosis. The fibrotic phenotype is often accelerated and exacerbated by acute inflammatory events. Although the initiating factors are often unknown, exposure to environmental stressors and air pollution are recognized contributors of this phenotype. Ozone (O₃) is a ubiquitous toxicant known to produce immediate but reversible epithelial damage, loss of alveolar function, airway hyperreactivity, and inflammatory cells infiltration and activation in healthy individuals. The effects in the susceptible or fibrosing lung are not fully established. We have previously described and developed a murine model of inflammatory exacerbations triggered by the induction of a PF-linked mutation in the Surfactant Protein C (*SP-C*^{T73T}) gene. We therefore set to investigate the

effects of O₃ exposure occurring at various times of an *SP-C*^{T73T}-induced exacerbations. **Methods:** Mutant *SP-C* induction is achieved through tamoxifen oral gavage (175 mg/kg, corn oil used as a control). O₃ exposure (800ppb, 3 hours) conducted on day 4 post tamoxifen gavage. A separate paradigm involved two O₃ exposures 48 hours apart from each other, prior to tamoxifen gavage. All experiments were terminated 7 days post tamoxifen. Bronchoalveolar lavage fluid was collected for cell counts, while perfused lungs were split for histological and histochemical analysis (right lobes) and flow cytometry (left lobe). **Results:** Histological evidence of septal injury and fibrosis induced by *SP-C* mutant induction were not significantly impacted by O₃, regardless of the timing of exposure. Mortality was significantly increased in the group receiving O₃ exposure following the initiation of an inflammatory exacerbation. Flow cytometric analysis showed that pre- or post-induction exposure does not have additive effects on myeloid and lymphoid cells relative abundance up to 7 days. This phenotype was characterized by increased neutrophilia and eosinophilia paired with drops in CD3⁺ lymphocytes and B cells. Histochemical analysis revealed changes in cellular proliferation (Ki-67) in the lungs of mice exposed to O₃ prior to an exacerbation. **Conclusions:** These results suggest that acute ozone exposure does not significantly alter the early trajectory of a fibrogenic exacerbation triggered by alveolar epithelial dysfunction in the *SP-C* mutant mouse. Further work will define any delayed effects and the impact of chronicity in this phenotype.

PS 3938 Alveolar Macrophage Temporal Transcriptional Plasticity in Response to Acute Ozone Exposure

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Background and Purpose: Ozone (O₃) is a ubiquitous urban air pollutant. Inhaled O₃ causes protein and lipid oxidation, and the formation of aldehydes and free radicals in the alveoli, leading to lung damage and inflammation. Evidence suggests that macrophages contribute to the initiation and resolution of tissue injury induced by O₃. This activity is mediated by distinct subpopulations of these cells. Herein, the cell-specific temporal transcriptional responses of macrophages were assessed. We hypothesize that macrophage transcriptional plasticity orchestrates cellular responses to O₃ exposure. **Methods:** Female mice were exposed to air or ozone (0.8 ppm) for 3 h. To map the dynamics of lung macrophages and their transcriptional signatures in the intraalveolar compartment of the mouse lung after O₃ exposure, bronchoalveolar lavage (BAL) cells were profiled by single-cell RNA sequencing (scRNA-seq), capturing the alveolar injury (24 h) and reparative resolution (72 h) phases. Single-cell libraries, prepared using the Chromium Single Cell 3' Reagent Kits v3.1, were sequenced on a NovaSeq 6000 (Illumina) in a 2 x 100 bp configuration, and data processed using Cell Ranger v5.0.1 (10X Genomics). Filtered feature matrix outputs from Cell Ranger were used for all downstream analyses performed in R Software v4.4.0, using the Seurat v5.2.1 packages. **Results:** Unsupervised clustering of batch-corrected datasets, followed by the application of cell-specific gene markers, distributed BAL cells into fourteen clusters, including macrophages. Subsetting and reclustering of the macrophages identified five transcriptionally distinct subclusters. Differentially expressed gene (DEG) and gene set enrichment (GSEA) analyses allowed for the identification of the generated macrophage subclusters, including cluster 0 comprising tissue resident or main macrophages involved in surveying and surfactant turnover, cluster 1 macrophages with upregulated mitochondrial activity or stressed macrophages, cluster 2 and 3 highly proliferative or cycling macrophages in S phase and G2-M phase respectively, and cluster 4 recruited antigen-presenting or poised inflammatory macrophages. The macrophage cluster composition changed both qualitatively and quantitatively in response to O₃, with a significant increase in overall numbers and a shift in cluster percentage distribution over time. Twenty-four h post-exposure, proliferative clusters 2 and 3 were widely depleted, but returned to pre-exposure levels by 72 h. The percentage of clusters 0 and 1 cells gradually increased at 24 h and peaked at 72 h. Cluster 4 cells appeared only at 72 h. In pseudotime trajectory analysis, proliferative clusters 2 and 3 were positioned at the beginning of the pseudotime, giving rise to macrophages in cluster 0. Consequently, cluster 0 macrophages transition into the cluster 1 cells, positioning this cluster at the end of the pseudotime. Cluster 4 was not developmentally connected to other macrophage clusters, representing the unique recruited macrophage population that appeared in the alveolar compartment only 72 h after O₃. NicheNet analysis of cell-cell communications during O₃ post-exposure revealed that macrophage intracellular signaling can be triggered by intercellular interactions with other alveolar cells. Monocytes, dendritic, and epithelial cells were the sender cells expressing genes with the highest ligand activity, which could target gene candidates in macrophages, affecting their responsiveness to O₃. In line, pathway analysis using Hallmark, Reactome, and Gene Ontology (GO) databases identified temporal macrophage cluster-specific metabolic, cell trafficking, immune, and proliferative responses. **Conclusions:** Lung macrophages are a transcriptionally diverse cell population and an integral component of the alveolar microenvironment responding to O₃ in a temporal cell-specific manner. Both O₃ exposure and non-macrophage signaling within the alveolar niche result in cluster-specific responses in the macrophage transcriptome. Specific gene and pathway signatures of the identified macrophage clusters can serve as a tool for further temporal interrogation and molecular targeting of macrophage subpopulations to prevent O₃-induced toxicity and promote the resolution of lung injury. **Support:** NIH Grants ES005022, ES004738, ES032473, HL171112.

PS 3939 Interleukin-1 Receptor 2 Transcription in a Subset of Alveolar Macrophages During Acute Lung Injury induced by Ozone and Endotoxin

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Background and Purpose: Ozone exposure has been linked to the development of acute respiratory distress syndrome (ARDS) in patients with sepsis. In a murine model, we demonstrated that inhalation of ozone prior to intravenous administration of lipopolysaccharide (LPS) to model sepsis, induces neutrophilic inflammation and acute lung injury (ALI). Resident alveolar macrophages (AMs) play a key role in resolving inflammation associated with ARDS. However, the heterogeneity of AM responses is becoming more apparent. In these studies, we performed single-cell RNA sequencing to elucidate transcriptional changes in AM subpopulations that may contribute to resolution of inflammation during ozone-induced ALI. **Methods:** Male C57BL/6J mice were exposed to 0.8 ppm ozone or filtered air for 3 h in a whole-body chamber. After 24 h, mice received LPS (3 mg/kg, i.v.) or PBS control. Bronchoalveolar lavage cells were collected 24 h post-treatment from pooled samples (2-3 mice/group) across 3 replicate experiments. Cells were processed for single-cell RNA sequencing using the 10x Genomics Chromium platform and sequenced with Illumina technology. Cell clusters were identified using a nearest-neighbor approach and visualized via Uniform Manifold Approximation and Projection (UMAP). Significant differentially regulated genes were further assessed using Integrated Pathway Analysis (Qiagen). **Results:** Among 22 identified cell clusters, 12 were classified as resident AMs based on characteristic gene markers (*Mertk*, *Mrc1*, *Itgax*, *Adgre1*, *Siglec1*). Mice exposed to ozone + LPS showed a marked decrease in resident AM frequency compared to air + PBS, air + LPS, or ozone + PBS exposure groups (29.0% vs. 86.2%, 86.6%, and 91.7%, respectively). Re-clustering within resident AMs revealed 14 subpopulations; with subpopulation 3 abundance increased only in mice exposed to ozone + LPS. Within this expanded subpopulation, pseudo-bulk and IPA analyses revealed the interleukin-1 family signaling pathway as the most likely upregulated pathway unique to ozone + LPS exposure (z-score 5.4; p = 0.0004). While genes with both pro and anti-inflammatory function were increased within this pathway, the gene with the highest expression was *Il1r2* (log2fold change 10.3) which encodes IL1-R2, a dummy/decoy receptor for IL-1. This was increased by orders of magnitude relative to *Il1b* (encoding IL-1β; log2fold change 7.8), *Il1a* (encoding IL-1α; log2fold change 3.7) and *Il1rn* (encoding IL-1 receptor antagonist; log2fold change 4.8). **Conclusions:** Both *Il1r2* and *Il1rn* transcription act to counterbalance proinflammatory IL-1 signaling. We identified a distinct subset of resident AMs that proportionally increase after exposure to ozone + LPS and exhibit markedly increased *Il1r2* transcription relative to *Il1rn*. While IL-1 receptor antagonists are clinically employed to treat macrophage activation syndrome, IL-1R2 supplementation has never been evaluated therapeutically. The preferential upregulation of *Il1r2* in this AM subset suggests that this decoy receptor may play an even more critical role than IL-1 receptor antagonists in resolving acute lung injury (ALI) during sepsis. These findings highlight IL-1R2 as a promising candidate for development as a therapeutic strategy to mitigate ozone-induced ARDS.

PS 3940 Ozone Exposure Alters Alveolar Macrophage Efferocytosis Receptor Gene Expression in Mice

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Background and Purpose: Neutrophils (PMNs) accumulate in the lungs following inhalation exposure to the oxidant air pollutant, ozone. These short-lived cells undergo apoptosis and are cleared from the lungs by resident alveolar macrophages (AMs) by a phagocytic process called efferocytosis. We previously reported that efferocytosis is impaired in AMs 48 hr after ozone exposure, leading to prolonged neutrophilia during sepsis. Efferocytosis requires recognition of apoptotic cells by macrophage receptors including MerTK, Axl, SR-AI/All, SR-BI, and CD36. Expression of these receptors is regulated by the transcription factor peroxisome proliferator activated receptor gamma (PPARγ). PPARγ signaling, which is downregulated following ozone exposure. However, the effects of ozone on genetic expression of efferocytosis receptors during the resolution of ozone-induced injury is not clearly defined, and this was assessed in the present studies. **Methods:** Male wild type C57BL/6J mice were exposed to air or ozone (0.8 ppm, 3 h) in a whole-body chamber. Mice were euthanized 48 h later, and lung cells collected by bronchoalveolar lavage (BAL) with gentle tissue massage. Cells were enriched for resident AMs by magnetic depletion of CD11b expressing cells and analyzed for expression of *Pparg* (encoding PPARγ), *Msr1* (encoding SR-AI/All), *Scarb1* (encoding SR-BI), *Cd36*, *Mertk*, and *Axl* using qPCR. Expression levels were normalized to the geometric mean of housekeeping genes *Actb*, *Gapdh*, or *18s*, and fold change values calculated using the $\Delta\Delta C_T$ method. Data were analyzed by a Kolgorov-Smirnov test. **Results:** Exposure to ozone resulted in a decrease in *Pparg* expression in AMs (fold change = 0.37) this was associated with decreased transcription of *Mertk* (0.60) and significantly decreased transcription of *Cd36* (0.61), with no change in *Scarb1* (1.31) or *Axl* (0.99)

expression. Conversely, *Msr1* transcription was significantly increased (3.83) following ozone exposure. **Conclusions:** These data suggest that decreased transcription of macrophage efferocytosis receptor genes contributes impairment in AM efferocytosis following exposure to ozone. SR-AI/All is known to promote efferocytosis by augmenting MerTK signaling in macrophages. Upregulation of SR-AI/All may represent a compensatory mechanism for decreased *Mertk* transcription. We further speculate that increases in SR-AI/All are insufficient to compensate for this impairment.

PS 3941 The role of CD163-mediated cell free hemoglobin clearance in ozone-induced pulmonary injury and inflammation

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Background and Purpose: Inhalation of tropospheric ozone (O₃) induces pulmonary inflammation in part through the production of damage associated molecular patterns (DAMPs), which bind to pattern recognition receptors (PRRs) and activate pro-inflammatory pathways in innate immune cells. However, different members of the scavenging receptor (SR) class of PRRs can either augment inflammation or protect against inhaled oxidants. CD163 is a SR responsible for clearance of cytotoxic, cell free hemoglobin (CFH) that accumulates in the lungs following sterile lung injury. Additionally, CD163 internalization and CFH metabolism upregulate anti-inflammatory and antioxidant pathways. However, the relevance of CD163 in scavenging CFH and promoting anti-inflammatory responses in O₃-induced lung injury is not currently understood. We hypothesize that CD163-mediated CFH clearance is necessary for resolution of pulmonary inflammation caused by acute O₃ inhalation. **Methods:** Female, wild type (WT) and CD163^{tm1(KOMP)Vlcg} (CD163^{-/-}) knockout (KO) mice were exposed to either filtered air (FA) or 1 ppm O₃ for 3 hours, which models an environmentally relevant O₃ exposure hazardous to humans. A separate cohort of WT and KO mice were given a single oropharyngeal (o.p.) dose of CFH (1 mg/ml, 100 µg/mouse) or vehicle control. Mice were necropsied 12- and 24-hours following exposure to collect bronchoalveolar lavage (BAL) and lung tissue. Gene expression was measured by real-time polymerase chain reaction. Lung injury and permeability were measured by BAL total protein and albumin, respectively. Lung inflammation was measured by airspace cell differential. CFH clearance was measured by BAL concentration of scavenging proteins, hemopexin (Hx) and haptoglobin (Hp). Airspace oxidative stress was measured by BAL electron paramagnetic resonance (EPR). **Results:** CD163 expression was significantly increased in WT mice 12 hours after O₃ exposure, concomitant with lung neutrophilia, BAL Hp concentration, and oxidative stress. Additionally, markers of lung injury and inflammation were significantly augmented in CD163^{-/-} mice, 12 hours after O₃ exposure. At 24 hours, O₃ significantly increased BAL neutrophilia and oxidative stress in CD163^{-/-} mice compared to WT controls; however, there was no difference in markers of lung injury. Compared to vehicle controls, airspace total protein and immune cell numbers were significantly increased in both WT and CD163^{-/-} mice 24 hours after CFH administration, with no difference between WT and CD163^{-/-} mice. CD163^{-/-} mice exposed to CFH had significantly greater airspace neutrophilia compared to WT mice exposed to CFH. CFH exposure led to significantly increased BAL Hp in CD163^{-/-} mice compared to WT mice after 24 hours and significantly increased oxidative stress in CD163^{-/-} mice only. **Conclusions:** These findings indicate that pulmonary injury, inflammation, and oxidative stress are augmented in CD163^{-/-} mice exposed to O₃ or CFH. Additionally, these data suggest that CFH clearance is impaired in CD163^{-/-} mice, supporting a role for CFH scavenging by CD163 in resolution of O₃-induced pulmonary injury and inflammation. Further research will examine CD163 expression by different lung cell populations, the role of soluble CD163 (sCD163) in response to O₃ exposure, and translation to human studies.

PS 3942 Synergistic effects of ozone and heatwave exposure on lung inflammation in young mice: Evidence and Mechanism of Lung Stem Cells

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Background and Purpose: Chronic respiratory diseases, the fourth leading cause of mortality globally, result a substantial public health burden. Concurrent extreme events are projected to increase with climate change, with ozone (O₃) and heatwaves (HW) extremes posing significant threats to respiratory health, particularly in children. Lung stem cells are crucial for maintaining homeostasis and regeneration of lung tissue. However, the mechanisms of lung stem cells in O₃ and HW-induced respiratory inflammatory diseases remain unclear. This study investigated the combined effects of O₃ and HW on lung inflammation, focusing on lung stem cell function in young mice, to inform adaptation strategies for concurrent events. **Methods:** C57BL/6 SPF male mice (4-6 weeks) were randomly assigned to four groups (n=10): control group (filtered air) and three treatment groups exposed to 36°C, 0.6 ppm O₃, and 0.6 ppm O₃+36°C for 21 consecutive days (3h/d). After exposure, lung function, pathological changes and inflammatory cytokines (TNF-α, IL-1β, CC16) were assessed. Club cells, key lung stem cells, are involved in the

maintenance of lung epithelium function and repair after injury. *Lkb1* (Liver Kinase B1) may affect the proliferation and differentiation of lung stem cells, so *Lkb1* expression in lung tissue and club cells was determined. To evaluate *Lkb1*'s effect on club cells, we used *CCSP^{CreER};Lkb1^{fl/fl}* mice (*Lkb1* was conditionally deleted in club cells) and *Lkb1^{fl/fl}* mice, measuring Ki67 (proliferation marker), Clca1 (goblet cell marker), and Foxj1 (ciliary cell marker) in club cells. Then we used an FACS-based method to separate club cells for transcriptomics analysis, with results validated by RT-qPCR. **Results:** Co-exposure to O₃ and HW significantly exacerbated lung inflammation, with a synergistic effect on lung function impairment and elevated inflammatory cytokines, while *Lkb1* expression was reduced in young mice. Lack of *Lkb1* in club cells significantly aggravated the lung inflammation and impaired club cells proliferation and differentiation, specifically suppressing ciliated cell generation while promoting goblet cell hyperplasia. Single-cell transcriptomic analysis revealed that the differentially expressed genes were significantly enriched in metabolic pathways, with lipid metabolism being the most prominently altered. Persistent dysregulation of key lipid metabolic genes was confirmed by qRT-PCR, including significant downregulation of *Bbox1* and upregulation of *Fbp1* and *Them5* ($P < 0.05$). **Conclusions:** Co-exposure to O₃ and HW synergistically aggravated lung inflammation by impairing club cell function in young mice, likely through *Lkb1*-driven lipid metabolic disruption.

PS 3943 Peripheral and Lung Cytokine Profiles Are Associated with Ozone-Induced Lung Inflammation in Humans

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Background and Purpose: Ozone (O₃) is a criterion air pollutant that causes airway inflammation and epithelial injury. However, individual O₃-induced lung injury and inflammation responses are highly variable, and there is a need for less invasive measures, which correlate with pulmonary responses to assess variability. This study examined whether assessments of peripheral immune cell populations and circulating cytokine levels correlate with O₃-induced lung inflammation in humans. **Methods:** Healthy adults (18-45 years; n=16) underwent a crossover-challenge exposure to filtered air (FA) and O₃ (200 ppb for 2.25 h). Peripheral blood was collected immediately after exposure and at the time of bronchoscopy to obtain bronchoalveolar lavage fluid (BALF; approximately 20 hours post-exposure). Flow cytometry was used to quantify immune cell populations, and cytokines were measured in BALF and serum using Mesoscale Discovery V-PLEX Proinflammatory panels. Bayesian modeling (COMPASS) was applied to assess proportional changes in immune cells, while Kruskal-Wallis and Spearman correlation analyses evaluated relationships among cytokines and cell populations. **Results:** O₃ exposure did not significantly alter peripheral immune cell proportions at either post- or 24-hour post-exposure time points (all $p > 0.1$). Functionality scores derived from COMPASS modeling similarly showed no association with lung inflammation ($p > 0.1$). Alternatively, IL-6 and IL-10 BAL cytokine levels were significantly elevated in participants exhibiting BAL inflammation as defined by BAL neutrophils ($p = 0.012$) and ($p = 0.022$). For levels of peripheral cytokines and peripheral cell populations, correlations were observed based on the timepoint following exposure. Immediately following O₃ exposure, IL-1 β was positively correlated with CD8⁺ T cells ($p = 0.0033$). IL-4 was negatively correlated with PMNs ($p = 0.007$), and IL-12p70 was positively correlated with eosinophils ($p = 0.001$). Alternatively, at 20 hours post-exposure, IL-8 was positively correlated with other T cells ($p = 0.034$), IL-12p70 remained positively correlated with eosinophils ($p = 0.023$), and TNF- α was negatively correlated with neutrophils ($p = 0.022$). For peripheral cytokines and lung bronchoalveolar lavage cell populations, at 24 hours post-exposure, IL-4 was negatively correlated with PMNs ($p = 0.043$), and IL-8 was negatively correlated with CD8⁺ T cells ($p = 0.041$). **Conclusions:** While no consistent peripheral immune cell changes were detected following acute O₃ exposure, cytokine-cell correlations, particularly involving IL-6 and TNF- α associations indicate inflammatory immune responses following acute O₃ exposure. These findings suggest that cytokine profiles, rather than cell proportions, may serve as more sensitive biomarkers of O₃-induced lung inflammation and could help to define inter-individual variability in susceptibility to air pollutant exposures.

PS 3944 Diesel exhaust particle inhalation-induced exacerbation of murine lupus nephritis may be mitigated with probiotic supplementation.

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Background and Purpose: Lupus nephritis (LN) is the most common clinical manifestation of systemic lupus erythematosus (SLE), a chronic autoimmune disease in which the immune system creates autoantibodies against nucleic acid-based autoantigens. Autoantibody-autoantigen immune complexes (ICs) form, travel through systemic circulation, and become trapped in the glomerulus and adjacent tubules of the nephron. LN is characterized by the accumulation of ICs in the nephron, which leads to persistent localized inflammation and damage to the glomerular filtration barrier, thus reducing kidney function. Traffic-generated air pollution exposure is known to exacerbate general kidney diseases and has been proposed to do the same in LN, given the increased incidence in urban areas. Thus, this study is focused on investigating whether inhalational exposure to vehicular air pollutants contributes to

more advanced LN pathophysiology. Conventional pharmacological treatments of SLE/LN often have adverse side effects and may not be effective due to the heterogeneity of disease activity between individuals. This has led to increased interest in identifying adjunct methods of disease management, such as the use of probiotics, due to their known immunomodulating properties. Thus, this study is also focused on investigating the effectiveness of probiotic supplementation in mitigating LN severity. **Methods:** To achieve both of these aims, 18-week-old female NZB/WF1 (Jackson Labs) LN-model mice were exposed to either 35 μ g of diesel exhaust particles (NIST SRM #2975; DEP, n=24) suspended in 30 μ l 0.9% sterile saline or sterile saline only (CON, n=24) via oropharyngeal aspiration and received either 0.3g (7.5 x 10⁸ CFU) Ecologic® Barrier 849 probiotic (PRO, n=24) or nutrient-matched placebo (PLC, n=24) daily in drinking water for 50 days. Deposition of IgG and complement 3 (C3) and expression of TNF- α were measured using immunofluorescent microscopy, and Masson's trichrome staining was utilized to measure collagen deposition in frozen kidney sections. The average kidney-to-body weight ratio was also assessed, and statistical analyses were performed using two-way ANOVA to determine significance. **Results:** Deposition of IgG and C3 was significantly increased in DEP PLC compared to CON groups, and Masson's trichrome showed increased collagen deposition in DEP groups. These alterations reflect greater accumulation of ICs with DEP exposure, leading to fibrosis in the glomerulus and adjacent tubules of the nephron. IgG deposition was significantly decreased in DEP PRO compared to all other experimental groups, and TNF- α expression and kidney:body weight ratio were significantly reduced in CON PRO compared to all other experimental groups. These alterations suggest that probiotic supplementation has mitigative effects on LN severity, both with and without DEP insult. **Conclusions:** These results indicate that inhalational exposure to diesel exhaust particles contributes to more advanced lupus nephritis and that probiotic supplementation may be beneficial as an adjunctive treatment. Funded by NIEHS 2R15ES026795 to AKL.

PS 3945 Sex Differences and Estrogen Dependent Cytokine Release in Human Small and Large Airway Epithelial Cells Exposed to Peat and Eucalyptus Wood Smoke Condensates

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Background and Purpose: Wildfires and biomass combustion are growing global health concerns due to the release of particulate matter (PM), which impairs respiratory function and contributes to cardiopulmonary disease. These risks are amplified by the increasing prevalence, severity, and geography of wildfires. Prior studies have shown that smoke chemistry can vary substantially by fuel type (e.g. eucalyptus (chaparral, southern California) and peat (peatland/coastal, midwestern and south-eastern US)) and studies from our lab and others have shown that by varying fuel type, respiratory immune responses can also be altered. However, there is limited research exploring these effects in primary respiratory epithelial cells from differing compartments of the respiratory tract. Beyond fuel type, sex-specific differences in airway disease pathogenesis also shape susceptibility and outcomes following wood smoke exposure. For example, in asthma and chronic obstructive pulmonary disease (COPD), females exhibit greater small airway inflammation, while males are more prone to alveolar destruction. The role of sex hormones in respiratory response to wood smoke exposure also remains understudied, although studies indicate that estrogen can modulate the respiratory immune system through estrogen receptors present on epithelial and immune cells in both small and large airways. Together, these factors suggest that interactions between wood fuel type, sex, and airway compartment influence immune responses to wood smoke exposure *in vitro*, though the mechanisms remain unclear. **Methods:** To investigate these factors, primary human small airway epithelial (SAE) and large airway epithelial (LAE) cells derived from male and female donors (n = 6M, 6F) were cultured at air-liquid interface for 4 weeks. To assess the role of estrogen in wood smoke induced inflammatory responses, a subset of cells were treated with 1 nM 17 β -estradiol (E2) throughout differentiation, equivalent to circulating levels in females of reproductive age. These cultures were then exposed to wood smoke condensates (WSCs) generated from peat and eucalyptus biomass at 25 μ g/cm² for 4 hours. Cytokine secretion was measured using the MSD V-Plex Human Cytokine multiplex ELISA. Significant values were determined in comparison to individual donor PBS-treated controls to account for inter-individual variability. **Results:** The data revealed several region-, fuel- and sex-dependent effects. IL-1 β was consistently up regulated across conditions, indicating a core inflammatory response to biomass smoke, while VEGF was selectively induced by peat, suggesting unique activation of airway remodeling pathways. TNF α was consistently elevated in males but increased in females only with peat, indicating sex- and fuel-specific pro-inflammatory signaling. Females had unique up regulation of IL-2, IL-6, and IL-13 for both peat and eucalyptus, consistent with a Th2-biased immune phenotype. IFN γ induction was found in female LAEs but suppressed by E2 treatment in SAEs. In males, E2 reversed cytokine responses in LAEs: peat shifted Eotaxin-3 and TARC from up- to downregulation, while eucalyptus shifted IL-4 and IL-12p40 from down- to upregulation. E2 exerted opposite effects on TARC by airway region in males, highlighting compartment-specific estrogen modulation. **Conclusions:** Together, these findings demonstrate that wood smoke elicits both

biomass fuel-conserved and sex-specific immune responses, with estrogen status and airway compartment serving as significant modulators. Peat and eucalyptus fuels also showed distinct cytokine profiles in some mediators measured, highlighting the role of fuel source in shaping inflammatory outcomes. This research provides insight into factors that may influence individual susceptibility to wood smoke exposure, such as local ecology, sex, level of circulating sex hormones and potentially provides targets for future research into treatments or preventative measures by sex or specific to location of exposure or smoke source.

PS 3946 Associations between PM_{2.5} and self-reported cognitive decline in the United States, 2015 to 2022: Evidence from the behavioral risk factor surveillance system (BRFSS)

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Background and Purpose: Research indicates that self-reported or subjective cognitive decline (SCD) is a major risk factor for mild cognitive impairment and eventually dementia. Environmental factors, such as air pollution, have emerged as significant influencers of cognitive abilities. Olfactory loss, cognitive decline, and dementia incidence all have been independently associated with exposure to air pollution in epidemiological studies. Recent data indicate that the odds of dementia diagnosis was 10% higher for every 1 µg/m³ increase in three-year average wildfire particulate matter (PM) concentration. The goal of current study is to understand how long term fine particulate matter (PM_{2.5}) levels influences SCD among adults from the Behavioral Risk Factor Surveillance System (BRFSS). **Methods:** We aimed to answer the question: Is there a relationship between PM_{2.5} and reported/subjective cognitive decline from 2015 to 2022 on a state-by-state basis. We hypothesized that there would be a positive association between PM_{2.5} levels and SCD. Approximately 14,135 survey results from all 50 states, District of Columbia and Puerto Rico were included in this study. We measured SCD from participants' self-reported answers to the Cognitive Decline Module, a module which identifies individuals, age 45 years and older, with worsening memory loss. Data for PM_{2.5} were obtained from the US Environmental Protection Agency. These data were cleaned to retain only state identifiers and average 24-hour concentrations, then grouped by state to calculate yearly means. For SCD, data from the BRFSS (2015-2022) were filtered to exclude "Don't know" and "Refused" responses, and a binary variable was created to indicate the presence of SCD, and only states that participated in the optional module each year were included. We used linear mixed models (LMM) to assess any associations, with state as a random intercept though to account for the repeated measures from each state. Additionally, for each model, a 1, 2, and 3-year lag for PM_{2.5} levels was run. We also employed imputation for missing data and compared results for observed and imputed data and significance was set at p<0.05. **Results:** Over the 2015 - 2022 period, Indiana had the highest average PM_{2.5} while Wyoming had the lowest PM_{2.5}, with averages at 10.3 µg/m³ and 4.21 µg/m³ respectively. Adult males in Oklahoma and Tennessee reported the most cognitive decline, 15.5% and 15.0% respectively while adult females in Arkansas and Alabama each reported an average cognitive decline of 14.4% per year. Adult males and females in Puerto Rico reported the lowest cognitive decline per year, 7.03% and 5.43% respectively. LMM results indicated that, on average, cognitive decline occurs each year by approximately 0.6% and with each lagged value of PM_{2.5} (p<0.05). This implies that even after accounting for year, there is a significant increase of the percent of people reporting cognitive decline when they are exposed to higher PM_{2.5} levels in the one (p=0.020), two (p<0.001), or three (p=0.002) years prior. To assess the sensitivity of these models, states with less than three years' worth of SCD data were excluded from the analysis. Model results from this limited dataset showed that there was still a significant increase in the percent of adults reporting cognitive decline when exposed to higher PM_{2.5} levels in the one (p=0.030), two (p=0.005), or three (p=0.005) years prior. Imputed data for all states showed similar and statistically significant trends. We did not observe statistically significant results by sex (p>0.1). **Conclusions:** Regardless of the region in the US, higher PM_{2.5} concentrations was associated with an increased risk of having SCD among adults who participated in the BRFSS. Particulate air pollution-based interventions should be prioritized to lessen the impact of cognitive decline in older adults.

PS 3947 Diesel Exhaust Particles Modulate Splenic Cytokine Expression in Juvenile Mice

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Background and Purpose: Diesel exhaust particles (DEP), a major component of fine particulate matter, can induce extrapulmonary oxidative stress and inflammatory responses. Preliminary data from our laboratory showed that juvenile mice exposed to DEP induced leukocyte infiltration in the lungs and neuroinflammation. The spleen is the largest peripheral immune organ and plays a central role in systemic immune responses. Therefore, the objective of this study was to assess if DEP exposure modulates cytokine expression and induces oxidative damage in the spleen.

Methods: Male and female C57BL/6J mice were exposed intranasally to increasing doses of DEP from postnatal day (PND) 25 to 33. Spleen tissues were collected six days after the final exposure and homogenized with RIPA buffer. Cytokines and chemokines expression were quantified with a multiplex bead-based immunoassay (23-plex) using a Luminex MagPix instrument. Oxidative alterations in the spleen were evaluated with Fourier Transform Infrared Spectroscopy (FTIR). Characteristic bands corresponding to lipids (2850-2923 cm⁻¹), proteins (amide I and II: 1650 and 1545 cm⁻¹), and nucleic acids (1080 cm⁻¹) were quantified to assess oxidative and structural changes. **Results:** Preliminary data showed a significant increase in IL-1α levels but significant decreases in IL-3, IL-9, and IL-13 levels. FTIR analysis revealed subtle alterations in spectra across exposure groups; however, no statistically significant differences were detected. Ongoing lipid peroxidation and protein carbonyl measurements will determine whether changes in FTIR spectra correlate with biochemical markers of lipid and protein oxidation. **Conclusions:** The results suggest that DEP exposure modulates splenic activity by inducing pro-inflammatory pathways and suppressing adaptive immunity. The observed spectral tendencies, combined with cytokine dysregulation, suggest that oxidative damage and inflammation occur concurrently as part of a systemic immune response to DEP.

PS 3948 Surgical Smoke in the Operating Room: Evaluating the Role of Smoke Evacuators on Surgical Staff Exposure

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Background and Purpose: Surgical smoke, which is the smoke generated by the burning of tissues during surgery due to the use of electrocautery devices, is known to contain harmful air pollutants such as particulate matter (PM) and volatile organic compounds (VOCs). Gaps remain in our understanding of the exposure experienced by surgeons and operating room staff to air pollutants in surgical smoke and the potential health impacts of exposure. Smoke evacuators can help remove surgical smoke from the surgical theater, potentially reducing exposures, though they are not used in all surgeries. Therefore, this study aimed to evaluate the role of smoke evacuators on PM air pollutant concentrations during procedures with frequent electrocautery use. **Methods:** PM_{2.5} concentrations and elemental compositions were analyzed from 20 surgical cases from two hospital systems, the Birmingham Veterans Affairs Medical Center and the University of Alabama at Birmingham Hospital in Birmingham, AL. The surgical cases included mastectomies, lumpectomies, and amputations. Operation details, room conditions, electrocautery use, smoke evacuator presence, and 1-minute averaged PM measurements were recorded. PM_{2.5} concentrations were measured from two locations within each operating room. The first location was near the surgical field, and the second location was at the room's periphery. PM_{2.5} concentrations were recorded using PDR-1500 DataRAM air monitor with an internal Teflon filter. Surgical cases were categorized based on smoke evacuator use. Peak and average concentrations were compared using t-tests and chi-square tests. PM collected on Teflon filters were analyzed using X-Ray Fluorescence (XRF) to evaluate the PM's elemental component levels. **Results:** Twenty surgeries were analyzed, with mastectomies being the most common (n=11, 55%). Smoke evacuators were used in 6 cases. The average electrocautery duration was 75 minutes. PM_{2.5} levels without a smoke evacuator were statistically significantly higher than PM_{2.5} levels with a smoke evacuator (22.6 vs 5.3 µg/m³, p<0.0001). While the mean differences in PM_{2.5} concentrations did not appear drastically different, cases without a smoke evacuator experienced significantly more peaks over 50 and 100 µg/m³. The frequent high peaks exhibited during cases without a smoke evacuator resulted in more acute exposures to surgical staff. Cases with a smoke evacuator had significantly smaller and less frequent peaks. Although the mean concentration was not high, cases without a smoke evacuator had a total of 197 peaks above 50 µg/m³ while cases with a smoke evacuator had a total of one peak above 50 µg/m³. The average number of peaks per case above 50 µg/m³ for cases without a smoke evacuator was 14.1 per case. The average number of peaks per case above 50 µg/m³ for cases with a smoke evacuator was 0.17 per case. Furthermore, in cases without a smoke evacuator, the peak sizes were larger than in cases with a smoke evacuator. The elemental analysis using XRF showed lower elemental chromium, cobalt, selenium, and iron concentrations in cases with a smoke evacuator than cases without a smoke evacuator. **Conclusions:** Electrocautery use without smoke evacuators resulted in statistically significantly higher PM_{2.5} concentrations and frequent pollutant peaks. The frequent and pronounced peaks demonstrated surgical staff experience more acute exposures in cases without the use of a smoke evacuator. The differences indicate the importance of using smoke evacuators during electrocautery procedures for reducing surgical staff exposure.

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Background and Purpose: Diesel exhaust particles (DEP), a major component of urban air pollution, pose significant health risks, including potential impacts on brain function and behavior. Mental health disorders are characterized by clinically significant disturbances in cognition, emotional regulation, and behavior with an average age of onset of 14.5 years. Therefore, this study investigated whether DEP exposure induces anxiety-related behaviors in prepubertal mice, used as a model of early adolescence. **Methods:** Both female and male C57BL/6J mice were exposed intranasally to increasing doses of DEP between postnatal days (PND) 25 to 33. Three days after the last exposure (PND36) mice locomotion, exploratory and anxiety-related behaviors were evaluated using the open field test under controlled environmental conditions. Behavioral activity was recorded for five minutes and analyzed using ANY-maze software to quantify grooming, rearing, and spatial zone preferences, among other variables. **Results:** DEP-exposed mice exhibited increased mobility, speed, grooming, and rearing behaviors compared to controls, indicating heightened stress and exploratory activity. Track plot analysis further revealed altered spatial distribution, with DEP-exposed mice spending more time in the peripheral zone. **Conclusions:** Adolescence is a transitional life stage between the ages of 10 to 19 years, in which significant cognitive and socioemotional development occurs. During this life stage, key processes in brain development might be susceptible to environmental insults. Our findings suggest that DEP exposure may influence exploratory and stress-related behaviors in prepubertal mice, reflecting potential behavioral effects of particle pollution.

PS 3950 Lipid Compositional and Spatial Alterations Associated with Pulmonary and Systemic Toxicity Following Silica Exposure

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Background and Purpose: Inhaled exposures, including ambient particulate matter and engineered nanomaterials, have been shown to disrupt lipid metabolism both locally and systemically. Despite these observations, comprehensive assessments of lipid dysregulation following inhalation exposures remain limited, and lipids are often overlooked in studies of systemic toxicity and disease. Among these exposures, silica particles (SiO₂), naturally occurring and frequently generated through human activities such as mining, construction, and manufacturing, are of particular concern. Respirable crystalline silica is a well-established cause of silicosis, chronic obstructive pulmonary disease, and lung cancer. Respiratory SiO₂ exposure has also been associated with extrapulmonary effects, contributing to chronic kidney disease, cardiovascular disease, and autoimmunity. Traditional lipidomic analyses rely on homogenized tissue preparations, an approach that may obscure localized effects since particles deposit heterogeneously within the lung. Desorption electrospray ionization mass spectrometry (DESI-MS) imaging overcomes this limitation by providing spatially resolved lipidomic data, thereby enhancing sensitivity and revealing site-specific lipid alterations. We hypothesized SiO₂ exposure induces dose- and time-dependent lipid dysregulation in both pulmonary and systemic tissues mediating health outcomes. **Methods:** To evaluate this hypothesis, mice were exposed to 0.25, 0.5, or 1 mg of SiO₂ by oropharyngeal aspiration, with controls receiving water (n=5/group). At 24 hours or 7 days post-exposure, bronchoalveolar lavage fluid and lung, liver, and kidney tissues were collected for quantitative real-time PCR of inflammatory and lipid metabolism genes. DESI-MS imaging was performed on tissues to evaluate SiO₂-induced lipid alterations while H & E staining enabled histological evaluation. **Results:** Gene expression analyses demonstrated significant upregulation of the pro-inflammatory mediators *CCL2*, *CXCL-1*, *CXCL-2*, *IL-6*, and *IL-1β* in the lung 24 hours post-exposure, with return to control levels by 7 days. While systemic tissues exhibited limited inflammatory changes, several lipid metabolism genes were altered compared to controls. Notably, *COX-2* was induced across the lung, liver, and kidney at 24 hours after exposure. DESI-MS imaging revealed dose-dependent SiO₂-induced lipid dysregulation, most prominently within the lung, with histological overlays suggesting lipid alterations localized to terminal bronchioles. MS imaging of systemic tissues demonstrated lipid alterations that may mediate extrapulmonary responses to inhalation exposures. **Conclusions:** Collectively, these findings demonstrate spatial lipidomics can provide unique insights into SiO₂-induced toxicity, uncovering lipid disruptions that may underlie exposure consequences. This approach offers a more holistic framework for understanding and ultimately mitigating the health risks of inhaled exposures. Alterations in lipids suggests they could be a regulator of systemic responses occurring following inhalation exposure.

PS 3951 Soluble Epoxide Hydrolase Inhibition Alleviates AgNP-induced Pulmonary Inflammation in a Metabolic Syndrome Mouse Model.

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Background and Purpose: Evaluations of nanoparticle safety have concentrated on healthy models. Epidemiological studies have shown that individuals with metabolic diseases are more vulnerable to particulate air pollution. Our previous research identified specific alterations in lipid mediators involved in inflammatory regulation in a metabolic syndrome (MetS) mouse model, as well as an increase in lung inflammation following exposure to silver nanoparticles (AgNPs). This heightened response was associated with elevated pulmonary gene expression levels of epoxide hydrolase 2 (*Ephx2*), which encodes soluble epoxide hydrolase (sEH). sEH hydrolyzes anti-inflammatory epoxy fatty acids, facilitating their clearance. Inhibiting sEH may be beneficial for resolving inflammation by decreasing the removal of lipid mediators responsible for resolution signaling. We hypothesize the application of a sEH inhibitor, 1-Trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU), will effectively improve the bioavailability of epoxyeicosatrienoic acids (EETs) thereby reducing enhanced AgNP-induced pulmonary inflammation due to MetS. **Methods:** C57BL/6J male mice were fed either a healthy or a high-fat Western diet for 14 weeks. Subsets of mice were treated with 0.15% DMSO (vehicle control) or TPPU (10mg/L) via drinking water *ad libitum* for one week prior to AgNP exposure to inhibit sEH. After one week, mice were exposed to either 50 µl of water (control) or 50 µl of water containing 50 µg of 20 nm AgNP via oropharyngeal aspiration. Endpoints of pulmonary inflammation and toxicity were evaluated 24 hours following AgNP exposure. **Results:** MetS mice exposed to AgNPs and receiving 0.15% DMSO treatment exhibited significantly exacerbated pulmonary inflammatory responses compared to exposed healthy mice. In mice exposed to AgNPs and treated with TPPU, neutrophil-exacerbated infiltration decreased in the MetS model. This decrease in neutrophils was associated with lower expression levels of pro-inflammatory cytokine genes, such as *IL-6*, *CCL2*, and *CXCL2*. In the DMSO-treated AgNP-exposed mice, the expression of the resolution inflammatory cytokine gene *IL-10* was reduced. However, TPPU treatment increased *IL-10* levels in both mouse models. **Conclusions:** Overall, inflammatory responses induced by particulate exposure may be treated via modulation of lipid metabolism to enhance resolution signaling thereby benefiting susceptible subpopulations.

PS 3952 Linking Airborne Particulate Exposure to VOC Emission Changes: The Role of TET1 and Oxidative Stress in HBEcs

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Background and Purpose: Exposure to wildfire generated fine particulate matter, less than 2.5 micrometers, (WFPM2.5) poses a significant health concern, particularly among individuals with asthma. Acute exposures to WFPM2.5 are linked to increased asthma exacerbations, emergency department visits, and short-term disease morbidity. Chronic exposure to ambient PM2.5 has been shown to induce oxidative stress signaling, impacting asthma pathophysiology. We have detected some of these PM2.5 related exposure changes in normal lung epithelial cell emissions by capturing and measuring volatile organic compounds (VOCs). The goal of our study was to test if similar changes, related to acute WFPM2.5 exposures, are detectable in VOCs and whether TET1 is involved. TET1 is an epigenetic regulator that impacts DNA methylation, histone modification, and other epigenetic mechanisms. Previous studies from our lab have demonstrated that TET1 expression is protective in severe asthma, based on association studies in children and observations from animal models of allergic asthma. Altered TET1 expression in human bronchial epithelial cells (HBEcs) and genetically modified animals, post traffic-related airway pollution (TRAP) or house dust mite exposure, has been found to down-regulate aryl hydrocarbon receptor (AhR) signaling and downstream detoxifying enzymes, resulting in increased airway inflammatory signaling and hyperresponsiveness. The objective of our study was to test the hypothesis that HBEcs exposed to WFPM2.5 emit a distinct volatile organic compounds (VOC) signature compared to unexposed HBEcs and this difference can be partly explained by decreased TET1 activity and subsequent increased oxidative stress responses. **Methods:** We cultured primary HBEcs from donors under air-liquid-interface conditions to recapitulate the pseudostratified mucociliary phenotype seen *in vivo*. We then utilized established methodology from our lab to sample, measure, and analyze VOCs emitted from the HBEcs. Briefly, well-differentiated cells were treated on the apical surface with 100µL of phosphate-buffered saline (control) or resuspended WFPM2.5 (100 µg/mL) extracted from filters following the 2018 California Campfire. After 5 days of repeated WFPM2.5 treatment and removal, headspace VOCs were collected onto Twisters® (sorbent-covered stir bars) and analyzed via gas-chromatography mass spectrometry (GC-MS). Principal component analysis (PCA) was used to create a table of all detected VOCs, and a partial least squares-discriminant analysis (PLS-DA) model was built for each experimental group to filter the list of VOCs to identify compounds related to the exposure. Basolateral media was collected to assess pro-inflammatory cytokine secretion and RNA was isolated from the cells to measure the expression of *tet1* and TET1-regulated genes. **Results:** Our PLS-DA model demonstrated that VOCs emitted from the HBEcs clearly separated the WFPM2.5 from the PBS treated groups with 0.90 accuracy, 0.78 predicted sensitivity, and 0.85 predicted specificity. Some

of the highly upregulated compounds dictating this separation were 4,6-dimethyl-dodecane, 1,3,4 Thiodiazol-2-amine, 5-methyl-, Decane, 5,6-dimethyl and Dimethyl phthalate. These compounds generally belong to the following superclasses: heterocyclic compounds, hydrocarbons, esters, and carbonyl compounds. We also found a trend of increased amphiregulin and IL-6 cytokine production with the onset of WFPM2.5 exposure that was not sustained. The expression of *TET1* demonstrated a downward trend and increased variability after 5 days of WFPM2.5 exposure. We also observed a significant decrease in AhR mRNA (*AHR*) and *CYP1B1* expression ($P=0.046$ and 0.031 , respectively) while the expression of *CYP1A1* remained significantly elevated ($p=0.011$). **Conclusions:** We have shown that HBEcs repeatedly exposed to WFPM2.5 affects *TET1* expression, downregulates AhR mRNA expression, and produces unique VOC signatures that may serve as markers of oxidative stress. We propose that *TET1* plays a protective role against oxidative stress induced by WFPM2.5 and downregulating *TET1* expression could be a key signal contributing to oxidative stress damage present in asthma. Further research will provide more information on the relationship between WFPM2.5 exposure and *TET1* activity and how this potentially affects VOCs emissions.

PS 3953 Mass spectrometry imaging (MSI) elucidates regional distribution of metabolomic and lipidomic changes in the brain following exposure to wildfire smoke in mice

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Background and Purpose: Metabolomic and lipidomic methods using liquid chromatography-mass spectrometry (LC-MS) have revealed persistent changes in neurochemistry following exposures to wildfire smoke in mice. These findings are limited, however, because localization of metabolites and lipids in tissues cannot be determined in homogenized samples. To address the regionspecificity of metabolomic and lipidomic alterations from wildfire smoke, matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI) was applied to visualize regional distributions of metabolites and lipids in mouse brain tissue sections. **Methods:** 21-month-old female C57BL/6 mice were exposed to either filtered air or simulated wildfire smoke (WFS; 500 $\mu\text{g}/\text{m}^3$, 4 hours every other day for 14 days) and sacrificed 1-day post-exposure. For LC-MS untargeted metabolomics and lipidomics, homogenized brain tissues were analyzed using a Thermo Vanquish UPLC-Exploris 240 Orbitrap MS system and mass spectrometer equipped with an electrospray ionization (ESI) source. For metabolomics, untargeted data from 70 to 1050 m/z were collected while for lipidomics, untargeted data from 100 to 2000 m/z were collected. For MALDI MSI analysis, we utilized a Bruker timsTOF Flex MALDI-2 mass spectrometry imaging instrument to establish a mass spectrometry imaging (MSI) method for spatial distribution of metabolites and lipids in mouse brain tissue sections. N-(1-naphthyl) ethylenediamine dihydrochloride (NEDC) and 2,5-dihydroxybenzoic acid (DHB) matrices were applied on the brain tissue sections for metabolomics and lipidomics, respectively, with HTX M3+ Sprayer. MSI data was acquired in negative ionization mode for metabolites and in positive ionization mode for lipids, in 50 μm resolution and with ion mobility separation mode on. Each whole brain sections were selected as regions-of-interest. MALDI MSI data were analyzed using SCI LS Lab, MetaboScape, and LIPID MAPS. **Results:** Phenylalanine, tyrosine, and tryptophan biosynthesis was downregulated in WFS-exposed mice, and purine metabolism was upregulated in WFS-exposed mice. Similarly, phenylalanine, tyrosine, and tryptophan biosynthesis and purine metabolism were downregulated and upregulated, respectively, in advanced aged mice. Multiple downregulated and upregulated lipids and metabolites, including neurotransmitters and amino acids, were observed in the hippocampus, cortex, and cerebellum of WFS-exposed mice. **Conclusions:** This MALDI MSI method can be used in environmental health to study regional metabolic and lipid changes in tissue sections following exposure to environmental pollutants. Wildfire smoke-induced neurochemical changes in specific regions can now be related to other phenotypes, including behavior and persistent neuroinflammation.

PS 3954 Removal of volatile organic compounds by chemical filters significantly inhibited the development of atopic dermatitis symptoms in mice: Potential implications for Air Conditioning Systems in Healthcare Environments

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Background and Purpose: In recent years, there has been increasing concern about the impact of indoor air quality on human health. Volatile organic compounds (VOCs) are indoor air pollutants that contain various chemical substances and are emitted as gases from solvents, detergents, building materials, and consumer or hobby products. Because indoor VOC concentrations often exceed outdoor levels, Japan has established an indoor air quality guideline of 400 $\mu\text{g}/\text{m}^3$ for total VOCs. Although the health risks of VOC exposure have been widely studied, their contribution to skin diseases such as atopic dermatitis (AD) remains unclear. The present study aimed to

measure VOC concentrations in medical environments and to elucidate the effects of VOC exposure on AD development using both cellular and animal models. **Methods:** As part of the field survey, total VOC concentrations were measured in three different types of veterinary hospitals. To examine the effects of VOCs on inflammatory skin diseases, *in vitro* studies were conducted using human epidermal keratinocytes exposed to standard VOC solutions. Cytotoxicity and production of inflammatory cytokines were measured. Furthermore, at concentrations that did not induce cytotoxicity with VOCs alone, the combined effects of co-stimulation with TNF- α (10 ng/mL) were evaluated. For *in vivo* experiments, female NC/Nga mice were used to establish an AD model by repeated topical application of house dust mite ointment. During the experimental period, a VOC mixture (10 $\mu\text{g}/\text{mL}$) was applied topically. AD symptoms, transepidermal water loss (TEWL), and skin thickness were measured weekly. At the end of the experiment, auricular lymph nodes and skin tissues were collected for immunological and histological analyses. The efficacy of a chemical filtering system for VOC removal was verified by both field investigation and whole-body inhalation exposure using the AD mouse model. **Results:** The field investigation revealed variations in indoor VOC concentrations among hospitals, with some facilities exceeding the Japanese standard value of 400 $\mu\text{g}/\text{m}^3$. Direct exposure of HaCaT cells to VOCs showed cytotoxicity at high concentrations. In addition, co-stimulation with TNF- α significantly increased the production of IL-8 and TARC. In the AD mouse model, topical application of VOCs significantly exacerbated AD symptoms and increased ear skin thickness. The number of CD4⁺ T cells in auricular lymph nodes and IL-13 gene expression in auricular tissues were significantly elevated compared with the AD control group. Moreover, the developed chemical filtering system effectively removed VOCs from actual medical environments. Inhalation of VOC-filtered air in the AD mouse model significantly reduced AD scores, skin thickness, and TEWL. Immunological analyses showed that CD4⁺ T cell populations and inflammatory cytokines (IL-4, IL-17) in auricular lymph nodes, as well as serum IgE levels, were decreased. Expression of the flaggrin gene, associated with skin barrier function, was significantly upregulated in the VOC-filtered air exposure group. In contrast, the therapeutic application of the chemical filtering system showed only limited immunological improvement. **Conclusions:** These findings indicate that elevated indoor VOC concentrations may contribute to the onset and exacerbation of AD by promoting inflammatory cytokine production and impairing epidermal barrier function. Air conditioning systems equipped with VOC-removal filters can effectively reduce VOC exposure, thereby improving allergic inflammation and skin barrier integrity. Incorporating such filtration technologies into clinical and domestic environments could represent a promising preventive strategy for managing allergic and inflammatory skin diseases associated with poor indoor air quality.

PS 3955 Repeated pharyngeal aspiration of cloth debris may cause chronic tissue damage through immune suppression

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Background and Purpose: Textiles and clothing are a primary source of microplastic pollution, which releases microfibers. **Methods:** In this study, real tornado washing machine lint (TWML) samples were obtained from ten volunteers and pooled into a single test sample to investigate biological and cellular responses to inhaled TWML. **Results:** The dispersion contained irregularly shaped or fibrous particles and various heavy metals, including arsenic, copper, chromium, bromine, and antimony, as well as microplastic particles. 90-day repeated pharyngeal aspiration of TWML (50 μg) decreased the number of WBC and the proportion of neutrophils in WBC in male and female mice, respectively, whereas the proportion of RET in red blood cells decreased in both male and female mice. The total number of immune cells in the lungs increased with dose, accompanied by an increase in the proportion of lymphocytes and alveolar macrophages aggregating around TWML. In addition, among the inflammatory mediators measured in this study, only CXCL-1 and GM-CSF levels showed significant increases in the lungs of male and female mice, respectively. Aggregation of alveolar macrophages, infiltration of inflammatory cells, and hyperplasia of mucous cells in the bronchial epithelium were found in TWML-treated mice. When incubated at a concentration of 40 $\mu\text{g}/\text{mL}$, MH-S cells (alveolar macrophages) were aggregated around the fibrous particles, as was observed in the lungs. The production of cell signaling-related secondary mediators was significantly enhanced in TWML-treated cells. NGS analysis also indicated that the plasma membrane, cell periphery, and cell projections were among the most affected cellular components and that phosphatidylserine binding was the most affected molecular function. Expression of mitochondrial dynamics- and cellular iron uptake-related proteins was inhibited following exposure to TWML, and expression of anti-oxidant response-related proteins was clearly enhanced in the cells. **Conclusions:** Taken together, we suggest that TWML-induced immune suppression may be due to the incomplete phagocytosis of macrophages and that TWML may disrupt cellular function through oxidative stress.

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Background and Purpose: Prescribed burns manage wildfires by weakening fuel loads which lowers intensity and fire duration. The burning of trees is a significant contributor to air pollution, releasing fine particulate matter (PM_{2.5}) composed of harmful chemicals including black carbon and other hazardous air pollutants. Prescribed burns are common in the southeastern United States mainly for wildfire prevention and ecosystem management purposes with Georgia burning 1.4 million acres annually. This study aims to investigate PM_{2.5} and black carbon concentrations in Georgia during 2015, specifically comparing concentrations during the prescribed burn (October 1- April 30) and burn-ban (May 1-September 30) seasons. **Methods:** PM_{2.5} was collected on Teflon filters by the Environmental Protection Division of the Georgia Department of Natural Resources (GDNR) throughout 2015 at 8 locations in Georgia every 3 days. PM_{2.5} concentrations were determined through gravimetric analysis by the GDNR. All collected filters were then analyzed for black carbon at 880nm. Average PM_{2.5} and black carbon levels were compared between sites locations were compared between seasons (burn vs. burn ban). **Results:** Site Differences: Preliminary data for four of the sampling sites (Albany, Macon, Atlanta, and South Dekalb) showed average PM_{2.5} concentrations across locations were 11.37 ± 3.87 µg/m³. Average black carbon concentrations across locations were 2.18 ± 2.21 µg/m³. PM_{2.5} concentrations were significantly different between Atlanta and the Albany and South Dekalb sites. Black carbon concentrations were also significantly different between Atlanta and all other locations (one-way ANOVA, p ≤ 0.05). Seasonal Differences: Macon and South Dekalb showed variations between burn-ban and burn seasons in PM_{2.5} concentration, with increases in PM_{2.5} concentration in burn-ban months (two-way ANOVA, p ≤ 0.05). However, Albany, Macon, Atlanta, and South Dekalb all showed seasonal differences for black carbon concentrations which were elevated during the burn season (two-way ANOVA, p < .001). Data collection and analysis for four additional sampling locations is currently underway and will be compared to the preliminary data described above. **Conclusions:** Though seasonal differences in PM_{2.5} were not present in all sites, there was significant seasonal variation in black carbon at all sites with elevated black carbon during the burn season. This demonstrates that exploring individual components of PM_{2.5}, such as black carbon, may provide more insight into the pollutants released during prescribed burns than measuring PM_{2.5} alone. Future directions include using prescribed burn records to compare PM_{2.5} and black carbon data at specific sites during prescribed burn events and expanding ambient characterization to more sites throughout the state.

PS 3957 Carbon Black particles induce emphysema and inflammasome activation: a whole-mouse lung proteome landscape

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Background and Purpose: Chronic exposure to ultrafine particles (UFP) is one of the leading causes of morbidity and mortality worldwide. The accumulation of UFP is cleared by the engulfment of these particles by alveolar macrophages (AMs). AMs are the first line of defense against pathogens, as well as also have an anti-inflammatory function for the resolution of inflammation. The effect of nCB exposures on the anti-inflammatory functions of macrophages is not explored in detail. We are exploring the effects of nCB on the anti-inflammatory function of AMs, efferocytosis, for the homeostasis, and resolution of inflammation. In our preliminary studies, we have developed a mouse model of chronic nCB exposures and its effect on the lung morphology and injury. We have performed the proteomics analysis to identify the dysfunctions and mechanisms caused by nCB exposures. **Methods:** C57BL/6 mice (n=5) were exposed to 2 doses of nCB (by oropharyngeal aspiration) each other day during one week. Nanosized Carbon Black (nCB) (15 to 75 nm) was utilized to perform these exposures. After treatment, all mice were euthanized, and lungs were harvested for histopathology and whole-proteomic analysis using mass spectrometry LC/MS. All animal protocols were approved by Institutional Animal Care and Use Committee at the University of Alabama at Birmingham (UAB). **Results:** *In vivo* study showed 1) increased inflammation by increased presence of lymphocytes in the lung, 2) alveolar macrophages full of engulfed nCBs, 3) increased alveolar space (emphysema) in the nCB lungs, and increased cell death. Our preliminary proteomic data identified 1894 total proteins across samples. We identified 178 significantly differential expressed proteins in the lung proteome exposed to nCB particles. The histology and proteomic analysis both demonstrated emphysema and increased cell death-related pathways. The functional enrichment analysis showed that nCB top differentially expressed proteins are involved in inflammasome activation, reduction of vesicle trafficking, endosome formation, and calcium signaling process. This proteomics analysis showed nCB differential expressed proteins are associated with emphysema development, such as cell death, dysregulation of ubiquitination and ROBO signaling pathways. **Conclusions:** UFP lung exposures induced a differential protein expression pattern, showing there's saturation of macrophages' capacity to clear nanoparticles and tissue damage. Inflammasome activation and suppression of endosomal and lysosomal-related proteins suggested an exhaustive of lung

macrophages' function after nCB exposure. After these studies, we will perform in-depth studies to determine the role of nCB exposures in the development of COPD and emphysema.

PS 3958 The Protective Role of Nlr1 in Air Pollution-Exacerbated Acute Lung Injury

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Background and Purpose: Exposure to air pollution is associated with the development and severity of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). Epidemiological and experimental studies demonstrate that air pollutants increase respiratory vulnerability, resulting in a more pronounced inflammatory response and tissue damage. However, the molecular mechanisms underlying this effect remain unclear. Evidence suggests that exposure to air pollution can "prime" the immune system, leading to exaggerated inflammation and lung injury following subsequent insults. NOD-like receptor X1 (Nlr1) is a mitochondria-associated protein that regulates inflammation, mitochondrial homeostasis, cell death, autophagy, and reactive oxygen species (ROS) production. Nlr1 has been shown to protect against inflammatory diseases. We hypothesize that subacute exposure to environmentally relevant levels of ozone (O₃) and ultrafine carbon black (CB) aggravates ALI, while Nlr1 mitigates these effects by maintaining mitochondrial function and reduce excessive inflammation. **Methods:** Eight- to ten-week-old male C57BL/6J wild-type (Nlr1^{+/+}) and Nlr1 knockout (Nlr1^{-/-}) mice were exposed to a mixture of 250 ppb ozone (O₃) and 1 mg/m³ carbon black (CB) for 3 hours per day, 5 days per week, over a 3-week period using whole-body inhalation. After 3 weeks of exposure, ALI was induced by intratracheal instillation of 1.5 units of bleomycin (BLM), and the mice were euthanized 14 days post-BLM exposure. Survival, lung function, inflammatory response, and lung remodeling were assessed. **Results:** Exposure to air pollution primed for a greater mortality in bleomycin-induced ALI, with this detrimental effect being more pronounced in Nlr1^{-/-} mice. Mucous cell metaplasia and type 2 alveolar epithelial cell hyperplasia were induced by bleomycin exposure and were not altered by prior air pollution exposure in both genotypes. In Nlr1^{-/-} mice, combined air pollution exposure and bleomycin challenge resulted in heightened inflammatory responses, more severe impairment of lung function, and enhanced collagen accumulation in lung tissue. **Conclusions:** In summary, our results indicate that Nlr1 plays a critical protective role in protecting the lungs from air pollution exposure-induced priming for acute lung injury.

PS 3959 Repeated Exposure to Great Salt Lake Dust Induces Asthma-Like Phenotypes in Mice

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Background and Purpose: Air pollution impacts human health. Understanding the mechanisms driving adverse effects can improve the management of health and facilitate the development of treatments for those affected. High wind events in the Salt Lake City urban area expose residents to particulate matter (PM) originating from the shrinking Great Salt Lake. We refer to this as GSL dust or GSLD. GSLD is a mixture of heavy metals, metal oxides, microorganisms, and various natural and man-made chemicals that have accumulated over time. Few studies have investigated the impact of GSLD on human health outcomes. We previously found that acute high-dose inhalation exposure, mimicking a dust storm, caused robust neutrophilic pulmonary inflammation in mice. Inflammatory responses were replicated in human lung cell models, driven in part by interactions with the PM sensing ion channels transient receptor potential vanilloid-1 and vanilloid-3 (TRPV1 and TRPV3). This study tested the hypothesis that repeated exposures to GSLD at a low concentration, mimicking potential daily exposures, would promote chronic inflammation and phenotypes consistent with allergic asthma. **Methods:** Here, humanized *Trpv1*^{N606D} mice were exposed to GSLD (PM_{2.5} ~5mg/m³) for 2h per day, for 15 or 25 days, using the SCIREQ inExpose inhalation delivery system. Lungs and bronchoalveolar lavage fluid (BALF) were collected 72h after the last day of exposure. Lung immune cell populations were quantified in BALF using flow cytometry and manual cell counting. Lungs were collected for histology and analysis of gene/protein expression. **Results:** Exposure for 15 and 25 days increased eosinophils, B-cells, and dendritic cells in BALF, and triggered goblet cell hyperplasia in female >male mice. Effects were exacerbated in a challenge model (3 weeks exposure, 3 weeks no exposure, 1 week exposure). **Conclusions:** To conclude, phenotypes characteristic of allergic asthma developed in female >male mice following 15 or 25 day exposure to GSLD and upon rechallenge, versus transient neutrophilia observed in prior acute high dose studies. Future studies will investigate the sex-based differences and assess pulmonary function to confirm airway hypersensitivity. Further, understanding the role of TRPV1 and TRPV3 could yield a novel therapeutic strategy for managing health effects associated with GSLD and possibly other forms of PM.

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Background and Purpose: Specific activities performed in a veterinary clinic may result in the release of dust and fine particulate matter. These contaminants contribute to higher rates of asthma and other respiratory diseases among veterinary clinic workers compared to other occupational workers. Since Ammonia is a by-product of animal waste, exposure to ammonia is higher in veterinary workers and can irritate the lungs and airways. Aerosolized particulates of small sizes, such as PM_{1.0}, PM_{2.5}, and PM₁₀, can penetrate deeper into workers' airways, thereby irritating and exacerbating the pathogenesis of asthma and respiratory inflammations. The goal of the study was to assess particulate matter with aerodynamic diameters ranging from 1.0 to 10 µm and ammonia gas levels in different sections of a large veterinary clinic, in relation to workers' varying activities. **Methods:** The veterinary clinic is situated in a rural, tropical environment and is a high-traffic area, with an average of 35 pets seen daily. The mass concentrations and particle number concentrations were measured over a two-month period using the DustTrak aerosol monitor and the CEM DT-9881 particle counter during various activities and sections of the veterinary clinic. Samples were collected biweekly, on Mondays and Fridays, during working hours, in both the morning and the afternoon. A sensor measured the ammonia gas. All air samples were collected at breathing height, and environmental factors, including temperature, relative humidity, dew point, and wind velocity, were also measured. **Results:** The preliminary data across all sections showed that, combined, particulate matter concentrations of PM_{1.0}, PM_{2.5}, and PM₁₀ were significantly higher in the morning than in the afternoon. Correlating with the increased activity performed in each section, such as grooming, washing, blow drying, and handling of pets. The mean concentrations of the morning sessions for the particulate matter numbers counted for PM_{1.0}, PM_{2.5}, and PM₁₀ were 7038.81 ± 12,089.85 /m³ (mean ± SD), 1060.17 ± 1915.30 /m³ (mean ± SD), and 78.50 ± 124.1 /m³ (mean ± SD), respectively. The afternoon levels significantly dropped to 1141.3 ± 1086.42 /m³ (mean ± SD), 260.39 ± 317.72 /m³ (mean ± SD), and 78.31 ± 251.06 /m³ (mean ± SD), respectively. The total mass concentration and individual levels of PM_{1.0}, PM_{2.5}, and PM₁₀ showed a different trend of data from the particulate counter from the morning concentrations were lower which were 0.326 ± 0.166 mg/m³ (mean ± SD), 0.274 ± 0.136 mg/m³ (mean ± SD), 0.285 ± 0.135 mg/m³ (mean ± SD), respectively. The total mass concentration and individual levels of PM_{1.0}, PM_{2.5}, and PM₁₀ increased in the afternoon, coinciding with a reduction in activity, and the levels were 2.954 ± 37.012 mg/m³ (mean ± SD), 0.805 ± 7.875 mg/m³ (mean ± SD), 67.079 ± 981.82 mg/m³ (mean ± SD), and 0.625 ± 4.897 mg/m³ (mean ± SD). Ammonia levels varied between the morning and the afternoon. The levels of the morning were 0.321 ± 0.333 ppb (mean ± SD) and 0.457 ± 0.221 ppb (mean ± SD). Although particulate number concentrations (PM_{1.0}, PM_{2.5}, PM₁₀) were substantially higher in the morning, consistent with intensive grooming and handling activities that generate large quantities of fine, respirable aerosols, the total mass concentrations demonstrated an opposite trend. Mass values increased in the afternoon despite lower particle counts, suggesting fewer but significantly larger particles resuspended from accumulated dander, fur, and dust as animal traffic decreased and surfaces dried. The data indicate that workers are exposed to high fine-particle loads during active operations and elevated coarse-particle mass during periods of lower activity. **Conclusions:** Preliminary data showed that PM concentrations were significantly higher in the morning during increased activity and in certain areas where those involved in longer animal-handling activities (grooming and washing) were exposed to higher levels of particulate matter. To mitigate such exposures, workers should consider using respiratory protection in those sections. All this information will be valuable for identifying specific tasks that increase workers' exposure to fine particles and ammonia in veterinary clinics.

PS 3961 Wildland-urban interface fires: toxic physicochemical properties of emitted particulate matter and impacts on lung macrophages

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Background and Purpose: Due to growth of urban areas in close proximity to wildlands, wildfires increasingly burn both biomass and man-made materials. The physicochemical properties and hazards of particulate matter (PM) from such 'wildland-urban interface (WUI)' fires may differ substantially from those of pure wildland fire PM. However, the properties and hazards of WUI fire PM have not been studied. **Methods:** We employed our wildfire simulator (WiFS) to reproduce biomass and WUI fires by combusting pinewood and a 1:1 mixture of pinewood and polyethylene, respectively. The generated PM were size fractionated to obtain PM_{0.1} fractions, designated PM_B (biomass) and PM_W (WUI), which were comprehensively characterized and their impacts on the health and function of THP-1 macrophages evaluated. THP macrophages were exposed for 24 h to PM_B or PM_W at doses equivalent to 0.5, 5, and 50 day inhalation exposures at 375 µg/m³. **Results:** PM_W contained more than twice the concentration of toxic high molecular weight polyaromatic hydrocarbons (PAHs) as PM_B, as well as significant amounts of highly bioactive oxygenated PAHs,

which were absent in PM_B. Although both PM_B and PM_W caused significant cytotoxicity, PM_W produced greater reductions in viability and mitochondrial membrane potential than PM_B. Moreover, whereas PM_W decreased phagocytosis of 1 µm beads by ~50%, PM_B had no effect. In addition, PMW exposure caused dysregulation of 803 genes, whereas PM_B caused dysregulation of only 3 genes. **Conclusions:** These findings underscore the heightened toxicological risk associated with wildfire-urban interface particulate matter, revealing that exposure to PM_W can impair macrophage viability, mitochondrial function, and innate immune responses more severely than pure biomass wildfire particulate matter. Because macrophages represent the first line of defense against inhaled pathogens and particles, the observed impairment of THP-1 macrophage phagocytosis following exposure to PM_W (but not PM_B) suggest that WUI PM exposure could increase susceptibility to or severity of respiratory infections. Together these results raise important concerns about human health impacts from inhalation of PM generated during wildfires, particularly in regions affected by mixed biomass and plastic combustion, and highlight the need for further research into the mechanisms of toxicity and long-term health consequences of such complex environmental exposures.

PS 3962 Prenatal exposure to ultrafine particulate matter dysregulates offspring lung function and blunts IL-13 responses in asthma-like model

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Background and Purpose: Fine particulate matter (PM_{2.5}) and ultrafine particles (UFPs ≤100 nm) are major public health concerns due to their ability to reach deep into the lung airways, systemic distribution, and association with cardiopulmonary diseases. While numerous studies link PM exposure to asthma exacerbation and respiratory morbidity, the developmental impact of prenatal UFP exposure on later allergic airway responses remains poorly understood. Fetal development represents a critical window of susceptibility, during which inhaled toxicants can alter immune and respiratory programming. Using the LDLR^{-/-} mouse model, which exhibits airway hyperreactivity and Th2-biased responses, this study investigated whether prenatal UFP exposure alters postnatal pulmonary function and cytokine regulation in response to an asthma-like challenge. Specifically, IL-13 expression was evaluated as a key Th2 mediator of airway constriction and remodeling. **Methods:** Pregnant LDLR^{-/-} mice were exposed for 19 days to either filtered air or UFPs (3.74e⁴ particles/cm³) throughout gestation using a versatile aerosol concentration enrichment system. Offspring (F1) were evaluated at postnatal day 28 (PND 28) using whole-body plethysmography (vivoFlow®, Scireq, Canada) to assess baseline respiratory parameters and methacholine (0-50 mg/mL) induced airway responsiveness. Afterward, offspring were re-exposed to either air or UFP for two months. A subset from each prenatal exposure group was sensitized to ovalbumin (OVA) to induce an asthma-like phenotype. Pulmonary function was reassessed following methacholine challenge, and lung tissue was collected for IL-13 quantification using an ELISA immunoassay and lung histology analysis. **Results:** Prenatal exposure to UFPs resulted in significant alterations in offspring respiratory function following methacholine challenge compared with air-exposed controls. UFP-exposed offspring exhibited higher enhanced pause (Penh; 2.47 vs. 1.3, *p* = 0.0046) and longer inspiratory time (75.7 vs. 63.6 msec, *p* = 0.003), accompanied by a reduced breathing frequency (321 vs. 365 breaths/min, *p* = 0.018). Similar trends were observed after re-exposure and ovalbumin (OVA) sensitization, where both UFP-only and UFP + OVA groups showed greater functional impairment than the Air + OVA controls. Lung IL-13 levels were significantly decreased in UFP-exposed and UFP + OVA offspring, suggesting suppressed Th2-related cytokine expression associated with prenatal and postnatal UFP exposure. **Conclusions:** These findings indicate that prenatal exposure to ultrafine particulate matter disrupts normal respiratory regulation, leading to altered breathing patterns and reduced pulmonary function following airway challenge. The observed decrease in lung IL-13 protein levels in UFP-exposed and UFP + OVA offspring suggests that prenatal exposure may suppress Th2-related cytokine activity, potentially dampening immune mechanisms that support epithelial repair and airway tissue integrity.

PS 3963 Particulate matter containing environmentally persistent free radicals (EPFRs) induced oxidative stress responses in lung organoids exposed at the air-liquid interface

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Background and Purpose: The thermal remediation of hazardous wastes can lead to the release of particulate matter (PM). During these treatment processes, aromatic hydrocarbons chemisorb onto metal oxide surfaces, leading to the formation of surface-stabilized environmentally persistent free radicals (EPFRs). These PM containing EPFRs are capable of continuous redox cycling, generating reactive oxygen species (ROS) that drive oxidative stress, inflammation, and subsequent epithelial injury in the lung following inhalation exposure. Recently, new approach methodologies (NAMs) are being recognized as key complementary approaches to provide advanced platforms for *in vitro* inhalation toxicology. These NAMs include 3D lung organoids and air-liquid-interface (ALI) exposures. Since airway epithelial

cells and macrophages act as the first defensive barrier to inhaled toxicants, initiating upon injury oxidative and inflammatory signaling, ideally *in vitro* studies replicating inhalation exposures would include these two cell types in a same model. Therefore, this study was designed to test whether EPFR exposures, conducted under submerged or pseudo-ALI conditions, would activate in a similar manner the Nrf2-Keap1-p38 MAPK antioxidant and inflammatory pathway in a lung organoid model. **Methods:** A lung organoid model was established in Matrigel extracellular matrix, which supports spheroid formation and promotes relevant epithelial-macrophage interactions. Lung alveolar type 2 epithelial (AT2) cells (A549 cells) and phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1-derived macrophages, were embedded together. Organoids were exposed to EPFRs under submerged (50 µg/cm²) or pseudo-ALI conditions (ALI Cloud system, Vitrocell) (EPFR nebulized suspension 35 mg/mL) for 24 hours. Gene expression (*NRF2*, *KEAP1*, *NQO1*, *HMOX1*, *AHR*, *p38-MAPK*, *CD14*, *CD68*) was quantified by qPCR. Extracellular oxidative stress and cytotoxicity markers [ROS, nitric oxide (NO), lactate dehydrogenase (LDH)] were measured in culture media. Organoid organization and spatial distribution of AT2 cells and macrophages were confirmed using CellTracker fluorescence confocal imaging. **Results:** Deposited dose of EPFRs at the ALI (42 µg/cm²) was similar to that of the submerged condition (50 µg/cm²). Macrophage activation markers were elevated, including CD14 and CD68, which expression increased by ~1.5-fold (*p*<0.05) in both conditions, indicating differentiation of monocytes to macrophages in the lung organoid. Confocal fluorescence imaging confirmed the successful co-localization of AT2 cells and macrophages within lung organoids, indicating that both cell types were maintained together in the culture system. EPFR exposure activated oxidative and inflammatory signaling in the AT2-macrophage lung organoids. Gene expression analysis showed upregulation of *NRF2*, *KEAP1*, *HMOX1*, *NQO1*, *AHR*, and *p38-MAPK* in both submerged and pseudo-ALI conditions (*p*<0.05), indicating activation of the oxidative stress and xenobiotic response pathways. Notably, under pseudo-ALI exposure, *HMOX1* increased by ~1.8-fold (*p*<0.05) and *KEAP1* increased by ~1.7-fold (*p*<0.05), reflecting stronger engagement of the Nrf2-Keap1 antioxidant axis compared to submerged exposures. These results suggest that EPFR deposition at the surface of the organoids triggers enhanced oxidative damage. Consistent with these transcriptional patterns, extracellular injury markers were significantly elevated under both submerged and ALI conditions (NO, ROS, and LDH; *p*<0.05). **Conclusions:** EPFR exposures activated oxidative and inflammatory signaling in lung organoids under both submerged and pseudo-ALI conditions, primarily through the Nrf2-Keap1-p38 MAPK pathway. This novel human-relevant *in vitro* lung organoid model, with exposures at the ALI, provides a mechanistic platform to study EPFR-induced oxidative injury and inflammation, offering a biologically relevant complementary tool to *in vivo* inhalation models.

PS 3964 Activation of AhR and NF-κB Pathways Links Wildfire Smoke Exposure to Lymphoma Development

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Background and Purpose: Exposure to wildfire smoke (WFS) has been linked to cancer development in animal models and associated with elevated risks of non-Hodgkin's lymphoma (NHL) and multiple myeloma risk in epidemiological studies. We previously demonstrated that both vehicular emissions and WFS activate the aryl hydrocarbon receptor (AhR). Our recent work further shows that particulate matter (PM) and ash collected from active California wildfires contain polycyclic aromatic hydrocarbons (PAHs), dioxin-like compounds, pathogens, and metals that activate both AhR and NF-κB signaling pathways. **Methods:** Building on earlier findings that activation of AhR by dioxin promotes lymphomagenesis in mice, we investigated whether WFS exposure might similarly drive B-cell malignancies. **Results:** Mice exposed to WFS PM exhibited enlarged cervical and axillary lymph nodes, as detected by PET/CT imaging. In contrast, control animals showed no evidence of tumor development at the same time point. At necropsy, cervical, axillary, popliteal, and inguinal lymph nodes from exposed and control mice were removed. Cervical and inguinal lymph nodes were enlarged in exposed mice (~4X size of control mice), due to marked expansion of the cortex. Supported by immunohistochemistry analysis, we found that enlarged lymph nodes from WFS exposed mice stained strongly with Pax5 supporting hyper expansion and B-cell proliferation. To further characterize these changes, we performed *in situ* single-cell RNA sequencing (scRNA-seq) on post-exposure lymph nodes. Gene set enrichment analysis (GSEA) identified transcriptional patterns consistent with early B-cell transformation which was consistent with the GSEA done on *in vitro* WFS PM-transformed primary B cells. *In situ* spatial expression analysis revealed hyper expansion of CD19+ B cells and a significant increase in proliferation. **Conclusions:** Together, these findings suggest that WFS exposure induces B-cell proliferation and transformation, potentially initiating lymphomagenesis mediated through activation of AhR and NF-κB pathways.

PS 3965 Effects of Acute Copper Ultrafine Particle Inhalation in APP/PS1 Mice

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Background and Purpose: Ambient particulate matter (PM) air pollution is a ubiquitous environmental health concern associated with respiratory, cardiovascular, and neurological disorders, including Alzheimer's disease (AD). Epidemiological studies suggest that exposure to redox-active transition metals, such as copper (Cu), may exacerbate neurodegenerative processes through inflammatory or oxidative mechanisms. Cu-containing ultrafine particles (Cu-UFPs, <100 nm) model the reactive metal fraction of ambient ultrafine PM and deposit in the alveolar region of the lung, where high surface reactivity may promote local and systemic effects. The purpose of this study was to evaluate acute pulmonary inflammation and biodistribution of Cu following short-term Cu-UFP inhalation in an AD-relevant mouse model. This work addressed the hypothesis that inhaled Cu-UFPs translocate from the lungs to distal organs, eliciting inflammatory and oxidative responses that may contribute to AD-related pathology. **Methods:** Male and female APP/PS1 transgenic and wild-type (WT) mice were exposed via whole-body inhalation to filtered air or Cu-UFP aerosols (mass concentration: 196 ± 11 µg/m³ (standard error, SE) number concentration: 8.05x10⁶ ± 1.35x10⁵ particles/cm³; count median diameter: 38.8 ± 1.6 nm; geometric standard deviation: 1.7 ± 0.03) for four hours per day over three consecutive days. Bronchoalveolar lavage (BAL) fluid and tissues were collected 24 hours after the final exposure. BAL samples were analyzed for total and differential cell counts, viability, total protein content, and lactate dehydrogenase (LDH) activity to assess pulmonary inflammation and cytotoxicity. Cu tissue burdens in BAL fluid, BAL cell pellets, and major organs (lung, liver, kidney, spleen, and brain) were quantified via inductively-coupled plasma mass spectrometry. **Results:** Cu-UFP exposure induced mild but statistically significant pulmonary inflammation, indicated by elevations in BAL neutrophils (8.1 ± 2.6% vs. 0.14 ± 0.05% in air-exposed mice) and protein content (0.21 ± 0.01 mg/mL vs. 0.18 ± 0.01 mg/mL in air-exposed mice) relative to air controls, with no significant changes in LDH activity or total BAL cell counts. Cu concentrations were significantly elevated in BAL fluid (48.9 ± 2.7 ng/g vs. 31.1 ± 4.1 ng/g in air-exposed mice) and cell pellets (25.4 ± 3.5 ng/g vs. 13.4 ± 2.2 ng/g in air-exposed mice) of Cu-exposed mice, confirming particle deposition and partial dissolution in the lung. Cu concentrations in extrapulmonary organs did not differ significantly between exposure groups, suggesting limited systemic translocation within 24 hours post-exposure. Independent of exposure, APP/PS1 mice exhibited significantly lower Cu concentrations in the frontal cortex (4.98 ± 0.58 mg/g vs. 7.41 ± 0.33 mg/g in WT mice). **Conclusions:** Acute Cu-UFP inhalation produced modest pulmonary inflammation without substantial Cu accumulation in distal tissues. These findings may suggest that the high solubility of Cu-UFPs promotes effective pulmonary clearance and limits acute systemic distribution. Transient lung inflammation may still represent an initiating event in the propagation of inflammatory and oxidative signaling to the brain during repeated exposures. These findings identify the lung as an important interface for metal-containing UFP toxicity and support a mechanistic link between reactive metal PM exposure and neurodegenerative disease risk through pulmonary inflammatory pathways.

PS 3966 Diesel exhaust particulates disrupt cardiometabolic and endocrine signaling associated with altered vascular AhR/TLR-4/Notch pathways and inflammation in female C57BL/6 mice.

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Background and Purpose: Air pollution exposure has been linked to increased risk of cardiometabolic disease, obesity, and vascular dysfunction. Epidemiological and animal studies indicate that females are more susceptible to particulate matter (PM)-induced outcomes, yet the mechanisms driving these effects remain poorly defined. We hypothesized that diesel exhaust particulate (DEP) exposure induces systemic endotoxemia and endothelial activation through inflammatory and xenobiotic-sensing pathways, leading to hormonal disruption and vascular dysfunction that are further amplified by a high-fat diet (HF). Following exposures, we analyzed vascular endpoints, including the toll-like receptor (TLR)-4, aryl hydrocarbon receptor (AhR), angiotensin II type 1 receptor (AT1), as well as inflammatory signaling via tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β. **Methods:** Female C57BL/6 mice (4-6 weeks old) were fed either a low-fat (LF) or HF diet and exposed via oropharyngeal aspiration to 35 µg DEP suspended in saline or saline alone (CON) twice weekly for 30 days. Circulating lipopolysaccharide (LPS) was quantified by chromogenic ELISA. Aortic gene expression of TLR-4, AhR, AT1R, TNF-α, and TGF-β was assessed by RT-qPCR. Endothelial activation and Notch signaling were evaluated by immunofluorescent co-localization of von Willebrand factor (VWF) and HES1. Circulating metabolic and sex hormones were quantified by multiplex immunoassay. **Results:** DEP exposure, particularly when combined with a HF diet, disrupted systemic and vascular homeostasis. Circulating LPS was markedly elevated by HF diet (*p* < 0.0001), indicating diet-driven endotoxemia modulated by DEP exposure. Aortic TLR-4 and AhR expression increased with DEP, consistent with inflammatory and xenobiotic-sensing activation. AT1R expression was significantly influenced by diet (*p* = 0.04), and TGF-β increased with DEP exposure (*p* = 0.008),

supporting vascular remodeling. VWF/HES1 co-localization was enhanced in HF + DEP groups, suggesting Notch-mediated endothelial activation. Metabolic multiplex analysis revealed broad endocrine disruption. Glucagon (diet $p = 0.0002$; exposure $p = 0.0008$), GIP ($p = 0.04$), GLP-1 (diet $p = 0.0002$; exposure $p = 0.0055$), leptin (exposure $p = 0.0061$), pancreatic polypeptide (PP) ($p = 0.0078$), and PYY ($p = 0.0024$) were significantly altered, reflecting impaired metabolic hormone regulation. Sex hormone analyses showed elevated progesterone ($p = 0.0018$) and disrupted estradiol signaling (diet $p = 0.0256$; interaction $p = 0.0053$). T3 increased with both diet ($p = 0.0043$) and exposure ($p = 0.0132$), while T4, insulin, and cortisol were unchanged. **Conclusions:** These findings demonstrate that DEP exposure may promote vascular inflammation via AhR/TLR-4/Notch activation, accompanied by systemic endotoxemia and endocrine dysregulation in female mice. High-fat diet exacerbates these effects, with coordinated alterations in glucagon, GLP-1, leptin, and estradiol signaling, suggesting a convergence of environmental and dietary stressors on vascular and metabolic pathways. Together, these results highlight sex-specific vulnerabilities to air pollution and provide mechanistic evidence linking particulate exposure to cardiometabolic disease risk. *Funded by NIEHS R00ES016586 and R15ES026795 (A.K. Lund).*

PS 3967 Late Adolescent Exposure to Concentrated Ambient Fine Particulate Matter Air Pollution (PM_{2.5}) Increases Seminiferous Tubule Degeneration in Adult Mice, While Having no Effects on Testicular/Epididymal Sperm Counts

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Background and Purpose: Our recent work revealed that exposure of late adolescent female mice to concentrated ambient PM_{2.5} for significantly decreased primordial ovarian follicle numbers by about 30% and 36% in wildtype and late-onset Alzheimer's Disease (LOAD) mice, respectively. In male mice, our study showed that adolescent exposure to PM_{2.5} did not significantly decrease testicular spermatid or epididymal sperm counts in early or late adulthood. However, the effects of PM_{2.5} exposure on testicular seminiferous tubule degeneration are still unknown. Herein, we test a hypothesis that PM_{2.5} exposure during adolescence is a sensitive window for testicular germ cell damage in adulthood. **Methods:** Adolescent wildtype and LOAD male mice at 8 weeks of age were exposed to concentrated ambient PM_{2.5} for 5h/day, 4 days/wk for 12 weeks. Testes were harvested at 5 months (24 hours after last exposure, Experiment 1) or at 14 months (9 months after last exposure, Experiment 2) of age and fixed in Bouin's solution overnight, washed in 50% ethanol and stored in 70% ethanol until being embedded in glycolmethacrylate (GMA) resin. Testis blocks were sectioned at 20 μ m and stained with Periodic Acid-Schiff (PAS) for histopathological evaluation. The tubules/testis with abnormalities (multinucleated giant cells, Sertoli cell cytoplasmic vacuolation, and exfoliation of germ cells into tubular lumen) were graded. **Results:** The percentages of tubules with abnormalities were calculated for data analysis. Damage in seminiferous tubules were not significantly increased by 12-week exposure to PM_{2.5} at 5 months of age in either wildtype or LOAD mice (N=6/group). At 14 months of age, vacuolation was significantly increased by exposure to PM_{2.5} in LOAD mice. Multinucleated giant germ cells and exfoliation were not significantly affected by exposure to PM_{2.5} in either wildtype or LOAD mice. **Conclusions:** Our preliminary results show that exposure to concentrated ambient PM_{2.5} during adolescence increases damage in seminiferous tubules in late adulthood, while not significantly decreasing testicular or epididymal sperm counts.

PS 3968 Virtual Impactors as Tools for Increasing Concentration of Particulate Matter in Air Sampling to Improve Chemical Characterization for Assessing Exposure to Air Pollutants

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Background and Purpose: Air pollution is a major cause of chronic disease globally, responsible for more than 10 million disability-adjusted life years annually. Physico-chemical properties of air pollution, such as chemical composition, particle size, and concentrations of dust and other particulate matter (PM), can vary substantially depending on the sources of pollution and environmental conditions. Understanding the size-dependent composition of the PM in polluted air is vital for understanding its toxicological impact on respiratory health; however, in many environments, the particles of interest are too dilute for size-fractionation and chemical analysis. Our overarching goal is to develop and optimize tools to facilitate such research. **Methods:** Recently, our group has developed a high-flow-rate virtual impactor (VI) concentrator system can be used to concentrate air particles in a wider size ranges than the previously designed VIs (less than one order of magnitude in diameter). Our system concentrates particles from an intake flow of $>1000 \text{ L min}^{-1}$ to a highly concentrated sample flow of $<10 \text{ L min}^{-1}$, and it enables concentration increases for particles ranging in size from 300 nanometers to 10 micrometers. In this study, we tested and validated this system using aerodynamic particle samplers (APS) to calculate concentration factors. **Results:** The particle number density of air from a low particle concentration environment was measured using the APS with and without the VI concentrator. A concentration factor was calculated for each particle size in the APS sampling range, sub 500 nanometers to 19 micrometers. The VI system was able

to generate a highly concentrated sample in excess of 500 times the concentration without the VI system. The system also showed a dynamic range from below one micrometer up to ten micrometers in particle size. **Conclusions:** The highly concentrated sample flow that was generated using the VI concentrators demonstrated the ability to rapidly collected and measure particles even in low concentration environments. This provides an opportunity to use these tools in with other instruments like Anderson impactors to separate particles based on size. These VI systems will allow for more in-depth analysis of air pollutant particulate matter even in low concentration environments.

PS 3969 Early biomarkers of metabolic dysfunction-associated steatotic liver disease in juvenile mice exposed to diesel exhaust particles

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Background and Purpose: Metabolic dysfunction-associated steatotic liver disease (MASLD), previously known as non-alcoholic fatty liver disease, is the most common chronic liver disease among children and adolescents. If untreated, MASLD can progress to metabolic dysfunction-associated steatohepatitis (MASH), MASH with fibrosis and cirrhosis. Although childhood obesity is often associated with MASLD, there is a gap in knowledge about the genetic and environmental factors that contribute to the onset and progression of this liver disease. Air pollutants, such as particulate matter (PM), may play a role in the development of MASLD by inducing metabolic alterations, oxidative stress and inflammatory responses in the liver. Therefore, the objective of this study was to identify early molecular changes in the liver of juvenile mice exposed to diesel exhaust particles (DEP). **Methods:** Female and male C57BL/6J mice (n=8/group) were exposed intranasally with increasing concentrations of DEP during postnatal days 25 to 33. Mice were euthanized six days after the last exposure and major organs were dissected. The median lobe of the liver was used for metabolomics profiling and the right lobe for Attenuated Total Reflectance Fourier-Transform Infrared (ATR-FTIR) spectroscopy, lipid peroxidation (TBARS assay) and protein carbonyl (FTC assay) analyses. **Results:** A total of 53 metabolites were identified and the main groups classified as amino acids (n=19), short-chain acids (n=14), unsaturated fatty acids (n=5) and saturated fatty acids (n=4). At the lowest DEP concentration, a significant increase in 4-hydroxyproline was observed when compared to controls. In addition, the levels of glyoxylic acid, a reactive intermediate of 4-hydroxyproline catabolism, had a dose-dependent increase. In contrast, serine levels significantly decreased in a dose-dependent manner. Significant changes in the FTIR spectra were also observed, with 9 of 11 prominent peaks in the lipid, amide I, amide II and nucleic acid regions having a significant decrease in peak intensity in mice exposed to DEP when compared to controls. However, there were no changes in lipid peroxidation or protein carbonyl levels. **Conclusions:** Overall, the results suggest that exposure to DEP during postnatal development can alter early biomarkers of liver dysfunction. Epidemiological studies have reported that amino acid metabolism is altered in adolescents with MASLD. Serine deficiency has been associated with MASLD and 4-hydroxyproline has been associated with metabolic disorders and fibrinogenesis. Therefore, exposure to DEP during childhood might contribute to the onset of chronic liver disease.

PS 3970 Impact of short-term inhalation of Great Salt Lake Dust Particles (GSLD PM_{2.5}) on Dravet Syndrome pathophysiology

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Background and Purpose: Harsh climatic perturbations and the resultant increase in air pollution levels are a global public health emergency. The water level in Great Salt Lake, Utah, USA, is at record low levels, which increases the risk of exposure to dried lakebed sediments. Great Salt Lake Dust (GSLD) particle (2.5-micron size, PM_{2.5}) contains a mixture of metals, lipopolysaccharides, natural/anthropogenic chemicals, and bacteria. A landmark study demonstrated the adverse effects of GSLD exposure, including oxidative stress and inflammation in the lungs. Neurological disorders associated with central breathing disruption, like epilepsy, multiple sclerosis, and Parkinson's, may present a unique risk associated with respiratory pollutants. Dravet Syndrome (DS) is a catastrophic, childhood epilepsy caused by mutations in the *Scn1a* gene that encodes a voltage-gated sodium channel subunit (NaV_{1.1}). Clinical features of DS include drug-resistant hyperthermia-induced and spontaneous seizures, cardiorespiratory issues, and a high mortality rate due to sudden unexpected death in epilepsy (SUDEP). Repeated seizure-spread to the brainstem that controls vital autonomic functions can lead to central apnea, respiratory distress, and cardiac dysfunction, and has been shown to be a leading cause of SUDEP. Hence, the DS population is at high risk to environmental toxicant exposure-mediated adverse effects owing to their predisposition to cardiorespiratory issues and high rate of mortality. No study thus far has evaluated the impact of exposure to GSLD PM_{2.5} on the DS population. Our goal was to evaluate the effects of short-term GSLD PM_{2.5} exposure on lung function and temperature threshold for hyperthermia-induced seizures in DS mice that contain a knock-in missense mutation in the *Scn1a* gene. **Methods:** Male and female heterozygous (HET, *Scn1a*^{A1783V/WT}) DS, and age-matched

wildtype (WT) mice were exposed to GSLD PM_{2.5} by inhalation (5mg/m³ dose) using the inExpose system (Scireq) for 2 hours every day for 4 consecutive days. The instrument was set to a feed rate of 15mm/hr, and the RPM of the brush at 1200. A group of HET and WT mice were exposed to room air. This inhalation paradigm was followed to model low and slow short-term human exposure to peak concentrations of GSLD PM_{2.5}. N=5/group. On the 4th day after exposure, lung function was assessed in mice by whole-body plethysmography. On day 5 (24h post-exposure), the threshold for hyperthermia-induced seizures was evaluated in HET mice exposed to air or GSLD PM_{2.5}. **Results:** *Scn1a*^{A1783V/WT} HET mice exposed to air had significantly ($p < 0.0001$) lower tidal volume (TV), minute ventilation (MV), and breathing frequency (f) rates compared to air-exposed WT mice. WT mice exposed to GSLD PM_{2.5} exhibited significantly ($p < 0.0001$) lower TV and MV compared to air-exposed WT mice. Interestingly, exposure to GSLD PM_{2.5} significantly decreased ($p < 0.05$) breathing frequency in HET mice compared to air-exposed HET mice. TV and MV were unaltered in HET mice exposed to GSLD PM_{2.5}. The temperature threshold for hyperthermia-induced seizures was not significantly different between HETs exposed to GSLD PM_{2.5} and HETs exposed to air. However, there was a strong trend towards a decrease in the temperature threshold and time to seize in GSLD PM_{2.5}-exposed HETs compared to air-exposed HETs. **Conclusions:** DS mice exhibit altered baseline lung function parameters compared to age-matched WT. Short-term, low-dose GSLD PM_{2.5} exposure adversely impacts breathing frequency and possibly the temperature threshold for hyperthermia-induced seizures in HETs. Ongoing studies are evaluating the effects of acute, high-dose GSLD PM_{2.5} exposure on DS mice.

PS 3971 *TET1* Protects Against Diesel Exhaust Particle (DEP) - Induced Airway Inflammation and Dysfunction

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Background and Purpose: Air pollution, particularly diesel exhaust particles (DEP), has been shown to exacerbate asthma severity. DEP exposure promotes oxidative stress and airway inflammation and is strongly associated with Th17-mediated immune responses, which increase the risk of severe and persistent asthma. However, the molecular mechanisms of air pollution-induced asthma, and specifically the involvement of epigenome-modifying proteins such as *TET1* (Tet Methylcytosine Dioxygenase 1), remains largely unknown. **Methods:** In this study, we investigated the role of *TET1* in DEP-induced airway inflammation and dysfunction, as well as evaluated therapeutic interventions targeting *TET1*-related pathways. *TET1* was knocked down in human bronchial epithelial cells (HBECS) via siRNA, and the cells were subsequently treated with DEP (10 µg/cm²). RNA-seq was used to examine transcriptomic changes. Additionally, *Tet1*-deficient mice (*Tet1*^{-/-}; HET) and wildtype controls (*Tet1*^{+/+}; WT) were used in a murine DEP exposure model (intratracheal challenge of 150 µg DEP × 9 treatments over 3 weeks) to assess the effect of *Tet1* deficiency on airway hyperresponsiveness, lung pathology, immune cell counts and cytokine levels in bronchial alveolar lavage fluid (BALF), and lung tissue mRNA expression. We also sought to examine potential therapeutic interventions to mitigate the effects of *TET1* loss in both *in vitro* and *in vivo* DEP exposure models. Three potential interventions were selected to target different mechanistic pathways: Dimethyl-2-ketoglutarate (DMKG) - supports *TET1* enzymatic activity, CH223191 - an aryl hydrocarbon receptor (AhR) antagonist, and SS-31 (Elamipretide) - a synthetic mitochondria-protective peptide. **Results:** Transcriptomic analysis of HBECS revealed that both *TET1* knockdown and DEP exposure significantly altered gene expression, with the most pronounced changes in the combined *TET1* knockdown+DEP group (KDDEP vs DEP: 3,983 DEGs). Ingenuity Pathway Analysis identified multiple DEP-related pathways, including the Nrf2 oxidative stress response and cytokine signaling (IL-17, IL-8). The aryl hydrocarbon receptor (AhR) pathway was particularly disrupted during DEP exposure in *TET1*-deficient cells: pro-inflammatory mediators (*IL-1a*, *IL-1b*, *GSTP1*) were upregulated, while AhR cofactor (*ARN1*) and downstream detoxification genes (*CYP1A1*, *CYP1B1*, *ALDHs*) were suppressed. These changes suggest that *TET1* loss enhances inflammation while impairing xenobiotic detoxification responses. In the murine DEP exposure model, *Tet1*-deficient mice exhibited exacerbated lung inflammation and airway hyperresponsiveness (AHR) compared to wildtype controls. Bronchoalveolar lavage fluid (BALF) analysis showed elevated neutrophils and total immune cells, with increased expression of neutrophil-recruiting chemokines (CXCL5, CXCL15) and Th17 cytokines (*IL17a*, *IL17f*) in the combined HET+DEP group. Airway hyperresponsiveness strongly correlated with *IL17a* expression, supporting a role for Th17 responses in the functional phenotype. In HBECS, the three potential therapeutic interventions (DMAKG, CH223191, SS-31) demonstrated varied results in reducing cytokine production, with some treatments leading to more pronounced reductions in certain cytokines than others. *IL-1b* was most consistently reduced by all three treatments. **Conclusions:** These findings indicate a protective role of *TET1* in DEP-induced airway inflammation and dysfunction, acting through AhR and cytokine signaling pathways. Furthermore, while individual interventions may offer partial benefits, a combination approach targeting multiple pathways may be required for effective mitigation of pollution-induced airway disease pathogenesis.

PS 3972 Distribution and Deposition of Wildfire Smoke Chemicals into Brain and Olfactory Bulb of Rats after Intranasal Installation

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Background and Purpose: Epidemiological and experimental studies suggest wildfire smoke is a potential contributor to neurological dysfunction, accelerating onset of neurodegenerative diseases such as Alzheimer's Disease (AD), Parkinson's Disease (PD) and dementia. In a mouse model, wildfire smoke exposure induced neuroinflammation, characterized by proinflammatory phenotypes of microglia and peripheral immune cells in the brain. The protein pathologies used to diagnose AD and PD are positively associated with neuroinflammation. Using doses comparable to those encountered during intense wildfire events (200-300 µg/m³), we explore the absorption, distribution, metabolism, and elimination (ADME) and pharmacokinetics of representative members of major chemical classes (acid, phenol, PAH, aldehyde) in inhaled wood smoke condensates to determine components that reach the brain and examine the propensity of the compounds to cross the blood-brain-barrier. **Methods:** Male Sprague Dawley rats (n=7-10) were intranasally instilled with 50 µg/kg of ¹⁴C-palmitic acid (PA), ¹⁴C-benzo[a]pyrene (B[a]P), ¹⁴C-catechol (CAT) or 43 ng/kg ¹⁴C-benzaldehyde (BZ) reconstituted in 1mg/mL of smoldering eucalyptus woodsmoke extract (WSE). Controls received equal volume of saline. Serum was collected from 5 min to 2 weeks after dose and tissues were collected at 0.5, 2, 4, 24 h and 2 weeks after dose. Urine was collected from the 24 h exposure. Brain, olfactory bulb, esophagus, lung, liver, kidney, heart, spleen, and stomach were collected, rinsed in PBS and analyzed by accelerator mass spectrometry (AMS) for excess ¹⁴C. Aliquots of urine were counted using liquid scintillation and analyzed by reversed-phase HPLC to determine the presence of metabolites or breakdown products. **Results:** PA and B[a]P entered circulation slowly, reached maximum concentration (C_{max}) near 15 ng/mL at 2 h, and had circulating concentrations near 1/3 C_{max} 24 h after exposure. CAT and BZ rapidly entered circulation and were mostly cleared at 2 h. Excess ¹⁴C from all four chemicals was detected in olfactory bulb and brain over the first 24 h but only PA (or its metabolites) was retained in olfactory bulb, brain and kidney at 2 weeks post dose. All excess ¹⁴C was cleared from the lung at 2 weeks. Metabolite analysis of plasma (CAT) and urine (CAT, BZ and B(a)P) dosed samples did not detect any parent compound. **Conclusions:** Catechol and benzaldehyde were rapidly cleared from the biological tissues. The slower uptake and clearance of PA or B(a)P or their reactive metabolites when dosed with WSE in brain and olfactory bulb potentially provide greater opportunity for inflammatory response. This study suggests that wildfire smoke chemicals can enter the brain through two primary pathways; directly from the nasal cavity to the olfactory bulb, and via systemic circulation. This work performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344. LLNL-ABS-2013898.

PS 3973 Biochemical and Histopathological Evaluation of long-term exposure of copper oxide nanoparticles in Wister Rats

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Background and Purpose: Copper oxides nanoparticles (CuO NPs) are widely used for a variety of industrial and life science applications. This widespread use, however, has increased the risk of short-term and long-term toxicity to human health and the environment. **Methods:** The current study investigates the hepatotoxic effects of copper oxide nanoparticles in male Wister rats. Three groups of six male rats were orally administered CuO-NPs, once a day for 28 days with doses of 50, 100 and 200 mg/Kg, body weight. The control group was also made up of six rats. Blood and liver samples were collected 24 hours after the last treatment following standard protocols. Biomarkers of hepatotoxicity, such as Alanine aminotransferases (ALT), aspartate aminotransferases (AST), alkaline phosphatases (ALP), lactate dehydrogenase (LDH) in the serum sample, and oxidative stress assessment of reduced glutathione (GSH), along with histopathological evaluation of liver injury, were evaluated. **Results:** The investigated results revealed a statistically significant ($P < 0.05$) elevated levels of ALT, AST, ALP and LDH in the serum and a significant decline in GSH in the liver tissue of treated rats in a dose-dependent manner relative to the control group. Ultra structural assessment of liver showed significant morphological changes in accordance with biochemical parameters. **Conclusions:** The results of this study demonstrate that CuO-NP is hepatotoxic, and its toxicity may be mediated through oxidative stress. This was a preliminary study, and more mechanistic elucidation is needed before to use CuO-NPs widely in biomedical applications.

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Background and Purpose: Gadolinium-based contrast agents (GBCAs) are widely used in clinical MRI but provide weak MRI signal and pose risks such as nephrogenic systemic fibrosis and tissue accumulation. Manganese oxide (MnO) and iron oxide (Fe_3O_4) nanoparticles have emerged as promising alternatives due to their stronger magnetic properties and biodegradability. However, the impact of contrast agent design on off-target interactions with the innate immune system, particularly neutrophils and macrophages, remains underexplored. Macrophages play a central role in nanoparticle clearance and immune modulation, while neutrophils can release neutrophil extracellular traps (NETs)—web-like DNA structures that contribute to inflammation and thrombosis—when stimulated by certain nanomaterials. The goal of our study is to investigate how the metal content and surface coating of metal oxide nanoparticles modulates off-target interactions with macrophages and neutrophils. **Methods:** MnO and Fe_3O_4 nanocrystals were synthesized using thermal decomposition of Mn(II) acetylacetonate and Fe(III) acetylacetonate in dibenzyl ether and oleylamine. Metal oxide nanocrystals were encapsulated with fluorescent Cy5-dye, biodegradable poly(lactic-co-glycolic acid) (PLGA) and increasing amounts of PLGA-polyethylene glycol (PEG) including 0%, 2.5%, and 5%. To determine base polymer interactions with the cells, blank nanoparticles, with no metal oxide, were also synthesized. A murine macrophage cell line, RAW 264.7, and neutrophils isolated from the bone marrow of healthy female BALB/c mice femurs via magnetic bead separation were utilized for this study. T_1 -weighted and T_2 -weighted MRI at 1 Tesla was used to assess signal intensity in macrophages and neutrophils exposed to contrast agents for 1h including Gadavist as a clinical comparator against blank nanoparticles, Nano-Encapsulated Manganese Oxide (NEMO) particles, and Nano-Encapsulated Iron Oxide (NEIO) particles. Flow cytometry quantified nanoparticle uptake, intracellular ROS generation (via CellROX), and immune activation (CD80 for macrophages, CD11b for neutrophils) after 1h incubation. **Results:** MRI analysis revealed that Gadavist produced strong T_1 signal in both macrophages ($\% \Delta R_1 = 71\%$) and neutrophils ($\% \Delta R_1 = 160\%$). In contrast, NEMO particles generated lower signal intensities in both cell types ($\% \Delta R_1 = 40\%$), which decreased with increasing PEGylation. Blank nanoparticles had negligible immune cell MRI signal. T_2 -MRI studies are ongoing. Flow cytometry confirmed that PEGylation reduced nanoparticle uptake in both cell types, with 5% PEG formulations exhibiting the lowest internalization (<8% cell uptake). ROS generation and immune activation did not decrease uniformly with PEGylation. Neutrophils exhibited higher ROS levels than macrophages across most formulations. Overall, immune activation from contrast agent exposure was low (<16%). Macrophages showed higher activation (CD80 expression) than neutrophils (CD11b) for NEMO particles, which agreed with their greater nanoparticle uptake. **Conclusions:** While PEGylation consistently reduced MRI signal and cellular uptake, its effects on ROS generation and activation were variable and cell-type dependent. Macrophages had more activation and nanoparticle uptake compared to neutrophils, but neutrophils generated much higher intracellular ROS levels from contrast agent exposure. These results highlight the importance of evaluating multiple immune parameters when designing MRI contrast agents. PEGylated MnO nanoparticles, particularly at 5% PEG, remain promising candidates for safer imaging.

PS 3975 A House Dust Mite Allergen Corona on Multi-Walled Carbon Nanotubes Amplifies Pro-Inflammatory Responses of Murine Alveolar Macrophages *in vitro*

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Background and Purpose: Inhaled particulate air pollution is a major factor that exacerbates allergic asthma in humans. Advancements in nanotechnology world-wide have increasingly raised concerns over the toxicity of inhaled engineered nanomaterials and their potential to exacerbate the pathogenesis of allergic lung disease after occupational or environmental exposure. We previously reported that house dust mite (HDM) allergens adsorb to multi-walled carbon nanotubes (MWCNTs) to form an allergen corona that intensified lung inflammation in mice *in vivo*. In the current study, we utilized an *in vitro* cell culture model with murine alveolar macrophages to investigate possible mechanisms involved in the exacerbation of allergic lung disease by MWCNTs with HDM allergen coronas. **Methods:** Allergen coronas were prepared in a cell-free system by co-incubating MWCNTs (NC7000, Nanocyl Inc.) with HDM extract (Greer Laboratories, Inc.), followed by sequential rinsing and centrifugation of the MWCNTs to remove free HDM proteins. The resulting allergen corona was characterized by gel electrophoresis, western blotting and proteomics. For the *in vitro* study, mixtures of MWCNTs and HDM extract or MWCNTs with HDM corona were used to treat the MH-S mouse alveolar macrophage cell line (ATCC). After 4 and 24 hours, RNA extracted from MH-S cells were used for detection of cytokine mRNAs by RT-PCR and RNA-sequencing. **Results:** Approximately 7% of proteins in the HDM extract adsorbed to MWCNTs, including Der p 2 and Der p 1, which are associated with asthma in humans. Isolated mRNA from treated MH-S alveolar macrophages demonstrated significant changes in pro-inflammatory and pro-fibrotic mediator gene expression, including *Cxcl2* and *Il-1 β* , when comparing the

co-exposure treatment group and/or allergen corona treatment group to the control treatments. Also, RNA-seq results demonstrated key inflammatory pathways (i.e. STAT3 and STAT1) being activated upon co-exposure and allergen corona treatments. RNA-sequencing was completed using Qiagen IPA. **Conclusions:** The exacerbation of pro-inflammatory cytokine secretion in alveolar macrophages exposed to MWCNTs and HDM extract is enhanced via the allergen corona. The formation of nanoparticle biocoronas can be hazardous to individuals exposed via inhalation, and the intrinsic property of these nanoparticles provides new insight to the mechanisms through which inhaled nanoparticles exacerbate allergic asthma in humans.

PS 3976 Characterization of remaining titanium dioxide in Peyer's patches of rats after 90-day subchronic oral exposure

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Background and Purpose: Titanium dioxide (TiO_2) is widely used as a whitening and opacifying agent in pharmaceuticals, foods, and personal-care products. In 2021, the European Food Safety Authority (EFSA) concluded that TiO_2 containing more than 50% of nanoparticles (NPs; <100 nm in at least one dimension) can no longer be considered safe as a food additive, leading to a ban on its use in food in the EU from 2022. In a 90-day subchronic oral toxicity study of TiO_2 with a 6 nm crystallite size, we observed no adverse effects in male and female F344 rats (Akagi et al., *Part. Fibre. Toxicol.* 2023, 20; 23). Histopathological examination revealed deposition of TiO_2 agglomerates in Peyer's patches (PPs) of the small intestine, indicating incorporation of orally exposed TiO_2 into gut-associated lymphoid tissue. A subsequent study using TiO_2 particles with crystallite sizes of 6, 30, and 180 nm showed that deposition of TiO_2 agglomerates in PPs occurred regardless of crystallite size. Based on these findings, we investigated the post-treatment fate of TiO_2 agglomerates deposited in PPs at 8 weeks after the cessation of administration. **Methods:** Nano-grade (6 and 30 nm crystallite) and pigment-grade (180 nm crystallite) anatase TiO_2 were used as test materials. Dosing suspensions were prepared at 100 mg/mL using 0.2% disodium hydrogen phosphate as a dispersant. The test substances were orally administered to 6-week-old male F344/DuCrIj rats at a dose of 1,000 mg/kg bw/day for 90 days (10 animals per group). The control group received an equal amount of dispersant. At the end of dosing, five rats per group were necropsied, and the remaining five after an 8-week recovery period. TiO_2 particles in PPs were examined in hematoxylin-eosin-stained sections, and immunohistochemical analyses for immune-related factors, including CD68, TNF- α , and IFN- γ , as well as DNA damage marker γ -H2AX, were performed. **Results:** Administration of TiO_2 with median secondary particle sizes of 178.4, 362.1, and 252.3 nm and nanoparticle proportions of 27.2, 11.5, and 13.2% for the 6, 30, and 180 nm crystallites, respectively, did not cause treatment-related changes in mortality, clinical signs, body weights, or food consumption. Under microscopic observation, TiO_2 agglomerates were observed in PPs in all TiO_2 -treated groups, with occasional phagocytosis by macrophages but without associated inflammation or tissue injury. Notably, TiO_2 agglomerates remained detectable in all TiO_2 -administered groups after the 8-week recovery period. CD68⁺ cells contained TiO_2 particles, indicating macrophage phagocytosis of TiO_2 . In the recovery groups, focal accumulations of macrophages suggesting repeated phagocytosis were observed. No changes were observed in TNF- α , IFN- γ , or γ -H2AX staining, indicating the absence of excessive immune activation or inflammation-induced DNA damage. **Conclusions:** TiO_2 incorporated into PPs was still detectable after an 8-week recovery period. Although particle-laden macrophages were observed, no immune activation, inflammation, or DNA damage was detected. These findings suggest that TiO_2 remaining in PPs after 8 weeks of the cessation of administration was biologically inert.

PS 3977 Electrostatic vs. Covalent Doxorubicin Conjugation: Optimizing Chitosan Nanoparticles for Receptor-Mediated Cancer Cell Targeting.

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Background and Purpose: Nanotechnology represents a dynamic area in biomedicine with transformative potential, especially in cancer biology, for diagnostics and targeted therapeutics. Among the various nanocarriers, chitosan-based nanoparticles (CSNPs) stand out for their biocompatibility and biodegradability. However, their application has been limited by low solubility, premature drug leakage, and poor stability. As such, this study aims to improve the therapeutic potential of CSNPs by conjugating doxorubicin (DOX) either electrostatically or covalently, focusing on comparing treatments based on folate receptor targeting in HeLa and HepG2 cancer cell lines. **Methods:** We synthesized and characterized DOX-CSNPs using X-ray diffraction (XRD) and dynamic light scattering (DLS). Next, we conducted cytotoxic studies on HeLa and HepG2 cells using electrostatically loaded DOX-CSNPs. We are currently working on enhancing drug specificity by adding folic acid into the structure and covalently attaching DOX and folic acid to CSNPs. Then, we plan to further study drug release kinetics via the dialysis bag method, followed by comparative cytotoxic and mechanistic studies between the two cell lines. **Results:** Our

DLS characterization data showed that DOX-CSNPs had an average size of 18-23 nm. The Zeta potential values showed that the DOX-CSNPs that were also loaded with polyvinyl acid (PVA) were positively charged (+15 mV). The XRD measurements confirmed the semi-crystalline nature of the CSNPs having a low to moderate degree of crystallinity. Furthermore, preliminary cytotoxic studies revealed that DOX-CSNPs exhibited higher anticancer activity than free DOX, with similar effects in both cell lines. **Conclusions:** Our study aims to overcome limitations of CSNPs through covalent conjugation and folate modification to improve receptor specific targeting and pH-responsive drug release. These preliminary findings highlight the potential of CSNPs as a tunable platform for cancer therapeutics.

PS 3978 Folic acid tagged and carbon quantum dot loaded liposome for targeted drug delivery of doxorubicin to HeLa cervical cancer cells

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Background and Purpose: Current cancer treatments are nonspecific, evoking side effects for many patients. While many routes for targeting cancer cells exist, using cancer specific biomarkers like the folate receptor (FAR) in HeLa cervical cancer cells is a promising route. Creating a folate tagged drug delivery system will enhance cervical cancer targeting, increasing chemotherapeutic payoff and minimizing harrowing side effects. Encapsulating fluorescent, non-toxic, carbon quantum dots and doxorubicin in a liposome structure tagged with folic acid, allows for targeted drug delivery of an otherwise poorly soluble chemotherapeutic and real time imaging. **Methods:** Carbon quantum dots (CQDs) were synthesized using an autoclave. The subsequent CQDs were then characterized using DLS, zetapotential, UV-Vis, FTIR, Fluorescence, XPS, and TEM. The CQDs were tested for toxicity using the HeLa cervical cancer cell model and a XTT viability assay. Biocompatible liposomes were creating using hydrogenated soybean phosphatidylcholine (HSPC), cholesterol (CHOL), DSPE-PEG (2000)-Malate, and DSPE-PEG (2000)-Folate. One liposome included all lipids, and the other was constructed without the DSPE-PEG (2000)-Folate. Using active loading methods, the carbon quantum dots were encapsulated by the liposomes. Subsequent active loading will be carried out to incorporate doxorubicin and further viability assays will confirm cell specific targeting. **Results:** The synthesized carbon quantum dots exhibit high fluorescent yield in the red-light range, have an overall charge of -15.4 mV, and have an average size of 4 nm. The bare CQDs show no cytotoxicity towards HeLa cells. HSPC-CHOL-DSPE-PEG (2000)-Malate liposomes have an average diameter of 143 nm. **Conclusions:** Carbon quantum dots are a nontoxic, highly fluorescent nanoparticle with potential to be used as a bioimaging probe to track drug delivery. Liposomes comprised of HSPC-CHOL-DSPE-PEG(2000)-Malate were successfully formed. Further studies will focus on finalizing the drug delivery system and testing its efficacy in HeLa cells.

PS 3979 Pulmonary mTORC1 Disruption Mediates Metabolic Syndrome Associated Susceptibility to Nanoparticle Exposure by Regulating Lipid Signaling, Autophagy and Insulin Sensitivity

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Background and Purpose: Individuals with metabolic syndrome (MetS) exhibit exacerbated inflammatory responses following inhalation of particulates. MetS and other metabolic diseases cause disruption in mTORC1 signaling. mTORC1 activation mediates lipogenesis, inhibits autophagy, and causes insulin resistance. The presence of MetS may exacerbate nanoparticle (NP) exposure induced inflammatory responses and toxicity, via mTORC1 dysregulation. The potential role of increased susceptibility in individuals with MetS via mTORC1 dysregulation has not yet been elucidated. Macrophages are key components of immune system and acts as primary deposition site for inhaled NP. Macrophage-specific knockout of Raptor (mTORC1 component) will allow us to assess the contribution of mTORC1 signaling to the pulmonary response induced by inhaled NPs. Previously MetS mice with dysregulated lipid signaling has demonstrated exacerbated pro-inflammatory response marked by an influx of neutrophils and alterations in inflammatory mediators corresponding with increased production of inflammatory cytokines/chemokines on NP exposure. Additionally, cell culture studies have demonstrated upregulation of mTORC1 mediated pathways following NP exposure. Hence, we hypothesize that NP exposure may exacerbate MetS associated dysregulation of mTORC1, enhancing its susceptibility via inflammation, reduced autophagy, and insulin resistance. **Methods:** To investigate the impact of NP exposure on mTORC1 mediated lipid signaling and its susceptibility to inflammation, we utilized C57BL6/J mice with myeloid-derived cells (macrophages) lacking the mTORC1 specific component, Raptor, to examine mTORC1 dependent disruption of lipogenesis, autophagy, and insulin signaling. This model is produced by breeding Raptor^{fl} mice with LysM^{Cre} mice. An exploratory pilot study was performed to generate preliminary insights regarding mTORC1 mediated toxicity following exposures. In the primary study mice at 6 weeks of age were fed either a control diet or a high-fat western diet for 14-weeks. This generates 4 distinct groups healthy wildtype, MetS wildtype, healthy with macrophages lacking Raptor and MetS with macrophages lacking Raptor. Mice were exposed to silver nanoparticles (AgNP) via oropharyngeal aspiration or water (control). Altered mTORC1

signaling mediating endpoints of autophagy, inflammation and insulin resistance were evaluated 24 hours after exposure to capture potential variations. Genotyping assessment was performed to confirm the knockout (KO) model in myeloid-derived cells in animals of 4-5 weeks old prior to full metabolic and molecular assessments. **Results:** Genotyping assessment demonstrated removal of Raptor gene expression in macrophages of KO mice compared to wildtype (WT) mice, validating the model. Raptor expression was further reduced in isolated macrophages relative to whole lung tissue, confirming cell-specific KO. Gene expression of inflammatory markers chemokine ligand-1 (CXCL1), macrophage inflammatory protein-2 (CXCL2), interleukin-6 (IL-6), interleukin-1 beta (IL-1β), monocyte chemoattractant protein-1 (CCL2) were upregulated in WT and KO mice exposed to AgNP compared to respective control mice, demonstrating the role of mTORC1 in the AgNP inflammatory response. Pulmonary lipids are currently being evaluated utilizing an MRM profiling approach demonstrating the effect of mTORC1 mediated lipid dysregulation and induction of pro-inflammatory lipid mediators (arachidonic acid, PGE2, 12-HETE, LTB4 etc) following AgNP exposure. Samples from the MetS mice are currently being examined to determine the contribution of mTORC1 to MetS susceptibility to pulmonary exposures. **Conclusions:** Overall, our pilot study supported the role for mTORC1 in modulating the inflammatory profile in response to NP exposure. MetS is expected to result in inhibited autophagy, increased inflammation, and elevated insulin resistance following exposure to AgNP. However, mice with macrophages lacking Raptor, and thus mTORC1 activity, are anticipated to show less modifications in these endpoints, underscoring mTORC1's contribution to NP toxicity. Together, this work will provide mechanistic insights into mTORC1 dependent pathways and their contribution to MetS related susceptibility to particulate exposures.

PS 3980 A review of the association of pulmonary health effects and inhalation exposure to carbon nanotubes

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Background and Purpose: CNTs are used in several products, including but not limited to gas and energy storage, electronics, sensors, optics, and as additives in various nanocomposite material, for their high electrical conductivity, optical transmission, and extremely high tensile strength properties. CNTs are cylindrical hollow tubes made of carbon with a diameter in the nanometer range (1-100 nm). Studies have demonstrated structure-activity relationship in the fiber pathogenicity pathway whereby the physical characteristics of the CNT structure facilitates deep lung access, biopersistence, pleural access and retention, and risk of chronic injury. The diameter of CNTs is the primary determining feature of lung deposition. The length of CNTs determines the degree of fiber clearance by macrophages from the site of deposition in the lung. Shorter fibers are phagocytosed and cleared more readily by macrophages, while longer fibers are prone to be biopersistent and resist clearance. Functionalization of CNTs enhances solubility, reactivity, and compatibility with various matrices, and the purity, size, length, diameter, surface chemistry, morphology, and dispersion methodology are all factors that can determine and alter the levels of toxicity potential following exposure to f-CNTs. A comprehensive literature search was conducted: (1) to evaluate the potential for development of pulmonary fibrosis with exposure to carbon nanotubes (CNTs) by inhalation; and (2) to identify health outcomes associated with exposure to functionalized-CNTs (f-CNTs) via inhalation exposure. **Methods:** The literature search was conducted in PubMed and ProQuest and resulted in over 4,000 results. Following duplicate removal, the results were screened using inclusion criteria established a priori, and assessed for study quality, in accordance with the Office of Health Assessment Translation Risk of Bias Rating Tool, prior to data extraction. Grey literature was also evaluated for authoritative assessments of CNTs. **Results:** Following literature screening and study quality assessment, 61 studies underwent full-text review and data extraction. Safe Work Australia, European Chemicals Agency, International Agency for Research on Cancer, U.S. Environmental Protection Agency, and the U.S. National Institute of Occupational Safety and Health evaluated the association of human health hazards and exposure to CNTs and concluded that there is a potential association with exposure to multi-walled CNTs (MWCNTs) and the development of fibrosis. No authoritative safety conclusions have been made on association of fibrosis with exposure to single-walled CNTs or carbon fibers, or on the association of health effects with exposure to f-CNTs. **Conclusions:** There was sufficient evidence in the public literature of an association of *in vivo* SWCNT and MWCNT exposure by inhalation or intratracheal administration with lung fibrosis, or precursors to the development of lung fibrosis, including collagen deposition, myofibroblast formation, and interstitial thickening. There was suggestive evidence that CNTs with smaller diameter and longer length are associated with severity of pulmonary toxicity, including fibrogenic effects, compared to shorter and thicker CNTs, which are cleared by macrophages. There was suggestive evidence that covalent functionalization reduces cytotoxicity by improving water solubility, promoting renal clearance and excretion through urine, and enhancing biocompatibility. In contrast, there is suggestive evidence that acid-f-CNTs may be more toxic than covalent f-CNTs.

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Background and Purpose: Pregnancy induces fundamental changes in maternal physiology and morphology to meet the energetic demands of both mother and offspring. By mid-gestation, for example, the human heart enlarges and alters ventricular contractility to accommodate increased blood volume and shifting vascular resistance. Prior studies show that maternal inhalation of nano-sized titanium dioxide (TiO₂)—a common industrial compound—disrupts cardiac mitochondrial function and REDOX balance in developing fetuses, with lasting effects into adulthood. However, little is known about how such exposures affect maternal cardiac adaptation during pregnancy. Here, we evaluated cardiac and mitochondrial function in pregnant mice and assessed whether gestational inhalation of nano-TiO₂ modifies typical pregnancy-induced changes. **Methods:** Beginning on gestation day 5, dams were exposed to either nano-TiO₂ (12 mg/m³) or sham control air for 6 hours/day across 6 non-consecutive days (gestation days 5-13). Age-matched non-pregnant controls were included for both exposure conditions. Echocardiogram of the maternal was conducted under light isoflurane anesthesia at GD14. On GD15 Maternal hearts were collected and snap frozen for mitochondrial analysis. **Results:** Echocardiographic analysis at gestation day 14 revealed significant pregnancy-induced changes in heart size, contractility, and performance metrics including ejection fraction, fractional shortening, and mitral valve flow. TiO₂ exposure disrupted these adaptations either by significantly modifying pregnancy effects (e.g., altered slopes between pregnant and non-pregnant groups) or by eliminating differences altogether (pregnant animals resembled non-pregnant controls). Similarly, at gestation day 15, mitochondrial complex I and IV activities were elevated during pregnancy, but this increase was mitigated following TiO₂ exposure. **Conclusions:** These findings suggest that the maternal heart and its mitochondria undergo coordinated remodeling during pregnancy to support the maternal-fetal unit, and that mid-gestation exposure to nano-TiO₂ can impair or negate these changes. Given the ubiquity of chemical exposures during pregnancy, such disruptions may compromise maternal capacity to meet the physiological and metabolic demands of gestation, potentially affecting health outcomes for both mother and offspring.

PS 3982 **Probabilistic estimation of daily total nitrosamine exposures generated from drinking water and dietary nitrate and nitrite sources.**

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Background and Purpose: Research has suggested that exposure to nitrosamines can occur through endogenous generation via reaction between nitrate/nitrite and amines within the stomach, resulting in a comparatively larger exposure to total nitrosamines than to levels found in the diet and/or pharmaceuticals. Given the wide range of potential nitrate/nitrite exposure sources (e.g., drinking water, processed meats), information on the distributions of intakes, presence in food items, and constituent conversion rates are needed to understand the absolute and relative contributions of nitrosamine exposure estimates. The objective herein was to develop a probabilistic model to estimate population level exposures of daily total nitrosamines generated (DTNG) due to endogenous production from drinking water and dietary nitrate/nitrite sources. **Methods:** First, NHANES dietary survey data (2017-March 2020) based on dietary recall interviews was collected and reviewed for use within the model. Second, a targeted literature search was conducted to identify distributions of nitrate/nitrite, amine, and vitamin C content within various food groups, which were aligned to the food items reported in the NHANES dietary data. For each reported intake period in the survey data, the daily amount of nitrate/nitrite/amines/vitamin C was calculated as an input into the model. Additional literature searches were conducted to understand the range of nitrate-to-nitrite conversion rates within the saliva as well as the conversion of nitrite to nitrosamines (i.e., nitrosatability rate), the latter of which is dependent on the pH and the presence of potential inhibitors. Where possible, variables were incorporated into the model as distributions, or else accounted for via the pH change. Lastly, the model accounted for nitrosamine elimination upon exiting the stomach, where nitrosamine generation is inhibited by pH change in the small intestine. Based on available data, a range of N-nitrosodimethylamine (NDMA) half-life values was used as a surrogate for the total nitrosamine elimination distribution in the model. Ultimately, the model was run for each NHANES survey individual with random sampling from distributions for the variables indicated above. Since the survey included time of day for water and food ingestion, the model provides a minute-by-minute estimate of nitrosamine generation throughout the day for each survey participant and an estimate of the DTNG. **Results:** The probabilistic model estimated that the median DTNG for the simulated population was approximately 0.085 ug/day (IQR: 0.0159 - 0.397). The 95th percentile of estimated DTNG was 3.69 ug/day while the maximum DTNG was over 1,000 ug/day. The wide variability observed was driven by heavy tails of the distribution with much higher total daily nitrosamine generation. The drinking water and diet ingestion intervals (especially when intervals were <3-4hrs) impacted the amount of nitrosamines present at any given time due to elimination not occurring until after transiting the full stomach. A portion of the population did not ingest any

drinking water during the day (~15%) or ingested drinking water without also ingesting an amine source resulting in no nitrosamine generation. Although the model indicates a wide variability in behaviors and generated nitrosamines across the population, a range of approximately two orders of magnitude was observed for most of the population. **Conclusions:** This model uses population dietary survey data and published information on food chemical constituents and nitrate/nitrite conversion relationships to estimate the DTNG that may occur following intake of nitrate/nitrite from drinking water and the diet. Notably, the model shows that 95% percent of the population generally falls within a relatively narrow range of DTNG and, while slightly lower, is within the range reported with previous models. Importantly, this more sophisticated model offers more granularity and accounts for the variability in a large number of the model parameters compared with previous estimates or *in vitro* analyses. Furthermore, future analyses that define the relative contributions of drinking water vs. dietary nitrate sources to the generation of nitrosamines may be useful for informing regulatory standards of nitrate/nitrite.

PS 3983 **Development of a human PBMC TXG-MAPr as a mechanistic framework to interpret toxicodynamic variability across the human population**

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Background and Purpose: Accurately characterizing interindividual variability in toxicodynamic responses across key toxicity-related pathways is essential to improve chemical safety assessment and refine toxicodynamic uncertainty factors (UFs). However, our understanding of such variability remains limited, which emphasizes the need for human-relevant models that capture mechanistic diversity in chemical responses. Freshly isolated peripheral blood mononuclear cells (PBMCs) provide a physiologically relevant *in vitro* platform to investigate these mechanisms across the human population, as they integrate immune and stress signaling responses. The goal of this work was to establish and demonstrate the PBMC TXG-MAPr as a translational resource to explore mechanisms underlying human toxicodynamic variability and to support future refinement of toxicodynamic UFs. **Methods:** PBMCs from five healthy male donors (20-30 years) were exposed for 24 h to 45 mechanistically diverse compounds covering multiple key stress response pathways, such as ER stress, oxidative stress, DNA damage and inflammation. High-throughput whole transcriptome analysis (TempO-Seq) was performed, followed by differential gene expression analysis with DESeq2. Weighted gene co-expression network analysis (WGCNA) identified 397 gene networks (modules) of co-regulated genes representing shared biological responses. Modules were annotated using curated pathway databases to capture a wide range of responses, including cellular stress response pathways, immune signaling, and cell-cycle processes. An interactive TXG-MAPr interface enables visualization of module activation and comparison of donor- and compound-specific responses. **Results:** The human PBMC TXG-MAPr organized compound-induced transcriptomic responses into defined modules capturing coordinated gene expression changes across biological pathways. Concentration-dependent activation patterns were observed across donors, with modules corresponding to DNA damage, ER stress, and inflammation responding to compounds with expected mechanisms, such as mitomycin C, tunicamycin and PHA, respectively. Inter-donor variability was modest for key toxicity-related pathways with consistently low technical variability, demonstrating network robustness, reproducibility and the capability to characterize inter-donor response heterogeneity. Integration with benchmark concentration estimates showed transcriptional perturbations at biologically relevant concentrations, comparable to cytotoxicity measurements. The network also highlights less characterized pathways and novel modules responsive to specific chemical perturbations. As a case study, we applied the PBMC TXG-MAPr to transcriptomic data from six additional donors, further demonstrating its utility for assessing inter-donor variability in stress pathway responses. **Conclusions:** The PBMC TXG-MAPr provides a mechanism-focused framework for interpreting transcriptomic responses in the context of human toxicodynamic variability. By integrating co-expression structure, pathway annotation, and donor-specific expression profiles, the PBMC TXG-MAPr enables intuitive visualization of stress response mechanisms, supports cross-study comparisons, and aids interpretation of pathway activation across donors and compounds. This platform can support human-relevant chemical risk assessment and provide a bridge between *in vitro* data and risk characterization in human populations. Supported by the TD-TRAQ project funded by the European Food Safety Authority (OC/EFS/SCER/2021/03).

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Background and Purpose: Accurately accounting for interindividual differences in toxicodynamic responses is essential to improve chemical safety testing and risk assessment. However, our current understanding of human variability in sensitivity to toxicity-related pathways remains limited. Characterizing this variability is therefore critical for deriving refined toxicodynamic uncertainty factors (UFs). To address this gap, freshly isolated, minimally manipulated peripheral blood mononuclear cells (PBMCs) from healthy volunteers were used and combined with high throughput imaging and transcriptomics to study population-level toxicodynamic variability. In this study, we specifically investigated whether sex influences cellular stress responses to support quantitative evaluation of toxicodynamic variability. **Methods:** PBMCs from 100 healthy young volunteers (50 White males, 50 White females; age 20-30 years) were exposed to ten compounds in a wide concentration range covering activation of key stress response pathways, including ER stress, DNA damage and inflammation. Functional readouts on cytotoxicity and mitotoxicity were assessed at 24 and 72 h using high content confocal imaging. For six donors, high throughput whole transcriptome analysis (TempO-Seq) was performed to evaluate pathway-specific transcriptional activation. Differential expression analysis (DESeq2), benchmark dose modeling (BMDEExpress), and pathway/module activity scoring (PBMC TXG-MAPr) were used to quantify donor- and compound-specific transcriptional responses. **Results:** Functional imaging of 100 donors revealed concentration-dependent effects on mitochondrial potential and cell death induction. While individual donors showed variability in both the onset concentration and magnitude of both cytotoxicity and mitotoxicity, the extent of variability was compound dependent activating specific key stress response pathways. Despite compound- and donor-specific differences, average responses of male and female donors were comparable, indicating similar overall sensitivity. These phenotypic effects were reflected at the transcriptional level in a subset of six donors, with increasing concentrations leading to progressive activation of stress response pathways, including consistent induction of DNA damage, ER stress and inflammatory signaling modules across donors. The magnitude and direction of pathway activation were largely comparable, indicating limited interindividual variability in pathway/network-level responses within this initial population subset. BMD accumulation analysis of transcriptomics data from six donors showed clear concentration-dependent increases in the number of genes with determined benchmark concentrations. Overall, these results indicate that stress response activation patterns showed some difference across these six donors, with males and females showing comparable responses, supporting use of this framework to study interindividual variability in larger populations. **Conclusions:** Integration of high-throughput transcriptomic profiling and functional phenotypic readouts with quantitative population-based modeling enhances mechanistic understanding of interindividual variability in stress response activation. These data enable derivation of data-driven toxicodynamic variability factors (TDVFs) for key events within defined adverse outcome pathways (AOPs), supporting quantitative, mechanism-informed risk assessment. High throughput transcriptomic analysis will be completed for the initial cohort of 100 young male and female donors, while the study is currently being extended to an additional 100 donors encompassing diverse age groups (20-30, 40-50, 60-70 years) and ethnic backgrounds (White, Asian, Black/African American) to characterize variability across a broader human population. Collectively, these efforts provide a framework for refining chemical risk assessment by integrating empirically derived human toxicodynamic variability beyond default uncertainty factors.

PS 3985 Risk Assessment of Flame Retardant and Plasticizer Exposures from Mattresses Considering Body Weight and Body Temperature Impact on Emission Rates

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Background and Purpose: Studies suggests that infants and children less than 3 years of age may be exposed to a diverse array of semi-volatile organic compounds, including flame retardants and plasticizers, among other chemicals from the covers and foam inserts of crib mattresses. These exposure levels may be higher than those measured during the product's production due to the added effects of the child's weight, movements, and body heat on the expulsion of these chemicals into the sleeping microenvironment. The purpose of our poster is to calculate potential exposures to 2 different chemical substances, one from each of the above categories, using concentrations from analytical studies, and determine if they exceed established regulatory exposure limits when considering the sleeping duration, inhalation rates, and body weights representative of a 6-month old and 3-year old child. **Methods:** Concentrations of chemicals off-gassing from mattresses were identified under conditions intended to simulate body weight and temperatures of a child. Two representative substances that have been reported to be present in mattresses

used for this assessment, butyl benzyl phthalate (BBP) which is a plasticizer and tris (2-chloroethyl) phosphate (TCEP) which is a flame retardant, were further evaluated. Using the concentration data, we conducted an exposure assessment to determine the exposures from sleeping on the mattress using information for inhalation, body weight, and sleeping duration from the exposure factors handbook. Exposures at the mean and 95th percentile were calculated using the maximum concentrations measured in the sleeping microenvironment (SME) air. The inhaled exposures were compared to no effect levels determined from toxicity studies of the most sensitive endpoint of toxicity. **Results:** For a 6-month-old and 3-year-old child, sleeping time, inhalation rates, and body weights were derived from the exposure factors handbook. For a 6-month-old child, mean and 95th percentile values, respectively, were approximately 13 hours and 18 hours for sleeping, 0.225 and 0.333 m³/hour for inhalation rates (IR), and 9.2 and 11.3 kg for body weight (BW). The mean and 95th percentile values, respectively, for the 3-year-old child were approximately 11 and 14 hours/day for sleeping time, 0.421 and 0.575 m³/hour for IR, and 18.6 and 26.2 kg for BW. The maximum concentrations of BBP and TCEP based on a simulated study to account for body weight and temperature were reported to be 76 ng/m³ and 44 ng/m³, respectively. Exposures were calculated based on the following equation: Exposure = (concentration ng/m³ x IR m³/hour x hours/d of sleep)/bw = ng/kg/d. For BBP, the mean and 95th percentile exposures were calculated to be 24.2 and 40.3 ng/kg bw/d for the 6-month-old and 19.5 and 23.3 ng/kg bw/d for the 3-year-old. For TCEP exposures calculated at the mean and 95th percentile were 14.0 and 23.3 ng/kg bw/d for the 6-month-old and 11.3 and 13.5 ng/kg bw/d for the 3-year-old. For BBP, the most sensitive endpoint was determined to be effects on development with a derived maximum allowable dose level of 1,200 micrograms/d corresponding to 20.69 microgram/kg bw/d (20,690 ng/kg bw/d). For TCEP, effects observed at the lowest dose levels were kidney and liver organ weight changes and associated hepatotoxic and nephrotoxic effects. A reference dose of 0.007 mg/kg bw/d (7,000 ng/kg bw/d), below which adverse effects are not expected to occur, was reported for TCEP. The margin of exposure (MOE) for BBP ranged from approximately 513 to 1059 and the MOE for TCEP ranged from 300 to 619. **Conclusions:** The current risk assessment indicated that potential exposures to BBP and TCEP emitted from a mattress under simulated conditions of body weight and body temperature were below dose levels that would be expected to cause adverse effects. However, while there are benefits to use of these chemicals, manufacturers should be aware that exposures to the chemicals could increase as a result of the temperature and body weight of children and, given that children up to the age of 3 years spend the majority of the day sleeping, need ensure that concentrations of these chemicals used in mattresses are as low as possible for their intended purpose.

PS 3986 Enhancing genotoxicity risk assessment through an *in silico* workflow

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Background and Purpose: (Quantitative) structure activity relationship ((Q)SAR) systems are widely used to predict genotoxicity throughout the development lifecycle of a chemical, from prioritisation to regulatory submission. Models for bacterial mutagenicity are considered sufficiently sensitive that regulators accept predictions *in lieu* of Ames data under ICH M7. In recent years, there has been a growing interest in the development of models for predicting other genotoxicity endpoints, particularly chromosome damage. The interest in understanding the genotoxic mechanism of chemicals and regulatory requirements in the agrochemical industry is driven by the recent publication of EFSA's guidance on the establishment of the residue definition for dietary risk assessment. These models are built on *in vitro* chromosome aberration and micronucleus test data, for which there is limited availability in the public domain and there are known concerns about false positive responses in historic data. As a result, predictions from these models are used as of weight of evidence alongside, rather than *in lieu* of, assay data in regulatory assessments. **Methods:** To address these challenges, the chromosome damage model within Sarah Nexus was previously developed to support the assessment of individual predictions, included supporting examples, references to study results, and *in vivo* data to facilitate expert review. The latest versions of Sarah Nexus and Derek Nexus, statistical and expert knowledge-based (Q)SAR systems produced by Lhasa Limited, were used together to generate consensus predictions for chromosome damage. The predictive performance was tested against data extracted from eChemPortal. Improved decision support was achieved through implementation of over 50 statements to support automatic review of different prediction scenarios. Furthermore, a new workflow has been implemented to combine consensus predictions for mutagenicity and chromosome damage to provide a full genotoxicity assessment to support regulatory submissions. **Results:** Consensus predictions are beneficial for regulatory assessments as they increase the sensitivity compared to individual models and provide confidence to make negative predictions where no prediction is provided by Derek. The sensitivity of Derek and Sarah against data from eChemPortal increased from 32% (Derek) and 46% (Sarah) to 64% when combined as a consensus model for chromosome aberration data. For micronucleus data, sensitivity increased from 28% (Derek) and 44% (Sarah) to 59% with the consensus approach. The negative predictivity where Derek provided no alert increased to ~70% if Sarah is also negative compared to ~40% or ~50% if Sarah is outside domain or positive, respectively. Case studies demonstrate how these predictions can be used for genotoxicity risk assessments of agrochemical metabolites. **Conclusions:** By leveraging both expert

and statistical models, Derek and Sarah provide robust genotoxicity predictions across endpoints, supporting evolving regulatory requirements and decision-making in diverse industries including agrochemicals. The new combined genotoxicity workflow, supported by automated expert review argumentation, facilitates efficient and consistent chemical safety assessments even for challenging endpoints such as chromosome damage.

PS 3987 Screening-Level Cumulative Health Risk Evaluation: Exposures to Acetamiprid, Clothianidin, Dinotefuran, Imidacloprid and Thiamethoxam residues from Non-Agricultural Uses

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Background and Purpose: Neonicotinoid insecticides are widely used to control sucking and chewing insects on crops and companion animals. Due to their systemic activity, they are extensively used for soil applications, seed treatments, and foliar treatments. All neonicotinoids share a key structural feature with both nicotine and acetylcholine enabling binding to and blocking nicotinic acetylcholine receptors (nAChRs) in the insect nervous system. Mammalian signs of exposure include incoordination, tremors, decreased activity, reduced body temperature and at sufficiently high doses, death; the developing nervous system is the most sensitive target. In 2024, California Assembly Bill 363 (AB 363) directed the Department of Pesticide Regulation (DPR) to evaluate the cumulative impact on human health from non-production, non-agricultural outdoor use of multiple neonicotinoid pesticides. This cumulative analysis focused on five neonicotinoids (acetamiprid, clothianidin, dinotefuran, imidacloprid, and thiamethoxam) and considered multiple exposure pathways relevant to the bystander population, including children. **Methods:** This analysis was limited to acute exposures, consistent with the US Environmental Protection Agency's (US EPA) designation of neonicotinoids as a candidate common mechanism group (cCMG) for neurotoxicity under acute conditions only. Three exposure scenarios were evaluated: (1) dietary exposure from consumption of fruits and nuts from non-production trees (e.g., trees grown in a residential settings), (2) post-application bystander exposure from contact with treated turf, and (3) multi-pathway exposure combining dietary and bystander routes. These exposure scenarios were consistent with the legislation and the scope of the human health risk and exposure assessments that were conducted in parallel. DPR employed the relative potency factor (RPF) approach recommended by US EPA to calculate toxic potencies for all five neonicotinoid pesticides using acetamiprid as the index chemical. RPFs are the ratio of doses that cause similar magnitude of toxic effects between the index chemical and each CMG compound. Points of Departure (PODs) for each neonicotinoid were selected based on acute neurotoxic effects that could result from disruption of the nAChRs, including decreased motor activity and startle response, tremors and reduced brain morphometry observed in acute neurotoxicity and developmental toxicity studies in animals. The RPFs were used to convert exposures for each CMG chemical into a single index chemical equivalent, which were then summed to calculate combined exposure. A modified Tier 1 approach was used for dietary, incidental oral, and dermal exposures. The multi-pathway analysis aggregated all exposure routes for children 1-2 years old as the most sensitive subgroup. Human health risks were calculated as margins of exposure (MOE), a ratio of the critical POD of the index chemical to the estimated cumulative human exposure, which were then compared to a target MOE of 100. **Results:** All calculated MOEs exceeded the target MOE of 100, indicating no acute health risks. The 95th percentile acute dietary exposure for children aged 1-2 years was 8.965 µg/kg/day, corresponding to an MOE of 1,115. The highest cumulative exposure in the multi-pathway scenario was 79.986 µg/kg/day, corresponding to an MOE of 125. **Conclusions:** This assessment incorporated several assumptions, including full co-exposure to multiple neonicotinoids, high-end residue values, and default uncertainty factors. Even under conservative assumptions, complete co-occurrence of residues, and maximum exposure scenarios, no acute cumulative risks were identified from the combined neonicotinoid exposures from outdoor non-agricultural uses.

PS 3988 A Risk Assessment of Ethylene Oxide Release from a Sterilization Facility: A Data-Driven Case Study on Community Exposure and Risk

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Background and Purpose: Ethylene oxide (EtO) is a widely used industrial sterilant and chemical intermediate that has been subject to increasing scrutiny in recent decades regarding its potential carcinogenicity. Historically, EtO has been released into the atmosphere through both stack emissions and fugitive leaks from sterilization facilities, hospitals, chemical plants, and storage tanks. Prior to the implementation of enhanced regulatory controls, some facilities released untreated EtO into the air, potentially resulting in localized areas with elevated concentrations. Epidemiological studies have identified two main cancers that may be associated with EtO exposure at some dose: breast cancer and lymphohematopoietic cancer (primarily lymphoid leukemia). Despite industrial emissions, ambient EtO levels are generally consistent across different environments. According to EPA monitor-

ing data, average EtO concentrations in rural (0.16 ppb), suburban (0.17 ppb), and urban (0.17 ppb) settings are remarkably similar. This suggests that, while industrial sources contribute to localized emissions, EtO emissions in urban areas do not appear to be sufficient to alter background concentrations. **Methods:** This study evaluated residential and occupational exposure to EtO in a valley surrounding two point-source emissions in proximity (~11 meters). Air samples were collected in residential and industrial areas. Eight locations, ranging from 100 to 1700 meters from the center of the point-sources in varying directions, had average concentrations between 0.290 and 3.212 µg/m³ (0.16 to 1.8 ppb) and peak levels reaching 26.4 µg/m³ (15 ppb). Exposure scenarios were developed based on daily activity patterns, long-term residency, and estimates derived from historical emissions data. **Results:** Under the most conservative assumptions, including 40 years of occupational exposure during the periods of highest recorded emissions around the facility, the maximum estimated lifetime exposure found was 591 ppm-days. When compared to epidemiological studies of EtO-exposed workers from similar facilities (studies used by the EPA and IARC in their cancer risk assessments), the highest cumulative exposures observed (13,500+ ppm-days) were at least 23 times higher than our maximum estimated lifetime exposure value (591 ppm-days). Importantly, these high-exposure groups showed no statistically significant cancer incidences, particularly for breast and lymphohematopoietic cancers. **Conclusions:** Epidemiological studies have not demonstrated a statistically significant increase in the incidence of breast or hematopoietic cancers within the evaluated exposure ranges. While some studies have noted possible associations, these do not establish causality. The available epidemiological evidence is insufficient to infer a causal relationship between EtO exposure and the development of these cancers, requiring reliance on unverified or substantial assumptions. A comparison of stack emission data from the sterilization facility evaluated in this assessment with data from other EtO-emitting facilities across the United States revealed that EtO releases are generally comparable and, in many cases, lower. This assessment encompasses exposures representative of a broader population of sterilization and chemical facilities. The findings suggest that both current and historical EtO emissions from a substantial portion of these facilities have not posed a measurable risk of cancer to nearby communities.

PS 3989 A Conceptual Framework Using Weight-of-Evidence Incorporating New Approach Methods for Risk Assessment

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Background and Purpose: Weight of Evidence (WoE) analysis supports hazard and risk assessment while reducing animal testing by supporting the integration of New Approach Methods (NAMs). A globally harmonized WoE framework, grounded in the OECD's key principles, can foster broader adoption of NAMs. This can be achieved by establishing best practices for implementing the core principles of problem formulation, data collection, evidence evaluation and weighing, and evidence integration and reporting. In practice, these principles must be flexible to fit into regulatory contexts and to support integrating various data types. For example, when working with *in vitro* data, it is important to emphasize assay validity, reliability, and reproducibility, whereas integrating read-across data requires a focus on structural similarity, consistency, and concordance. Therefore, WoE as a conceptual framework can only be applied as 'fit-for-purpose'. This case example applies a globally harmonized WoE framework to the human health evaluation of a chemical metabolite, implementing a NAMs approach to avoid unnecessary animal testing. **Methods:** Data collection included Threshold of Toxicological Concern (TTC) approach with QSAR analysis for Cramer classification and structural alerts (OECD Toolbox; DEREK, and SARAH); chemical analog identification (RDKFingerprint and Tanimoto) for ID of structural analogs; comprehensive field trial residue data; and dietary exposure modeling (DEEM). **Results:** QSAR modeling and structural analogs indicated no alerts for genotoxicity, and the structure did not indicate potential for *in vitro* or *ex vivo* bioactivity. The metabolite was classified as Cramer Class III, with dietary exposure at <1% of the TTC. **Conclusions:** Based on the WoE, the metabolite was not included in the residue definition, and no additional animal testing was required. This case study illustrates the successful application of a WoE framework to a common regulatory scenario, providing a solid basis for safety assessment while promoting NAMs. It demonstrates how systematic evidence collection, evaluation, weighing and integration can inform decisions, minimize animal testing, and uphold scientific rigor in pesticide evaluations.

PS 3990 Integrating Epidemiological Evidence for Dose-Response Assessment of Inorganic Arsenic-Induced Non-Cancer Effects

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Background and Purpose: As a naturally occurring element, inorganic arsenic (iAs) is widely distributed in the environment, resulting in continuous human exposure. Consequently, an extensive body of epidemiological studies has investigated the association between arsenic exposure and various adverse health outcomes, form-

ing the principal evidence underlying recent risk assessments. In addition to certain types of cancers in humans (e.g., urinary bladder and lung cancer), exposure to high doses of iAs can cause non-cancer effects such as diabetes, which has received increasing attention in risk assessment. Modeling epidemiological dose-response data is challenging due to the diverse response metrics reported across studies, uncertainty in exposure measurements, and heterogeneity among populations. However, directly utilizing epidemiological data in dose-response assessment provides an opportunity to quantitatively characterize human variability and replace the traditional empirical uncertainty factor used in reference dose (RfD) derivation. Therefore, the purpose of this study is to develop a dose-response modeling framework that integrates epidemiological data from different populations to derive toxicity values, demonstrated using iAs-induced diabetes as an example. **Methods:** A Bayesian hierarchical benchmark dose (BMD) modeling approach was employed for meta-regression to derive an integrated dose-response relationship for BMD estimation, with uncertainty quantification of population heterogeneity. This approach utilizes the Hill model for continuous response to characterize the dose-response relationship between daily iAs intake levels and the adjusted relative risk (RR) or odds ratio (OR) of diabetes reported in various individual populations. The Hill model, within the Bayesian hierarchical structure that accounts for variability in background exposure and response sensitivity, was fitted to the integrated dose-response data and used for point of departure (POD) calculation. Five high-quality epidemiological studies (in terms of appropriate study design, clear documentation of data collection and reporting, etc.) with adequate dose-response data were identified through a systematic review and used to demonstrate the modeling approach. The same approach was applied to the epidemiological dose-response data of iAs-induced diabetes selected in the U.S. EPA's IRIS risk assessment of iAs, for the purpose of comparison and demonstration of the approach's adaptability. **Results:** Preliminary dose-response assessment suggested a POD estimate of 1.87 $\mu\text{g/kg/day}$ when the BMR was set at 0.5%, indicating that a 0.5% increase in incidence rate in the U.S. population can be expected at the BMD level. Various BMR options (from 0.05% to 1%) were attempted, and the results showed a relatively flat dose-response relationship in the low-dose region, suggesting that a threshold causing diabetes in the target population may exist. A sensitivity analysis regarding the selection of the dose-response model was performed, and it suggested that the choice of a flexible dose-response model would not have a major impact on the POD estimation. On the other hand, the same modeling approach generated lower POD values (from 0.04 to 0.15 $\mu\text{g/kg/day}$ at the BMR level of 0.5%) when different combinations of the four epidemiological studies selected by the EPA were used. **Conclusions:** This study demonstrates that a Bayesian hierarchical dose-response modeling structure with a flexible Hill model is an adequate approach for modeling epidemiological dose-response data, with the benefit of quantitatively characterizing the uncertainty and variability in POD estimation. It is also important to note that the POD estimation is highly sensitive to the selection of epidemiological studies modeled, highlighting the necessity of using high-quality epidemiological studies and considering modes of action obtained from toxicological studies as supporting evidence.

PS 3991 Risk and Exposure Assessment of Non-agricultural Uses of Imidacloprid

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Background and Purpose: Imidacloprid is a systemic and contact neonicotinoid insecticide. It is one of the most widely used pesticides worldwide, applied to soil, foliage and animals to control insects on crops, pets, and turf. Imidacloprid's pesticidal action is attributed to its binding to insect nicotinic acetylcholine receptor (nAChR) causing overstimulation and paralysis. In mammals, signs include incoordination, tremors, decreased activity, reduced body temperature, and at sufficiently high doses, death. The developing nervous system is the most sensitive target. In this assessment, the California Department of Pesticide Regulation (DPR) evaluated the potential human health effects and risks from occupational and non-occupational exposure to imidacloprid in non-agricultural settings. These included (1) professional handlers in landscape, residential, and recreational settings; (2) use of home (consumer) products; (3) post-application exposures to non-applicators; and (4) dietary and aggregate exposures. **Methods:** The critical points of departure (PODs) for characterizing risk from all exposure routes were derived from laboratory studies in animals. Short-term, seasonal, and annual exposures for professional handlers were estimated using surrogate data from the Pesticide Handler Exposure Database. Short-term post-application exposures were calculated for residential, household, and recreational scenarios. Probabilistic dietary and drinking water exposures were calculated for the US population and sensitive subpopulations, including infants, children aged 1-2, and women of childbearing age (13-49 years) using the Dietary Exposure Evaluation Model, consumption data from the 2005-2010 National Health and Nutrition Examination Survey dietary survey, and residue data from federal and state monitoring databases. Margins of exposure (MOEs) were calculated as ratios of the critical PODs to the estimated human exposures for various exposure scenarios and durations. They were compared to the target value of 100, which is equal to the total combined uncertainty factor. **Results:** The acute POD of 5.5 mg/kg/day was based on changes in physical dimensions of certain brain structures (corpus callosum and caudate putamen) in rat pups seen in an oral developmental neurotoxicity (DNT) study. The subchronic and chronic POD was 1.0 mg/kg/day based

on altered negative geotaxis (a measure of neuromotor and vestibular function in rat pups) following gestational and lactational exposure in a separate DNT study. Non-agricultural handlers exhibited the highest short-term, seasonal, and annual exposures. The non-Hispanic-other subpopulation exhibited the highest acute dietary exposure (7.22 $\mu\text{g/kg/day}$). Children aged 1-2 years had the highest chronic dietary exposure (0.57 $\mu\text{g/kg/day}$). Calculated MOEs were compared to the target MOE of 100. **Conclusions:** Potential risks (i.e., calculated MOEs <100) were identified for 5 of the numerous scenarios evaluated. These included dermal and inhalation risks for professional handlers involved in mixing/loading for aerial and groundboom applications. The only non-occupational risk was for incidental oral exposures to children aged 1-2 years from treated turf (eating grass and hand-to-mouth activities). Neither acute nor chronic dietary and drinking water exposures alone present a risk to human health for any subpopulation. In addition, for all occupational and non-occupational scenarios, aggregation with dietary exposure did not increase risks, including those for all post-application exposure scenarios for children.

PS 3992 Coexisting with Toxic Legacies: Assessing Soil Metal Levels, Sources, and Health Risks Associated with Living Amid Contamination Within a Superfund Site

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Background and Purpose: The Ringwood Superfund site in New Jersey (NJ) presents a controversial case of environmental injustice shaped by the interplay of mining legacy, hazardous waste dumping, and weak regulatory oversight. Uncertainty remains over the effectiveness of cleanup actions and ongoing health risks to the Indigenous Ramapough Lunaape Turtle Clan (RLTC), who maintains cultural, subsistence, and residential ties to the land. This study aims to quantify current soil metal levels in community spaces outside designated cleanup areas, delineate potential pollution sources, and estimate exposure-related health risks to support continued dialogue and cooperative remediation among stakeholders. **Methods:** Soil samples ($n = 23$) were collected from five zones in and around the Superfund site, each selected for proximity to former dumping areas or frequency of visit. Samples underwent acid digestion and were analyzed via ion-coupled plasma-mass spectrometry (ICP-MS) for 14 heavy metals. Contamination levels were quantified using the Contamination Factor (CF) and Geo-accumulation Index (I_{geo}), comparing ICP-MS results to NJ background levels. Source apportionment leveraged three receptor modeling methods, Absolute Principal Component Scores-Multiple Linear Regression, UNMIX, and Non-negative Matrix Factorization, for a balanced assessment. Ranges of non-cancer (Hazard Index [HI]) and cancer (Total Cancer Risk [TCR]) risks from metal exposure via oral, inhalation, and dermal pathways were modeled for children and adults using Monte Carlo simulations. **Results:** Among tested metals, mean soil concentrations, in mg/kg, for arsenic (22.3), chromium (62.6), and lead (270) exceeded U.S. Environmental Protection Agency (EPA) residential screening levels of 0.68, 0.95, and 200 mg/kg, respectively. CF and I_{geo} values for several metals exceeded natural baselines, with some beyond thresholds attributable to variability in geochemical background, thus implicating substantial human influence. Notably, across all samples, CF values (unitless) of cadmium (11-28) and antimony (7-15) placed these elements in the highest contamination tier (CF > 6). Despite the moderating effects inherent to I_{geo} 's (unitless) adjustments for background variability, cadmium remained within "heavy" to "heavy to extreme" categories ($I_{geo} > 3$). Other metal contamination levels fluctuated across sample zones, indicating spatial variability in the composition and relative intensity of geogenic and anthropogenic outputs. All three receptor models resolved four comparable sources. Source 1, defined by naturally abundant copper, manganese, and zinc, represented natural soil metal content. Source 2, characterized by elevated iron and iron-loving arsenic, cobalt, and vanadium near an old mine pit, was linked to the site's iron-mining and ironworks legacy. Source 3, contributing beryllium, cadmium, chromium, and molybdenum, denoted a mixed anthropogenic source arising from fuel combustion and weathered car parts and municipal waste strewn across the site. Source 4 was identified as automotive paint sludge dumped at the site, based on concurrent elevated levels of its signature metals, lead and antimony, in samples near the dumping area and in outlying areas where hydrologic connectivity and topography suggest contaminant migration beyond Superfund boundaries. Monte Carlo simulations showed that exposure to the measured heavy metal levels resulted in HI (unitless) across all pathways averaging 2.11 and 0.470 for children and adults, and exceeding EPA safety threshold (HI = 1) in 59% and 8% of simulated exposure scenarios, respectively. TCR (unitless) was higher for adults, averaging 5.59×10^{-5} compared to 3.79×10^{-5} for children, with, respectively, 12% and 10% of scenarios exceeding the threshold of acceptable excess cancer risk (TCR = 10^{-4}). **Conclusions:** Persistent contamination at the Ringwood Superfund site was associated with increased health risks beyond EPA safety thresholds for the RLTC. Source apportionment analyses suggested major contributions from the dumping activities to the measured metal levels. These findings highlight the need for sustained and community-centered intervention to safeguard both public health and environmental justice.

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Background and Purpose: Clothing dyes are widely available for use by consumers. Aniline is a common chemical intermediate - and sometimes a contaminant - in clothing dyes. Cordin et al. (2021) reported that synthetic indigo dye contains up to 0.6% aniline. However, the potential health risks from use of at-home clothing dyes, including potential exposures to aniline, have not been thoroughly studied. California's Office of Environmental Health Hazard Assessment (OEHHA) classifies aniline as a carcinogen under Proposition (Prop) 65, and set a No Significant Risk Level (NSRL) of 100 µg/day. However, to our knowledge, no publicly-available quantitative model is available to assess dermal exposure to clothing dye ingredients and contaminants such as aniline, making compliance with the aniline NSRL difficult. As such, the purpose of this study was to develop a model to quantitatively estimate the exposure and risk of cancer resulting from potential dermal exposure to aniline under a typical at-home dyeing scenario using a synthetic indigo dye. **Methods:** Potential dermal exposure to aniline from use of synthetic indigo was calculated for home users using exposure parameters from publicly-available sources in a deterministic model. The EPA Swimmer Exposure Assessment Model (SWIMMODEL) Version 3.0 was adapted for use in the risk assessment. The SWIMMODEL is typically used to model swimmers' exposures to chemicals in pools, but can be adapted to reflect immersion of individual body parts in water as long as the exposure time, surface area of exposed skin, concentration of the chemical in water, and chemical-specific permeability coefficients are known. The NSRL of 100 µg/day for aniline reflects exposures over all exposure routes. Aniline is assumed to have high systemic absorption after ingestion, up to 100%. According to the Australian Industrial Chemicals Introduction Scheme (2023), up to 38% of a single dermal exposure to aniline may be absorbed through the skin. This information was used to derive the adjusted dermal NSRL of 263 µg/day. The daily dermal exposure estimate for aniline was compared to the dermal NSRL for aniline using a Margin of Safety (MOS) approach. Specifically, the MOS was calculated as: MOS = Dermal NSRL / Daily exposure. **Results:** Typical consumer use parameters were applied to the exposure model. Specifically, it was assumed that at-home clothing dye users handle synthetic indigo twice per year for 15 minutes per event. The number of uses per year was averaged to the number of uses per day to estimate average daily exposure. The model also assumed that a user immerses two full, ungloved hands into the dye solution during these events. The concentration of aniline in the dye solution was calculated based on the reported concentration of aniline in synthetic indigo, the amount of dye used per event, and the amount of solution in which the dye is dissolved. To assess the dermal permeation of aniline, its permeability coefficient (Kp) was calculated based on aniline's Log Kow and molecular weight. These inputs were then used to calculate a potential daily dermal exposure in µg/day. Home users of synthetic indigo were estimated to have an average daily exposure to aniline of 0.3 µg/day. This corresponded to an MOS of 890 for home users when compared to the adjusted dermal NSRL of 263 µg/day. A MOS greater than 1 suggests that risk of adverse effects is not anticipated. **Conclusions:** Through this assessment, a quantitative model for exposure and risk posed by aniline in synthetic indigo dyes was developed. This analysis suggests that adverse effects due to aniline following use of synthetic indigo dyes are not expected under typical home use scenarios at the concentration evaluated. Multiple conservative assumptions were incorporated into this assessment, including that a user's two hands are fully immersed in the dye solution during the entire handling period, and that the user does not wear gloves while handling dye. Further work in this area may include refinement of data regarding consumer use of clothing dyes, development of a probabilistic model to incorporate the variability and uncertainty in exposure parameters, and application of the model to other chemicals in consumer clothing dyes. Nonetheless, this model presents a valuable tool to quantify exposure and potential risk corresponding to synthetic indigo dye use among at-home users.

PS 3994 California Proposition 65 NSRL Derivation for Aflatoxins

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Background and Purpose: Aflatoxins are a group of mycotoxins produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. These fungi flourish under moist and warm environments and contaminate crops (corn, wheat, rice), and nuts, spices, coffee beans, and dried fruits (among other food types). Food contamination usually involves aflatoxins B1, B2, G1, and G2 (crops) and M1 (dairy products). Aflatoxins have the potential to cause acute illness and liver injury, and multiple epidemiological studies in Africa and Asia indicate that dietary aflatoxin exposure increases the risk of developing hepatocellular carcinoma (HCC). Aflatoxins have been listed on California's Proposition 65 List of Chemicals Known to Cause Cancer or Reproductive Toxicity ("Prop 65") since 1988 due to aflatoxin's carcinogenicity potential. The California Office of Environmental Health Hazard Assessment (OEHHA) has not established a safe harbor value for aflatoxin. OEHHA defines a safe harbor value as a level of exposure to a listed chemical (in units of µg/day) that does not require a Proposition 65 warning. For carcinogens on Prop 65, the safe harbor value is known as the No Significant Risk Level (NSRL). The current project derives a NSRL for aflatoxins that may be used as part of a safe harbor assessment to determine whether exposure to aflatoxin-contaminated food items have safe harbor from Proposition 65 labeling. **Methods:** As defined by OEHHA, a Proposition 65 NSRL is equivalent to an

exposure level that results in 1 excess cancer case in an exposed human population of 100,000, assuming lifetime exposure at the level in question. OEHHA risk assessment guidance outlined in 27 CCR § 25703 (Quantitative Risk Assessment) was followed to derive a NSRL for aflatoxins following a step-wise approach: 1) The aflatoxin NSRL assumes that there is no carcinogenic threshold. 2) The NSRL builds on a 0.4 µg/kg-bw/day Benchmark Dose Lower Confidence Limit (BMDL10</sub>) for aflatoxins calculated by the European Food Safety Authority Panel on Contaminants in the Food Chain (EFSA CONTAM Panel) using data from a 105 week dietary feeding study conducted by Wogan et al. (1974). The Wogan et al study identified increased HCC in male Fischer rats exposed to Aflatoxin B1 at dietary doses up to 100 µg/kg. Fischer rats are considered to be the most sensitive species among those studied, which adheres to OEHHA guidance. 3) A Human Equivalent Dose (HED) at the BMDL10 was derived through the application of a dosimetric scaling factor: $(BW_{animal}/BW_{human})^{1/4}$, as specified in OEHHA guidance. 4) A cancer slope factor (CSF) in units of (µg/kg/day)⁻¹ was estimated based on a 10% benchmark response (BMR) and HED. 5) Aflatoxin's NSRL was derived from the CSF and 1 in 100,000 (10⁻⁵) increased cancer risk, adjusted to a body weight of a 70 kg, which is also specified in OEHHA guidance. **Results:** A 0.108 µg/kg/day HED was calculated from the BMDL10</sub> of 0.4 µg/kg-bw/day multiplied by a by dosimetric adjustment factor calculated by dividing a 0.380 kg body weight for adult Fisher male rats by a 70 kg body weight for adults, taken to the one-fourth power. A CSF of 0.92 (µg/kg/day)⁻¹ was derived by dividing a 10% benchmark response by the 0.108 µg/kg/day HED. Finally, a NSRL of 0.00075 µg/day (equal to 0.75 ng/day) was derived by dividing the level of acceptable lifetime cancer risk (1 in 100,000) by the CSF and multiplying the resulting quotient by a 70 kg body weight. **Conclusions:** Aflatoxin's small NSRL value of 0.75 ng/day evidences the carcinogenic potency of aflatoxins, and can be incorporated into safe harbor evaluations for aflatoxin-contaminated food items marketed in California. In instances where daily aflatoxin exposure exceeds this NSRL, a manufacturer must warn consumers through a Proposition 65 warning.

PS 3995 Derivation of an NSRL for vinyl acetate.

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Background and Purpose: Vinyl acetate monomer (VAM) is a synthetic chemical building-block used in the production of copolymers. These polymers are in turn used as the basis for manufacture of many industrial and commercial products, such as adhesives, textiles, and plastics. The general population may be exposed to low levels via contaminated air, food, or water. Occupational exposure occurs via inhalation of contaminated air in the workplace. Vinyl acetate has been detected at hazardous waste sites; therefore, populations living near contaminated sites may have increased exposure via ambient air, groundwater contamination, or vapor intrusion, compared to the general population. There is limited information on health effects following exposure to VAM. Studies have suggested that respiratory tract, gastrointestinal effects, and developmental effects are the likely primary targets of toxicity. Studies in animals have suggested that VAM is carcinogenic with effects likely attributable to the rapid transformation to acetaldehyde and acetic acid. IARC has determined that VAM is possibly carcinogenic to humans, and in 2024, California's OEHHA proposed to add VAM to the Proposition 65 chemical list. With the impending Prop 65 labeling requirements for VAMs, set to take effect in 2026, we propose to derive No Significant Risk Levels (NSRLs) for VAM. **Methods:** We conducted a comprehensive literature review to identify studies of potential interest. Because Prop 65 listings are based on carcinogenic potential, we focused on cancer outcomes as the primary health effect of concern. We identified principal studies and endpoints that demonstrated dose-response. There is uncertainty inherent in guidance values. We attempted to minimize uncertainty where possible. We used EPA BMDS to model the incidence data and provide recommendations on viable models that appropriately fit the data. We then collected the underlying variable estimates and standard errors for the various parameters for each model included in the standard BMDS package (e.g., gamma, Hill, etc.). This allows us to utilize the distribution of the values underlying the parameter estimates rather than discrete values. Using Oracle's Crystal Ball software to perform a probabilistic analysis, we iterated more than 10,000 Monte Carlo simulations for each of the 54 different mathematical models that BMDS had fit to the dose-response data set. This forecasted 54 unique probabilistic distributions of the benchmark dose associated with a 10% increased risk, and also 54 unique probabilistic distributions of the lower 95th percentile point estimate value (BMDL) associated with a 10% increased risk. We then applied model-weighting using AIC to generate 6 weighted functions that considers all sources of data variability and removes model selection bias. The 6 model-weighted functions pertained to analysis of the data set for each combination of exposure route (oral, inhalation) and sex (males, females, combined). The 5th percentile point estimates for each of these 6 model-weighted probabilistic functions was then selected as the POD for the NSRL derivation. **Results:** Following a literature review, we identified principal studies and critical health effects for oral and inhalation routes of exposure. We identified Umeda et al. (2004) and Bogdanffy et al. (1994) as the critical studies for oral and inhalation route. VAM tends to have portal-of-entry effects, so the primary health outcome identified were squamous cell carcinoma of the oral cavity and nasal tumors in for oral and inhalation exposure, respectively. Rats are typically the more appropriate species for extrapolation to humans. Tumor incidence across the database were relatively low. We extracted the data for males and females and ran the incidence data through BMDS for males only, females only, and combined. Following a probabilistic approach to selection of PODs, we then derived candidate oral and inhalation

NSRL values for VAM based on target risk estimates of 1 in 100,000 considering typical daily human intake for a 70-year statistical lifetime. **Conclusions:** In anticipation of the Prop 65 listing of VAM, we present here a proposed probabilistic approach to derive candidate NSRLs for inhalation and oral exposure. The approach taken here utilizes multiple modeling approaches to consider the effect of uncertainty and remove selection bias. We will then compare these candidate NSRLs to recent exposure estimates from various consumer product categories to estimate margins of safety.

PS 3996 A Review of EPA's TSCA Risk Evaluation for Site-specific Occupational Risk Assessment of Formaldehyde

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Background and Purpose: EPA released the final risk evaluation under the Toxic Substances Control Act (TSCA) for formaldehyde in December 2024. EPA evaluated human health risks from formaldehyde exposure under 63 conditions of use (COUs) during manufacturing, processing, distribution, industrial, commercial, and consumer use, and disposal for workers, consumers, and general population. EPA identified unreasonable occupational risk for 50 COUs. Formaldehyde is widely used in several industries, and research and EPA TSCA identification of unreasonable occupational risk for 50 COUs leads to risk management which can include site-specific assessment. Because site-specific occupational exposure is expected to be unique and different from the exposure concentrations and parameters used in EPA's assessment, risk assessment based on site-specific concentrations and exposure conditions can provide site-specific risks and concentration limits to assist in risk management decisions regarding formaldehyde. **Methods:** A site-specific approach was used to evaluate potential formaldehyde risk for workers at an aerospace facility (Site). The following approach was used: Step 1 - Identification of Site-specific Uses and Occupational Exposure Scenario (OES) Categorization - Several site-specific uses were identified at the Site. These uses were grouped and categorized into corresponding EPA TSCA OES categories. Step 2 - Identification of Exposure Concentrations and Exposure Parameters - High end site-specific exposure concentrations and exposure parameters were identified for the Site. In addition, exposure concentrations, exposure parameters, and toxicity parameters used in EPA's TSCA evaluation were identified for Step 1 OES. Step 3 - Calculation of Site-specific Risks - Consistent with EPA's TSCA evaluation, site-specific noncancer risks were calculated for worker acute inhalation, acute dermal and chronic inhalation, and cancer risks were calculated for chronic inhalation exposure scenarios and compared with EPA's TSCA evaluation risks. **Results:** Site-specific uses were grouped into four applicable EPA OES for risk evaluation: 1. Industrial - Use of coatings, paints, adhesives, or sealants (non-spray applications). 2. Industrial - Use of coatings, paints, adhesives, or sealants (spray applications). 3. Processing - Incorporation into Article - Adhesives and Sealant Chemicals in Wood Product Manufacturing; Plastic Material and Resin Manufacturing (Including Structural and Fireworthy Aerospace Interiors); Construction (Including Roofing Materials); Paper Manufacturing. 4. Commercial - Laboratory use. Site-specific acute inhalation noncancer risk were above benchmark margin of exposure (MOE) of 3 for three OES (Industrial - Use of coatings, paints, adhesives, or sealants [non-spray applications]; Industrial - Use of coatings, paints, adhesives, or sealants [spray applications]; Processing - Incorporation into Article - Adhesives and Sealant Chemicals in Wood Product Manufacturing; Plastic Material and Resin Manufacturing [Including Structural and Fireworthy Aerospace Interiors]; Construction [Including Roofing Materials]; Paper Manufacturing) and below benchmark MOE of 3 for one OES (laboratory use). Site-specific acute dermal noncancer risk were below benchmark MOE of 10 for all four OES. Site-specific chronic inhalation cancer risks were below the target cancer risk of 1×10^{-4} for all four OES and below 1×10^{-6} for one OES (Industrial - Use of coatings, paints, adhesives, or sealants [spray applications]). However, site-specific chronic inhalation noncancer risks were above benchmark MOE of 3 for all four OES. **Conclusions:** The potential risks associated with site-specific uses of products containing formaldehyde were significantly lower than EPA TSCA risk assessment results. Site-specific exposure concentrations in formaldehyde formulations contributed to lowering the exposure and risk for workers by at least 65-fold for dermal exposure and by at least 10-fold for inhalation exposure versus EPA TSCA risks. Site-specific exposure parameters for workers contributed to lowering the exposure and risk by at least an additional 30-fold versus EPA's TSCA risks. Therefore, site-specific human health risk assessment results for formaldehyde exposure can significantly aid in risk management decisions.

PS 3997 Acute (24-hour) Nickel Inhalation Reference Concentration for Use in Air Toxics Risk Assessment

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Background and Purpose: Acute inhalation exposure to nickel has been shown to cause immune and respiratory effects in animal and occupational epidemiologic studies. Several state air toxics control programs, including those of Minnesota, Texas and Oregon, assess potential hazards for peak 24-hour exposures to protect against acute effects. However, federal sources for acute standards use differ-

ing definitions of acute exposure. For example, the Agency for Toxic Substances and Disease Registry (ATSDR) acute inhalation Minimum Risk Level (MRL) is for exposures of 1 to 14 days, and the US Environmental Protection Agency (EPA) and National Research Council Acute Guideline Exposure Levels are for exposures up to 8-hours in duration. As a result, state agencies have taken a variety of approaches to develop acute values for specific exposure durations, including adopting federal values regardless of a common definition of 'acute', adjusting the exposure durations to meet the needs of their programs, and/or developing state-specific values, resulting in considerable inconsistency. For nickel, the federal and state acute inhalation reference values vary by more than 100-fold. **Methods:** The nickel acute RfC was derived by 1) reviewing data from authoritative sources and the scientific literature to determine sensitive Points of Departure (PODs), 2) developing and applying dose adjustment factors (DAFs) to account for differences in dosimetry between animals and humans, 3) applying time adjustment factors to calculate Human Equivalent Concentrations (HECs), and 4) applying appropriate uncertainty factors. Because the ATSDR (2024) recently concluded that the most sensitive endpoints for acute nickel exposure were immunotoxicity and respiratory irritation, our literature review focused on these endpoints. Authoritative sources for acute toxicity data for nickel included the ATSDR, EPA, Texas Commission on Environmental Quality (TCEQ), and California Office of Environmental Health Hazard Assessment (OEHHA). To determine the most sensitive POD, benchmark concentrations (BMCs) and LOAECs and NOAECs from the scientific literature were compared. To calculate the DAF, the multiple-path particle dosimetry (MPPD) model was run to estimate an HEC. **Results:** The literature review supported that immune effects are the most sensitive endpoint for acute exposures to nickel. Efremenko et al. (2017) evaluated transcriptomic responses to Ni inhalation exposure for 6 hours/ day, 5 days/week for 1 and 4 weeks and found that the lowest BMCs were observed for immune responses (0.047 mgNi/m³). The POD for acute immunosuppressive response was the NOAEC of 0.081 mg Ni/m³ reported by Buxton et al. (2021) in mice administered nickel chloride for 24 hours and challenged with sheep Red Blood Cells. Buxton et al. (2021) followed the Office of Prevention, Pesticides and Toxic Substances (OPPTS) guideline #870.7800 for immunotoxicity and was good laboratory practice (GLP)- compliant. To calculate the DAF, the MPPD model was run for the mouse and the human. MPPD only has two options for a mouse model species (BALB/c and B6C3F1); however, CD-1 mice were used in the Buxton et al. (2021) study. Thus, model runs were made with assumptions for each mouse species for comparison. Most input parameters are automatically calculated in the MPPD model. The resulting HECs ranged from 0.279 to 0.439 mg/m³, with a median value of 0.318 mg/m³. Using the median value as the PODHEC and a 30-fold uncertainty factor (ten for intraspecies sensitivity and three for interspecies kinetic variability) results in an acute RfC of 11 µg/m³ which is similar to that derived by the Minnesota Pollution Control Agency. **Conclusions:** The transcriptomic BMD at one week of exposure for immune effects is considerably lower than the LOAEC for respiratory effects as reported in Efremenko et al. (2020 µg/m³) which was used by ATSDR as the basis for the acute MRL. The acute RfC of 11 µg/m³ is also supported by the transcriptomic BMCs (47 µg/m³). Further, because it is based on a Guideline and GLP study, and provides a NOAEC for 24-hour exposures, the 11 µg/m³ acute RfC developed herein is less uncertain than values developed from other studies, including those reporting respiratory effects.

PS 3998 Application of a Bayesian Network to derive a probability of causality in support of weight of evidence assessments, with a case study on a drinking water risk assessment

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Background and Purpose: Evidence synthesis is a structured approach to identifying, appraising, and integrating findings from multiple studies to answer specific risk assessment questions. Weight of evidence, in the context of risk assessment, refers to the process of integrating multiple lines of evidence for assessing causality, typically during hazard identification. However, numerous qualitative and quantitative approaches are used for this process, often amounting to expert opinion, with little transparency in the weighting process. **Methods:** We advance ideas to dynamically create Bayesian Networks based on publication data reviewed and abstracted as part of a typical chemical risk assessment. Studies are considered within and across lines of evidence: epidemiologic studies, animal studies, and mechanistic studies. Observed evidence propagates through the Bayesian Network, which also incorporates recent ideas that introduce abstract causal indicators in the network, representing Bradford Hill considerations, such as support from the consistency of results, experimental evidence, statistical biological gradients, and temporality. Querying the resulting network provides an estimate of the probability of causality as study results are introduced, with estimates converging on 1 (certainly causal) or 0 (certainly not causal) if sufficient unambiguous and consistent evidence accumulates. **Results:** We describe a software tool created for this task, providing supporting information for risk assessors, with each piece of evidence introduced into the network, along with qualifying information such as risk of bias of individual studies and relevance of animal models for human health risk assessment. A case study is described on hazard identification using data from a recently published systematic review of human, animal, and mechanistic publications on asbestos in drinking water and stomach cancer. **Conclusions:** By incorporating probabilistic reasoning when considering evidence, Bayesian Networks can provide a sound means (enforcing

Bayes' Rule) of weighting evidence from published reports and addressing hazard identification questions in chemicals management. The approach can be used to support assessors facing challenging risk assessment problems.

PS 3999 Advancing Photoallergy Risk Assessment: Integrating the ROS Assay and UV-Vis Spectroscopy Under Full-Spectrum (UVA and UVB) Conditions for Tiered Screening of Fragrance Materials

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Background and Purpose: The Research Institute for Fragrance Materials (RIFM) applies a tiered photosafety strategy addressing both photoirritation and photoallergy. While photoirritation can be assessed using validated models, no standardized *in vitro* methods currently exist for photoallergy, making its evaluation particularly challenging. To fill this gap, RIFM identified ~100 fragrance materials with significant UV absorbance but limited photoallergy data. Significant absorbance at wavelengths of 290–700 nm, covering UVB, UVA, and visible light, is of regulatory concern, and the UV-Vis spectral analysis is first used to determine specific wavelengths of absorbance. Further, some test methods employ solar simulators equipped with filters that may attenuate certain wavelengths. Therefore, the absorbance spectra of each test chemical can help inform the selection of appropriate conditions for further photosafety testing. Given the resource demands of higher-tier *in chemico* and *in vitro* testing, RIFM is exploring the integration of the Reactive Oxygen Species (ROS) assay with UV-Vis spectroscopy to enable rapid, science-based screening and prioritization. **Methods:** UV-Vis spectroscopy, performed under the guidance of OECD TG 101, was first used to identify materials with significant UV absorbance. The spectra of each material, including identification of peaks of absorption were determined. These materials were then screened in the ROS assay, to determine photoreactivity potential, as described in OECD TG 495, and in alignment with the ICH S10 photosafety document (2015). The ROS assay quantifies singlet oxygen and superoxide anion generation after UV exposure. The UV exposure conditions for the ROS assay included solar simulators equipped with filters to attenuate UVB (>320 nm) or those which included UVB (>290 nm). Materials testing negative in the ROS assay were deprioritized based on low photoreactivity potential. ROS-positive materials were advanced to higher-tier photoallergy testing. Given that the ROS assay is highly sensitive but less specific, a negative result provides strong evidence of low photoreactivity. In contrast, a positive result indicates the need for further investigation rather than a definitive outcome. To increase confidence in the outcome of ROS assay, RIFM compared results obtained under UVA-only versus UVA+UVB exposure conditions. **Results:** Thirty-eight proficiency chemicals evaluated in the ROS assay showed strong concordance with published data. Among the 57 fragrance materials evaluated, 35 were classified as non-photoreactive, 14 as photoreactive, and 8 as inconclusive. Notably, nearly 60% of the tested materials showed no photoreactivity, substantially reducing the need for further in-depth evaluation. Sulfanilamide, a known photoallergen, was predicted to be non-photoreactive under UVA-only exposure conditions but predicted as photoreactive when UVB were incorporated into the exposure conditions. In contrast, other UVB-only absorbers such as methyl cinnamate and p-methoxybenzaldehyde remained non-photoreactive after testing with UVB incorporated into the exposures, demonstrating low photoreactivity potential. These findings highlight that full-spectrum exposure conditions reduce the likelihood of false-negative predictions and improve the confidence of non-photoreactive classifications. **Conclusions:** Integrating UV-VIS spectroscopy with the ROS assay offers a practical and efficient framework for photoallergy risk assessment of fragrance materials. Testing under full spectrum UVA and UVB conditions improves accuracy by ensuring that the true photoreactive materials, particularly UVB only absorbers, are correctly identified. Notably, a negative ROS result strongly suggests low photoreactivity, while a positive result does not indicate photoallergenicity but instead signals the need for higher tier testing. This tiered, data-driven approach improves efficiency, avoids unnecessary testing, and aligns with regulatory expectations. Extending the ROS assay to fragrance materials highlights its value as a robust, broadly applicable photosafety screening tool.

PS 4001 Inhaled Mycotoxin Doses from Fungal Growth in Water-Damaged Residential Exposure Scenarios Are Orders of Magnitude Below Tolerable Daily Intake Levels

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Background and Purpose: Airborne mycotoxins generated from hidden indoor proliferation of fungal species have been raised as a possible human health concern for “mycotoxicosis” and certain neurological and immunological adverse health complaints that appear to correlate with associated indoor dampness and musty odors. However, the most potent and well-studied mycotoxins are nonvolatile compounds adhered to fungal hyphae and/or spores; concentrations vary depending on species-specific growth characteristics and life cycle in conjunction with environmental conditions (e.g., temperature, humidity/saturation, and growth medium) that may or may not be capable of producing measurable airborne mycotoxin concen-

trations. In common residential scenarios where fungal growth occurs behind walls and in crevices related to hidden water intrusion events, airborne mycotoxin emissions are often limited for a variety of reasons. Our study goals included evaluation of upper bound indoor airborne mycotoxin exposure concentrations based on published studies of water-damaged buildings to assess inhaled dose in comparison to tolerable daily intake levels that are considered safe for the general population. **Methods:** Six studies published between 2003 and 2016 were utilized to identify the highest reported indoor air mycotoxin concentrations from *Stachybotrys*, *Aspergillus*, *Penicillium*, and other common fungi found in water-damaged buildings in the US and European countries (Spain, Holland, Finland, Denmark, and Belgium). Upper bound concentrations in ng/m³ were directly measured for Rodin E (0.08), Aflatoxin B1 (0.15), Aflatoxin B2 (0.02), Ochratoxin A (0.23), and Sterigmatocystin (1.8); Satratoxin G and Satratoxin H from *Stachybotrys* species were nondetectable in air and dusts of the water-damaged buildings studied. These upper bound airborne mycotoxin concentrations were then utilized in two illustrative residential exposure scenarios involving hidden water intrusion-associated fungal growth: (1) in a bathroom wall space and (2) in the intake plenum of a central air conditioning (AC) system. **Results:** The bathroom scenario assessed 80 kg adult exposures at the upper bound mycotoxin concentration for 1.5 hours daily at a breathing rate of 0.8 m³/hr assuming an exhaust fan-generated ventilation rate of 8 air changes per hour. The AC plenum scenario assessed similar 80 kg adult exposures for 16 h/day at a breathing rate of 0.8 m³/hr from upper bound mycotoxin concentrations continuously emitting from the intake plenum (0.224 m³) and transmitted into a home volume of 475 m³ at a ventilation flow rate generating 2 air changes per hour for the home. The inhaled total mycotoxin dose per day in each scenario was then compared to published tolerable intake levels for the most potent *Fusarium* trichothecenes (0.06 to 1 µg/kg-day) and for Ochratoxin A (0.014 µg/kg-day). For the bathroom scenario, the upper bound inhaled mycotoxin dose was 130,000-fold below the tolerable daily intake for Ochratoxin A. Similarly, the AC plenum scenario showed an upper bound inhaled mycotoxin dose 38,000-fold below the tolerable daily intake for Ochratoxin A. **Conclusions:** These findings demonstrate that upper bound exposures to airborne mycotoxins in water intrusion-impacted residential settings are several orders of magnitude below tolerable intake levels and hence are not a credible concern for mycotoxicosis or neurological/immunological adverse health complaints in water-damaged homes.

PS 4002 Considerations and Methodologies for Determining Systemic Health-Based Exposure Limits (HBELs) for Rare and Unconventional Routes of Pharmaceutical Administration

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Background and Purpose: Health-based exposure limits (HBELs) are essential tools for protecting workers and patients in pharmaceutical manufacturing. While robust methodologies to determine HBELs exist for compounds administered through traditional routes (e.g., oral, intravenous, or subcutaneous), the derivation of systemic HBELs for pharmaceuticals, particularly biologics administered through rare or unconventional routes (e.g., intravitreal, intrathecal, or inhalation), remains underdeveloped. The need to address this gap is becoming evident, as biologics represent an increasing focus of modern drug pipelines and novel delivery platforms expand the range of clinical applications. A refined and harmonized approach is needed to ensure both the safety of workers handling these drugs, and the safety of patients receiving the drug from residual carryover. The vast majority of drugs are administered orally or via intravenous (IV) or subcutaneous (SC) injection. For nearly all drugs, these routes achieve therapeutic exposure concentrations (C_{max}, AUC) in target tissues with acceptable dosing and safety profiles. However, some drugs, in particular biologics, must be administered through unconventional routes in order to achieve the requisite local therapeutic concentrations at the site of action. This may be necessitated by the need to bypass physiological barriers (e.g., the blood-brain or blood-ocular barriers) or to minimize systemic effects. Consequently, systemic exposure data [nonclinical and clinical toxicity, pharmacokinetics (PK) and pharmacodynamics (PD)] are often absent or incomplete, as development programs prioritize therapeutic endpoints over full PK/PD characterization. As a result, HBEL-setting utilizing traditional/default uncertainty factors originally developed for small molecules with well-characterized systemic exposure may lead to misestimation of risk. **Methods:** In this work, we propose general considerations for setting systemic HBELs for biologics delivered via uncommon routes. Limit-setting for systemic exposure when only uncommon route-specific data is available requires integrating traditional toxicological methods with biologic- and route-specific adjustments. These include determination of the critical effect and point of departure, assessment of route-specific uncertainties, integration of mechanistic models, and application of modification and uncertainty factors where appropriate. For data poor drugs, evaluating potential systemic effects may involve adverse outcome pathway (AOP) mapping, analysis of target expression patterns, and read-across from drugs with shared pharmacological targets. **Results:** For determining systemic HBELs for intravitreal and intrathecal routes, the typical approach often involves a volume-to-volume extrapolation; however, this approach may be overly generalized and lack compound-specific considerations. Using simulated case examples for each uncommon route of administration, we demonstrate that data-driven refinements incorporating receptor density, pharmacokinetic bridging, and barriers to entry (e.g., the blood-brain and blood-ocular barriers) provide a more realistic representation of expected systemic exposure estimates. For workers handling biologics, the typical

approach for establishing airborne HBELs often assumes an inhalation bioavailability factor of 1-5% based solely on molecular size; however, our analysis shows that integrating receptor distribution and respiratory tract deposition location can further refine systemic dose estimates and yield more accurate HBELs - differences that are operationally meaningful for occupational exposure control and cleaning validation. **Conclusions:** This framework advances scientific rigor and promotes consistency in HBEL setting for biologics administered via nontraditional routes, supporting both worker protection and patient safety in an evolving therapeutic landscape.

PS 4003 Case Study of Permissible Daily Exposure Limit Derivation for Butyl Oligomer (C₂₁H₄₀): Gradient vs. ICH Q3E

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Background and Purpose: Chemical characterization and toxicological qualification of leachable compounds from pharmaceutical containers/closures and packaging is an important component of drug product safety. The ICH published a draft guideline for extractables and leachables, Q3E, in August 2025. In the guideline, a safety assessment and toxicology-based permissible daily exposure (PDE) for a relatively common leachable, butyl oligomer (C₂₁H₄₀), was presented. No published toxicology exists for butyl oligomer; hence, the risk assessment and PDE required the use of read-across. We previously assessed and derived a parenteral PDE for butyl oligomer based on a read-across approach. The objective of this case study was to compare the read-across approaches and PDEs derived from two independently conducted safety assessments butyl oligomer. **Methods:** A combination of *in silico* analyses (using Toxtree v3.1.0, VEGA QSAR v1.1.5, OECD QSAR Toolbox v4.5, EPI Suite v4.11) and publicly available toxicology data were used to support the toxicity hazard profile of butyl oligomer. Due to the lack of toxicology data for butyl oligomer, surrogate chemicals with toxicity data were identified based on known or predicted physicochemical and toxicological properties. From these data, a PDE was derived in accordance with ICH Q3C and ICH Q3D methodology. **Results:** Due to lack of complete coverage of physicochemical properties and predicted reactivity by a single surrogate, we relied on two surrogates, (1R,2S,5R)-5-methyl-2-prop-1-en-2-ylcyclohexan-1-ol (isopulegol) (CAS No. 89-79-2) and 1-octene (CAS No. 111-66-0), for the toxicological assessment of butyl oligomer. In ICH Q3E, a single surrogate, 3,3,5,5-tetramethyl-4-ethoxyvinylcyclohexanone (CAS No. 36306-87-3) was used. Based on *in silico* predictions and surrogate data, butyl oligomer was concluded to not be mutagenic, sensitizing, or irritating in either assessment. In our assessment, the point of departure (POD) for derivation of the PDE was based on the results of a 90-day oral toxicity in rats in which a no observed adverse effect level (NOEL) of 38 mg/kg/day was identified based on kidney and hematological effects. In the Q3E assessment, the POD was based on a combined repeat dose/reproductive toxicity study in rats in which a NOEL of 100 mg/kg/day was identified based on kidney, liver, and spleen effects. Based on these POD values, a default body weight of 50 kg, and application of composite modifying factors 2,500 and 5,000, parenteral PDE values of 760 µg/day and 1,000 µg/day were derived by us and ICH Q3E, respectively. **Conclusions:** Independent derivation of a parenteral PDE for butyl oligomer (C₂₁H₄₀) by Gradient and ICH Q3E resulted in very similar exposure limits despite using different surrogates for read-across. This case study highlights that best practice approaches for surrogate identification and exposure limit derivation are robust if scientifically justified.

PS 4004 Evaluation of exposure associated with use of asbestos-containing gloves of the 1940s to 1960s era

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Background and Purpose: Asbestos-containing gloves were once common for high-temperature industrial processes until their discontinuation in the late 1970s. Despite their widespread use, few studies have provided quantitative, task-specific exposure data under realistic conditions. Prior investigations often lacked background controls, proper analytical verification, or representative work practices. This study improved on prior ones and evaluated airborne chrysotile concentrations generated during realistic simulated use of historical asbestos gloves and tongs to characterize occupational exposure potential. **Methods:** Three pairs of historical gloves (67-97% chrysotile) were analyzed in controlled simulations representing three work tasks - welding, hot material transfer, and handling laboratory equipment - along with a separate trial using asbestos-covered tongs. Simulations were conducted within a sealed 16 × 16 × 8 ft chamber ventilated at 3.5 air changes per hour to conservatively replicate typical working conditions. Personal and area samples were collected during 15-minute tasks and analyzed by both Phase Contrast Microscopy (PCM) and Transmission Electron Microscopy (TEM) to derive Phase Contrast Microscopy Equivalent (PCME) concentrations. **Results:** Average personal PCME concentrations were 0.074 f/cc for welding, 0.287 f/cc for hot material transfer, 0.092 f/cc for laboratory handling, and 0.004 f/cc for tong use. Area concentrations were approximately 22-35% of personal levels, consistent with bystander exposure models. When extrapolated to realistic durations of daily glove use, the estimated 8-hour time-weighted average (TWA) exposures were 0.018 f/cc, 0.014 f/cc, 0.005 f/cc, and 0.0001 f/cc, respectively. The average concentrations were substantially below the current

OSHA permissible exposure limit (PEL) of 0.1 f/cc. No significant differences were observed between gloves of differing asbestos content. **Conclusions:** These results showed that airborne asbestos concentrations from asbestos glove use are minimal, even under intentionally aggressive and repetitive conditions. The data align with earlier glove simulation studies (Longo and Hatfield, 2001; Cherrie et al., 2005) but incorporated improved analytical and procedural controls, including both PCM and TEM analyses, background sampling, and reproducible chamber conditions. The results provide quantitative evidence that historical uses of asbestos gloves produced airborne fiber concentrations severalfold below regulatory limits, both for workers and nearby bystanders (even under these unusually aggressive work practices). Simulated industrial use of asbestos-containing gloves and tongs yielded low airborne chrysotile concentrations under worst-case handling scenarios. Modeled daily 8-hr TWAs remained below OSHA's 0.1 f/cc PEL, suggesting negligible inhalation risk under typical historical work practices.

PS 4005 Need for Fit-for-Purpose Approaches that Integrate Dose, Mechanism, and Context of Use with Key Characteristics when Evaluating the Carcinogenic Potential of Widely Used Therapeutics: A Case Study of Acetaminophen

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Background and Purpose: The key characteristics of carcinogens (KCCs) can provide an organizational framework for grouping data by activity type during initial review stages, but meaningful conclusions about hazard and risk require further analysis through pathway-based approaches. Though complex, pathway-based are readily performed for drugs, such as acetaminophen (APAP), a substance with well-characterized biochemical activity related to therapeutic effects as well as potential toxicity. In such a case, simple groupings of biochemical properties by KCCs oversimplify complex biological processes that do not adequately represent the state of knowledge. The objective herein was to design an approach for evaluating mechanistic data for APAP that incorporates existing understanding of toxicity and efficacy pathways as part of an assessment of potential carcinogenicity involving KCC concepts. **Methods:** Literature reporting data related to biological pathways associated with APAP toxicity and therapeutic effects and applications of KCC to APAP was identified. Key events (KEs) and Key Event Relationships (KER(s)) from the pathways identified in the literature and/or previous assessments were inventoried. This inventory included an evaluation of each pathway or event in different model systems and in humans taking into account context of use, dose, toxicokinetics and relevance to humans with mapping to relevant KCCs, where applicable. An approach for assessing APAP mechanistic data for potential carcinogenicity within the context of established pathways for toxicity and efficacy was developed based on the collective assembly of pathways and mapping of KEs and KERs. **Results:** An extensive body of evidence related to biological pathways for efficacy at therapeutic doses and toxicity following supratherapeutic exposures were identified. Pathways for toxicity result primarily in liver injury and to a lesser extent kidney injury driven by CYP2E1 metabolism of APAP to NAPQI, depletion of glutathione, protein adducts and oxidative stress, mitochondrial dysfunction, and cell death at supratherapeutic doses. These KEs map to electrophilicity and genotoxicity (KCs 1, 2), oxidative stress (KC 5), and acute cell death (KC10); however, activity within and across KE and KERs are fundamentally distinct from chronic, DNA-damaging processes of classical carcinogenesis, nuanced by context-dependent conditions that do not result in carcinogenic outcomes. Pathways for pharmacological activity largely focused on biological events resulting in analgesic and antipyretic effects via COX1 and COX2 inhibition by acetaminophen itself and metabolism of acetaminophen resulting in interactions with other nociceptive pathways (e.g., TRPV1, CB1; direct or indirect) and inhibition of prostaglandin production. Pathways related to activity at these receptors appear to have some highly context-dependent immune modulatory and epigenetic effects. While these events could conceptually map KCs for epigenetic alterations (KC 4), immunosuppression (KC7) and receptor mediated effects (KC8), the activity within these KCs are time- and context-dependent, and transient and result in outcomes that are not adverse in humans. In some instances, there is evidence that acetaminophen may provide anti-tumor effects at higher doses (e.g., acting as a free radical scavenger that reduces oxidative stress). **Conclusions:** The assembly of the KEs and contextual attributes provide an acetaminophen-specific approach for organizing and assessing mechanistic data. Some of the KEs and KERs associated with acetaminophen at therapeutic and supratherapeutic exposures map to several of the KCCs. Given that these established pathways do not lead to carcinogenic effects, the collective results from the mapping and approach proposed herein highlight the importance of assessing context around KCC activity, warranting caution that not doing so may result in misleading conclusions about carcinogenic potential. The proposed approach can be applied by investigators interested in assembling and assessing mechanistic data for APAP using a KCC approach.

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Background and Purpose: The growing ubiquity of nano- and microplastics (NMPs) has raised concern about their potential toxicity to humans. Despite a rapidly expanding body of animal toxicity studies, no systematic approach exists to integrate findings for hazard characterization, hindering the development of reference values for human health and leaving risk assessments largely underdeveloped. **Methods:** Here, a structured, two-phase approach to derive benchmark dose (BMD) from recent mammalian oral exposure studies is illustrated through neurotoxicity as a proof-of-concept application. Phase 1 involved a PRISMA-guided systematic review using seven electronic bibliographic databases (n=41,784), screened for relevance using a fine-tuned large language model (LLM) and manual process. Phase 2 applied Bayesian dose-response modeling with a benchmark response of 10%, using Bayesian model averaging and strict exclusion of poorly fitted or extrapolated endpoints using three main metrics (i) precision, BMD/BMDL < 20; (ii) interval width, BMDU/BMDL < 50; and (iii) data support, BMD/ lowest non-zero administered dose < 10. For polymers with numerous qualifying endpoints (PET and PS), a statistical prioritization step was further applied to limit design bias and emphasize the most informative regions of the dose-response curve (lower end). Endpoints at or below the most likely BMD value were retained to capture data-dense, well-supported regions, while isolated high-BMD estimates were given less weight. **Results:** Phase 1 yielded 22 eligible oral-exposure studies on NMP-induced neurotoxicity among mammalian models, all of which passed Risk of Bias (RoB) and quality assessment and quality control QA/QC evaluations (average score = 82.85%) for inclusion in further analysis. Across these 22 studies, it is shown that exposure to NMPs disrupts neural integrity and function at molecular, cellular, and behavioral levels. These effects lead to dopaminergic neuron loss, reduced neurogenesis, and behavioral impairments such as memory loss, depression-like, and even Parkinson's-like symptoms. Phase 2 processed 134 statistically and/or biologically significant endpoints across polystyrene (PS; n = 98; sun-acute, sub-chronic, and chronic; MNP), polypropylene (PP; n = 5, sub-acute; MP), polyethylene terephthalate (PET; n = 23; sub-acute; MP), low-density polyethylene (LDPE; n = 3; sub-chronic; MP), and polylactic acid (PLA; n = 5; sub-acute; MP), encompassing neuropathological, neurochemical, behavioral, and developmental outcomes. Of the 134 modeled endpoints, 79 (59.0%) satisfied all three. Subsequent statistical and expert-based prioritization in Phase 2 refined these to 26 high-confidence endpoints suitable for quantitative comparison and hazard characterization. The benchmark dose lower confidence limits (BMDLs) for these prioritized endpoints ranged from 2.22×10^{-4} to $2.36 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, representing the most statistically and biologically robust findings. Among the evaluated polymers, PS contributed to the largest and most diverse dataset (30.8%), enabling meaningful comparisons between PS-NPs and PS-MPs. In contrast, comparisons across polymer types (PP, PET, LDPE, PLA) remain limited due to the highly uneven distribution of available studies and endpoints. **Conclusions:** In conclusion, findings suggest that MNP exposure may disrupt neural integrity and function across molecular, cellular, and behavioral domains, even at low exposure levels. The derived neurotoxic endpoints offer a robust foundation for expert elicitation within multi-criteria decision analysis platforms, facilitating their evaluation alongside other systemic endpoints (e.g., hepatic, cardiovascular, reproductive, immune). More broadly, the proposed framework provides a structured approach to assessing the toxicity of emerging contaminants by integrating dose modeling, evidence synthesis, and expert judgment to inform scientifically defensible reference values. It further supports transparent risk communication and highlights critical data gaps to guide future research.

PS 4007 **Systematic Evidence Reviews: When to Stop Screening?**

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Background and Purpose: Systematic evidence reviews and large-scale bibliographic screening are labor-intensive endeavors, often requiring reviewers to inspect thousands of titles and abstracts to identify the relevant literature. The software SWIFT-Active Screener introduces a dual-model approach, combining an active-learning prioritization engine with an integrated recall-estimation algorithm, to significantly reduce the manual screening burden and, critically, to provide a principled basis for **when to stop** screening. **Methods:** As screening proceeds, the tool uses a continuously updated active-learning model that reorders the unscreened document list, placing the most likely relevant items at the top based on past reviewer decisions. In parallel, it employs a statistical model (based on a negative-binomial distribution) to estimate how many relevant documents remain among the unscreened set, thereby generating a real-time estimate of cumulative recall (i.e., the proportion of all relevant documents identified so far). Once the estimated recall reaches a predefined threshold (for example 95%), the system alerts the review team that, under its model assumptions, the marginal yield of additional screening is likely to be very low. Thus, the software supplies a data-driven “when to stop” criterion, reducing wasted effort while maintaining high coverage of relevant records. **Results:** In validation across

26 diverse systematic-review datasets, the authors found that on average 95% of relevant documents were identified after screening only 40% of the total references; in larger projects ($\geq 5,000$ records) the average fraction screened to reach 95% recall dropped to 34%. Moreover, the recall estimator tended to be conservative: among simulated runs that stopped when the estimate reached 95%, the median **true recall** achieved was ~99%. These findings illustrate that integration of recall-estimation with active learning allows reviewers to stop far earlier than full-list screening while retaining strong confidence in achieving near-complete retrieval of relevant documents. **Conclusions:** SWIFT-Active Screener offers a scalable, principled framework for screening large bibliographic sets: active learning accelerates identification of relevant records, and the “when to stop” recall estimator provides reviewers with a defensible rule for terminating screening. By shifting from exhaustive manual review to targeted screening with a stopping threshold, the tool reduces resource requirements, speeds review timelines, and mitigates the key challenge of “how do we know when we’ve found enough?” Unlike tools that offer AI powered classification, where a black box algorithm classifies documents as include/exclude, our novel approach allows an expert human screener to make the inclusion decision and thus remain in control of this critical step in the literature review process while still saving a significant amount of time and achieving near perfect outcomes.

PS 4008 **Practical Predictive Risk Assessment Approaches for Chemicals**

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Background and Purpose: Regulatory directives to reduce animal testing in chemical safety assessments have created a need for improved and modern approaches to testing and assessment. A novel integrated framework for chemical safety assessment that combines New Approach Methodologies (NAMs) with weight of evidence and read-across analysis is one such approach. This framework, specifically designed for non-genotoxic agrochemicals with established pesticidal modes of action, provides a structured approach for predicting human and ecological toxicity risk while minimizing vertebrate animal testing. **Methods:** The framework employs a tiered integration of multiple lines of evidence including published data, regulatory databases, and physicochemical properties to address endpoint-specific regulatory needs. In addition, the RISK21 platform is implemented for exposure-based risk predictions and uncertainties of each line of evidence are systematically characterized. The framework focuses on chemicals within well-characterized chemical classes, utilizing data-rich safety profiles of analogues. A corresponding framework functionality survey evaluates the applicability of this approach to new chemicals. **Results:** We will present application of the survey and the framework with an agrochemical case study that leverages existing toxicological data from source analogues to evaluate dietary and occupational exposure scenarios. Comparison of the toxicological endpoints to predicted exposures as determined by regulatory authorities confirmed that the target chemical proposed uses were unlikely to pose human health risks. This case study illustrates how systematic evidence collection, evaluation, weighting, and integration can inform decisions while maintaining scientific rigor and minimize unnecessary animal use. **Conclusions:** The framework functionality survey was a valuable preliminary assessment tool, enabling evaluation of the framework’s applicability before full implementation. This combined approach provides structured guidance for predictive toxicity risk assessment while maintaining robust safety standards and offers a practical path toward regulatory compliance with reduced animal testing and demonstrates the viability of NAMs in regulatory safety assessment. This work represents a significant step toward meeting regulatory requirements while advancing the goal of eliminating reliance on vertebrate animal toxicology testing.

PS 4009 **A Structured Protocol for Developing Adverse Outcome Pathways: Ensuring Transparency, Reproducibility and FAIR compliance**

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Background and Purpose: For regulatory application, Adverse Outcome Pathways (AOPs) require systematic planning and robust protocol to ensure the integrity and consistency of the AOP framework. While the OECD’s guidance on AOP development is essential, additional strategies are needed to ensure transparency, reproducibility, and reliability. Central to this is adherence to the FAIR principles (Findability, Accessibility, Interoperability, and Reusability) which ensures that AOP elements, data, and processes are transparent, traceable, and usable by both humans and

machines. **Methods:** A PARC project case study was used to design a stepwise method for developing an AOP network that links organophosphate and organochlorine pesticide exposure to Alzheimer's disease. The methodology was defined a priori, before any data collection, by establishing a clear problem formulation and outlining a robust plan to guide subsequent steps and determine the scope of relevant evidence. This structured approach aims to ensure transparency, reproducibility, and confidence in AOP development. **Results:** The process for developing the AOP network was described in a protocol and can be summarised in four phases. The first step is to formulate the problem in order to define the objective, scope, evaluation criteria (biological plausibility, essentiality, empirical evidence and weight of evidence) and data sources. The second step is to define methods for mapping evidence and refining the AOP structure, starting from the official AOP repository. A decision tree has been developed to help search for specific events. One challenge is the replication of key events that address similar biological concepts, which complicates their reuse in the context of new AOPs. Early application of a protocol can guide decisions. The third step outlines whether a systematic or structured literature analysis will be conducted, paying particular attention to formulating search strategies. Collaborating with professional librarians is highly recommended to ensure effective literature retrieval. The fourth step involves appraisal tools to identify uncertainty and assess studies' reliability and relevance. Structured tools support this process. **Conclusions:** The approach has the advantage of being in line with FAIR principles and preventing data-driven judgements. This structured approach promotes stronger collaboration and coordination among contributors, as well as greater methodological consistency and transparency. A professional librarian-supported literature analysis ensures comprehensive coverage of all relevant information, providing transparent, reproducible documentation based on the best available evidence, thereby enhancing the credibility of the results. A formal appraisal of the evidence supports the overall assessment of the consistency and precision of the dataset, increasing confidence in an AOP. **Fundings:** "this work was carried out in the framework of the European Partnership for the Assessment of Risks from Chemicals (PARC) and has received funding from the European Union's Horizon Europe research and innovation programme under Grant Agreement No 101057014. Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union or the Health and Digital Executive Agency. Neither the European Union nor the granting authority can be held responsible for them".

PS 4010 Defining Best Practices for the Use of Sequence Alignment to Predict Across Species Susceptibility to Inform Taxonomic Domain of Applicability Descriptions in the Adverse Outcome Pathway Wiki

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Background and Purpose: Adverse outcome pathways (AOPs) map the progression from a molecular-level biological perturbation (often induced by hazardous chemicals in the environment) through several levels of biological organization to an adverse outcome—generally at the individual or population level—relevant for risk assessment. The Adverse Outcome Pathway Wiki (<https://aopwiki.org/>) provides a central repository for descriptions of AOP knowledge. A key aspect of the AOP framework is the definition of the taxonomic domain of applicability (tDOA) for each biological step—or key event (KE)—of a pathway, and the AOP as a whole. The tDOA captures evidence for understanding the species or taxa for which the KE is applicable. Although supporting data from *in vivo* studies carry the highest confidence in weight-of-evidence for defining tDOA, such methods require more resources and time than are practical—thus becoming restrictive in the development of comprehensive tDOAs for the AOP-Wiki. Computational approaches, such as the Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS) tool have the capacity to use existing empirical information from a single species to rapidly generate *in silico* evidence across the diversity of species to support tDOA descriptions. SeqAPASS compares primary amino acid sequences across species and generates predictions of chemical susceptibility based on protein conservation. Once a protein has empirical evidence (e.g. binding/affinity/activity assays, crystallography, molecular docking/dynamics etc.) for species in an AOP—or based on external literature review—SeqAPASS can rapidly evaluate whether that biology is conserved in other (typically untested) species. Those species can then be assigned as the biologically plausible tDOA with low confidence in the weight-of-evidence for the AOP. However, multiple steps in this process require guidance, including how to identify query proteins and accessions for an existing AOP, and how to analyze results from SeqAPASS. As such, case studies were performed to define best practices to standardize the use of SeqAPASS for tDOA descriptions in the AOP-Wiki. **Methods:** AOPs 545 and 548 were used as case examples. Protein accessions were identified for each KE using the AOP-Wiki, external literature review, and the NCBI Protein Database's canonical accession designation. Accessions were submitted to SeqAPASS Level 1 and checked for >10 orthologs. SeqAPASS susceptibility predictions were used to assign low weight-of-evidence tDOA predictions for KEs, and a series of logic statements written into R code were used to combine KE tDOAs across pathways into KER and then AOP tDOAs. **Results:** SeqAPASS predicted shared susceptibility for all mammals for both AOPs, as well as individual susceptibility predictions spanning a variety of clades for the KEs contained within. In all instances, AOP-Wiki/literature-identified accessions were identical to the NCBI canonical

accession, although two accessions had insufficient orthologs for SeqAPASS analysis. Canonical accession identification reduced the time required for SeqAPASS accession identification from ~40 hours to less than 1 hour. **Conclusions:** A flow-chart was developed from the results of the present study to guide future SeqAPASS application to identify AOP tDOAs. Importantly, if the AOP-wiki does not identify a protein accession for a KE, it is recommended to default to NCBI canonical accessions—rather than further literature review—due to accordance of literature-identified protein accessions with NCBI canonical accessions and significant time-saving. If future AOP authors are using isoform-specific information in KEs, NCBI accessions should be clearly identified in the AOP-Wiki text. Usage of the aforementioned flow-chart for accession identification and tDOA combination will increase efficiency and rigor of SeqAPASS application towards identifying AOP-Wiki tDOAs, and advance understanding of how species respond to adverse effects induced by hazardous chemicals.

PS 4011 A Novel Approach to Optimizing the Efficiency of Chemical Toxicity Testing Using Multi-Task Chemical Embeddings and Active Learning

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Background and Purpose: Military personnel are subjected to a wide range of complex and varying environmental factors that can negatively impact their health and mission readiness. Machine learning (ML)-based predictive models can be used to maximize the utility of limited data. These models aim to support rapid decision-making and reduce the need for time-consuming and costly animal testing. However, a major hurdle to their success is the lack of sufficiently large biological data sets for training. To address this issue, an active learning (AL) approach was adapted, which combines a multi-task (MT) ML pipeline with laboratory health and mission readiness. This approach allows for more strategic model development by identifying areas of uncertainty, conducting targeted experiments, and then retraining the model with new information. **Methods:** A directed message passing neural network (D-MPNN) learned a vector representation of molecular structure, which was used to train downstream random forest models for new tasks with insufficient data to train models from scratch. AL was performed on a low-data *in vitro* endpoint for inhibition of solute carrier proteins 6A2 and 6A3 (SLC6A2/3, norepinephrine and dopamine transporters). A specialized loss function was used to select groups of 10 novel chemicals for *in vitro* experiments, balancing the goals of expanding the applicability domain, reducing uncertainty, and maintaining class balance (Active/Inactive results). *In vitro* tests were performed using overexpression cell lines, and results were used to update the D-MPNN and select additional test chemicals. Two cycles of AL were conducted for SLC6A2/3 as well as a separate cycle for a task based on inhibition of monoamine oxidase A (MAO-A) that used a cell-free assay. Model performance was quantified using test set area under the curve (AUC) values for the receiver operating characteristic (ROC) and precision-recall curve (PRC). A statistical analysis of 100 model training seeds compared AUC values of the model before AL against versions of the model after 1 or 2 AL cycles. **Results:** In the first cycle of AL performed on the SLC6A2/3 endpoint, the model identified 10 test chemicals. Four (flutolanil, 2-bromodibenzofuran, 2-acetylaminofluorene, and N-phenyl-1-naphthylamine) were found to be Active in the *in vitro* assay at non-cytotoxic concentrations. The second AL cycle identified 4 additional active compounds: hexadecyl trimethyl ammonium chloride; 4-chloro-1,1'-biphenyl; paclobutrazol; and 1,6-dinitropyrene. The first AL cycle produced modest increases in model AUC. The second AL cycle produced greater improvement, highlighting the benefit of sequential AL rounds. On average, ROC AUC values increased by a modest 0.029 per iteration; PRC AUC values slightly increased by 0.010 per iteration. For the MAO-A AL cycle, the model selected 10 chemicals, of which 7 were Active *in vitro*. Model improvements for this task were marginal due to the smaller initial dataset compared to the SLC6A2/3 dataset. **Conclusions:** This project demonstrated utility of using AL model-directed experiments to fill data gaps and improve model performance. Integrating model development and data collection shows promise for expanding the model prediction range while minimizing the need for new data collection. The proposed AL approach coupled with the MT-ML pipeline provides a more generalized model of chemical space that is robust against data scarcity. While further improvements to the MT-ML model and refinements to the AL process are needed, the approach demonstrates a step forward in implementing modernized human risk assessment for complex operational exposures. *Disclaimer:* No DoD endorsement implied.

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Background and Purpose: Assessment of chemical toxicity is fundamental to risk evaluation, yet traditional approaches like Cramer's Decision Tree (CDT) and Expanded Decision Tree (EDT) rely on rigid, expert-defined rules, lack scalability and quantitative measures of uncertainty. We introduce a data-driven framework that predicts toxicity thresholds—such as Thresholds of Toxicological Concern (TTCs)—using a dual-fingerprint similarity search that captures both global molecular features and toxicity-relevant structural alerts. This approach provides mechanistic interpretability, built-in applicability domain assessment, scalability, and quantitative uncertainty estimation, overcoming key limitations of decision tree-based methods.

Methods: A reference database of compounds with No-Effect Level (NEL) values derived from *in vivo* experiments was used. For every target compound, a dual similarity measure was computed: (1) global molecular similarity, and (2) toxicity-relevant similarity from weighted structural alerts mined from experimental data. These were combined into a weighted similarity score to retrieve reference compounds above a threshold, forming a representative collection for the target compound. Cumulative NEL distributions were used to derive TTC values and identified alerts were used for assessing toxicity mechanisms. Predictions were benchmarked against Munro and Expanded Decision Tree datasets. **Results:** • Successfully retrieved representative collection for diverse query compounds, yielding robust TTC predictions. • Demonstrated mechanistic interpretability, with structural alerts identified in both query and reference compounds. • Achieved good correlation between predicted NEL at 5% cumulative probability and actual NEL values, supporting quantitative reliability (similarity cutoff = 0.7, $r^2 = 0.44$, coverage = 85%). • Demonstrated greater flexibility and quantified uncertainty than rule-based methods, providing confidence intervals for TTC predictions and flags for structurally novel compounds. • Comparative analysis revealed better adaptability than CDT/EDT approaches, while maintaining regulatory relevance. **Conclusions:** This framework provides a general, adaptable, and mechanistically interpretable method for deriving TTC values. By combining global general molecular features with toxicity-weighted structural alerts, it avoids the rigidity of rule-based trees, allows quick incorporation of new data, and introduces systematic mechanisms for uncertainty and domain applicability assessment. Limitations remain for compounds with novel structural features, but such cases can be flagged by applicability checks. Overall, this approach represents a next-generation alternative to Decision Tree-based methods for toxicity prediction.

PS 4013 Investigating the feasibility of deriving new environmentally relevant Thresholds of Toxicological Concern (TTCs) from estimated human equivalent benchmark dose (eBMD_{HED}) values

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Background and Purpose: The Threshold of Toxicological Concern (TTC) is a pragmatic approach used to establish safe thresholds below which there is likely no appreciable risk to human health. Kroes et al (2004) presented a tiered TTC approach that established several human exposure thresholds over several orders of magnitude, ranging from 0.0025 µg/kg-day to 30 µg/kg-day. The lowest tier is for substances that raise a concern of genotoxicity based on structural alerts for genotoxicity. For substances without structural alerts for genotoxicity, there are a series of non-cancer TTC tiers, which are based on the Cramer et al (1978) decision tree. Derivation of TTC values for each of these Cramer classes originates from work by Munro et al (1996) who compiled a dataset of NOELs for 613 substances that had been tested in subchronic, chronic and reproductive-developmental repeat-dose oral toxicity studies. In Nelms et al (2019), an evaluation was performed to explore whether TTC values derived from toxicity data on environmentally-relevant substances as compiled in the EPA Toxicity Values DB (ToxValDB) would give rise to comparable TTC values as those derived by Munro et al (1996). In that study, statistical outliers were removed, but no effort was made to review the quality of the individual studies. TTC values are calculated by taking the 5th percentile of the NOEL values and then adjusted 100-fold by default intra and interspecies uncertainty factors to derive a final value. More recently, efforts by Aurisano et al (2023) and Harrill et al (2025) have attempted to develop curated datasets restricted to effect levels, exposure routes, study designs and species relevant for deriving toxicity values. The effect levels are then adjusted to chronic human equivalent benchmark doses using the World Health Organization (WHO) framework which considers different conceptual models and magnitudes of effects for different types of endpoints within toxicity studies. **Methods:** In this study, the feasibility of deriving new oral TTCs was investigated using estimated human equivalent benchmark dose (eBMD_{HED}) values from EPA's ToxValDB v96.1. Study level eBMD_{HED} values were generated using the R code from https://github.com/USEPA/toxvaldb_dcapprep. Chemical level values, i.e. those representative of the study level eBMD_{HED} values, were derived by taking the 25th percentile of the fitted normal distribution. Chemical structural information was pulled from the EPA CompTox Chemicals Dashboard resulting in a dataset of 4076 substances. The chemical set was processed through a computational implementation of Kroes et al (2004) workflow. The 1271 substances assigned to the Cramer structural classes were then processed through the Toxtree Cramer module.

The 5th percentile of the empirical cumulative distribution function for each Cramer structural class was then derived. **Results:** The Cramer I class 5th percentile was estimated as 4.24 mg/kg-day. Applying a 10-fold intraspecies factor, resulted in a TTC of 0.424 mg/kg-day which was 14 times less conservative than the current TTC for Cramer I (0.03 mg/kg-day). The TTC derived for Cramer III gave rise to a value of 0.014 mg/kg-day, which was 9.4 times higher than the default Cramer Class III TTC value (0.0015 mg/kg-day) derived from the Munro dataset. Given there were only 56 substances in Cramer II, no TTC value was derived. **Conclusions:** Ongoing work will consider other ways to subcategorize the dataset beyond reliance on existing Cramer structural classes. TTC values derived from human equivalent chronic values (eBMD_{HED}) offer promise of establishing exposure thresholds that are more human relevant whilst still protective of human health.

PS 4014 Automating Structure-Based Analogue Selection for Read Across: The Power of Matched Molecular Pairs

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Background and Purpose: Read across is one of the most important animal alternative methods and is used to establish the safety of a data-poor target compound using toxicological data associated with a related data-rich analogue. The process of identifying related compounds starts by comparing the molecular structures of the target compound with those of potential analogues. The most common approach involves using algorithms to calculate the overlap of molecular fingerprints; however, this method's effectiveness hinges on the choice of fingerprints and may not accurately reflect analogue quality. To enhance this process, a matched-molecular pair (MMP) approach has been developed, linking small structural changes, defined as MMP transformation rules, to analogues suitable for read across. This poster presents a summary of the structural changes associated with analogues that are promising candidates for read-across predictions. It also illustrates how to encode these transformations using two different programming languages, ensuring that the method is accessible even to those without extensive chemistry expertise. **Methods:** MMP transformations associated with analogue quality have been summarized (Lester and Yan, 2021). Strategies and methods used to automate the approach using Pipeline Pilot and Python are presented. Matched target-analogue pairs of structures produced by one- or two-bond cuts are selected if they possess the same functional groups resulting in pairs that fall into defined structural categories. Encoding the algorithms for categorizing each target-analogue pair is described including challenges and best approaches. Lester, C. C.; Yan, G. A matched molecular pair (MMP) approach for selecting analogs suitable for structure activity relationship (SAR)-based read across. *Regulatory Toxicology and Pharmacology* **2021**, *124*, 104966. DOI: <https://doi.org/10.1016/j.yrtph.2021.104966>. **Results:** The programs output MMP transformation categories, which provide straightforward and transparent comparisons of molecular structures that are likely to exhibit similar toxicities. Each program first identifies innocuous salts of organic acids (carboxylic, sulfonic, phosphoric) or bases (amine) as well as stereoisomers. Remaining structural transformations identified include the following for aromatic compounds: addition/deletion of a small (C1-C4) alkyl group to the ring, change in alkyl substituent chain length or branching, different locations of the same substituents on the aromatic ring, or different numbers of the same substituents. For aliphatic compounds structural transformations include the following: change in alkyl chain length, branching or bond order, change in number of repeat units, or the addition of a functional group such as an OH group. To illustrate the simplicity and utility of the technique, case study examples are presented for each category type. **Conclusions:** The Matched Molecular Pair (MMP) approach for analogue selection has been successfully automated using Pipeline Pilot and Python. This automation generates output that categorizes target-analogue pairs, providing clear, structure-based analogues with strong potential for accurate read across predictions. Following selection, quantitative comparisons of similarities in metabolism, reactivity, and physicochemical properties are needed to validate the use of each analogue as a data source. This streamlined process enhances the reliability of toxicity assessments and supports informed decision-making in chemical safety evaluation.

PS 4015 Integrating Structural and Mechanistic Evidence to Select Relevant Analogs for Read-Across Assessment

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Background and Purpose: Risk assessment for food-use conventional pesticides generally requires toxicity testing on over 20 mammalian-based studies and is estimated to consume 10,000-15,000 animals per pesticide regulatory evaluation. In working towards animal reduction, read-across assessments are being increasingly utilized to evaluate if sufficient information is available from chemical analogs to inform data needs for a scientifically sound and credible characterization of a target chemical's risk profile. The work herein explores a new approach for analog selection and inclusion criteria applied to a data-rich pesticidal chemical class, acetyl-CoA carboxylase (ACCase) inhibitors. Structural, physicochemical, pesticidal mode of

action, and biological similarity were included to identify relevant analogs with existing toxicity data. This case study supports a precautionary, health-protective assessment of pesticides while reducing reliance on animal studies. **Methods:** An inventory of 798 pesticides was compiled from three sources: 212 from the Fungicide Resistance Action Committee (FRAC), 325 from the Herbicide Resistance Action Committee (HRAC), and 261 from the Insecticide Resistance Action Committee (IRAC). Structural similarity was assessed by calculating Tanimoto scores using aggregated ToxPrint (730 bits), MACCS (166 bits), and PubChem (641 bits) fingerprints. ToxPrint data were obtained from the CompTox Chemicals Dashboard, while MACCS and PubChem fingerprints data were generated using the rcdk v3.8.1 R package. After feature reduction, the total number of aggregated bits decreased from 1537 to 1017. Biological similarity assessment to refine the list of potential analogs was anchored on identifying those that do not induce activation of the peroxisome proliferator-activated receptor (PPAR). PPAR mechanistic assay outcomes for multiple PPAR isoforms were retrieved from the Integrated Chemical Environment (ICE v4.1.1). Seventeen PPAR activity assay endpoints were evaluated; pesticides showing less than 10 percent PPAR activity across tested assay endpoints were retained. Absorption, distribution, metabolism, and excretion (ADME) information, with an emphasis on metabolic sites and routes of excretion, was also extracted from regulatory public data sources and CompTox Chemicals Dashboard. Chronic toxicity studies were identified and compiled from regulatory document searches. Candidate analogs were then prioritized based on Tanimoto score, PPAR activity, chemical class, toxicological mode of action, ADME properties, and available chronic toxicity data. **Results:** The aggregated chemical fingerprints provided a broad coverage of structural features while emphasizing toxicologically relevant descriptors. PPAR activity was used to filter out PPAR-activating compounds that would be more sensitive in producing rodent tumors. Approximately 300 pesticides exhibited PPAR activity below 10 percent. Applying a distribution-based cutoff yielded eighteen analog candidates, compared to twenty-five without PPAR activity evaluation, with spirotetramat emerging as the closest relevant analog (Tanimoto score ≈ 0.7). Visual evaluation of structural similarity among the top identified analogs by PCA analyses confirmed overlap in chemical space, reinforcing their relevance. Of the analogs with publicly available data and regulatory reviews, many exhibited comparable ADME characteristics, including good absorption, hepatic metabolism, and primary urinary excretion. Of the 18 analogues retained for further read-across assessment, the 90-day rat NOAELs ranged from 1.69 to 1509 mg/kg day, while the dog NOAELs ranged from 0.5 to 1038 mg/kg day. **Conclusions:** An effective analog selection strategy for read-across assessment integrates structural, mechanistic, and empirical evidence in alignment with the latest international read-across guidance. By incorporating computational tools and similarity cutoff criteria, the approach enhances robustness and transparency to better support read-across assessment. Overall, this approach demonstrates a systematic and evidence-based method for selecting the most suitable analogs for regulatory read-across applications.

PS 4016 Physiologically Based Kinetic Model of Dibutyl Phthalate and Reproductive Hormones in Female Mice

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Background and Purpose: Phthalates are a group of chemicals used in the manufacturing of many plastic products. Dibutyl phthalate (DBP) and phthalates in general have been shown to have effects on the endogenous reproductive hormone levels of both sexes, and DBP historically is higher in women of reproductive age. Studies in male and female rats exposed to DBP utilized physiologically based kinetic (PBK) models to characterize the absorption, distribution, metabolism, and excretion (ADME) behavior of DBP in the body. However, to our knowledge, there are no PBK models that incorporate endogenous hormone changes due to DBP exposure. Thus, in support of our NIH Virtual Consortium for Translational/Transdisciplinary Environmental Research (ViCTER) grant, "In Vitro to In Vivo Extrapolation of Toxicant Effects on Ovarian Function" we developed a PBK model for female mice exposed to DBP orally. **Methods:** Our PBK model was designed to simultaneously simulate the behaviors of nine molecules: DBP, its metabolite monobutyl phthalate (MBP), 17 β -estradiol, testosterone, androstenedione, progesterone, inhibin B, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). To achieve this, we designed a conceptual model consisting of four tissue compartments (i.e., gut, liver, ovary and "other"), and blood. We formulated mass-balance differential equations for each compartment that account for the ADME of the nine molecules. We used COPASI to build and run the model. Model parameter values (e.g., body mass, organ volumes, blood flow rates, kinetic rate constants, partition coefficients, etc.) were measured directly by experiments performed for this project when possible, obtained from the literature, or scaled allometrically. Model predictions will be evaluated with experimental data as they become available. Goodness-of-fit will be determined quantitatively. **Results:** The PBK model comprises over 30 ordinary differential equations that are numerically solved simultaneously in COPASI, enabling it to predict concentrations of the nine molecules in each compartment as a function of time. Some unique features of this model include steroidogenesis pathway reactions in the ovary that lead to the production of hormones of interest, and regulatory feedback control of LH and FSH. **Conclusions:** This study is a first attempt to develop a PBK model for DBP in mice that incorporates changes in endogenous hormones.

Additional work remains to evaluate its ability to simulate other DBP exposure scenarios. The model's predictive abilities allow us to simulate different exposure scenarios and generate new hypotheses about the mechanism of action of DBP and MBP. Finally, this model will serve as a foundation for developing a PBK model for rhesus macaques exposed to DBP.

PS 4017 FINS: An Interactive Platform for Automated Zebrafish Image Analysis and Morphological Screening

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Background and Purpose: Zebrafish (*Danio rerio*) embryo assays are increasingly recognized as a powerful and scalable model for developmental and toxicological studies, as zebrafish embryos themselves exhibit optical transparency, rapid organogenesis, and strong genetic and physiological similarity to humans. However, traditional morphology-based phenotyping relies on manual scoring of abnormalities, which is time-consuming, subjective, and difficult to scale for high-throughput applications. The absence of standardized, automated, and interpretable tools has limited the reproducibility and regulatory acceptance of zebrafish-based screening. To address these challenges, we developed FINS (Fish Imaging and Neural Scoring), an interactive, web-based platform that combines deep learning with intuitive visualization and quality control (QC) tools for automated zebrafish image analysis and morphological screening. **Methods:** Zebrafish embryos were imaged in 96-well plates using high-resolution bright-field microscopy at 24 hours post fertilization (hpf) and 5 days post fertilization (dpf). FINS processes either single or multiple time-lapse images per well, aggregating predictions across views to ensure robust classification of developmental phenotypes. The platform employs a multitask ConvNeXt v2 architecture with dual heads: one for phenotypic classification (normal, mortality at 24 hpf (Mo24), mortality at 5 dpf (Mo5d), or abnormal at 5 dpf) and another for QC assessment (high vs. low image quality). The model was trained on a manually annotated dataset of 4,641 zebrafish embryo images, encompassing a range of morphological phenotypes, and evaluated on an independent test set of 576 images. The FINS backend integrates FastAPI and MySQL for data management, with a Django-based frontend for visualization. The interface includes a color-coded 96-well plate viewer, enabling rapid navigation between wells, visualization of convolutional neural network (CNN) confidence scores, and manual correction of predictions. Continuous model improvement is supported through MLflow, which automates experiment tracking, retraining, and deployment, allowing iterative refinement as new annotations become available. **Results:** The phenotypic classification head achieved high F1 scores across classes, 0.94 (Normal), 0.90 (Mo24), 0.79 (Abnormal), and 0.60 (Mo5d), reliably distinguishing normal, mortality, and abnormal phenotypes. The QC head attained an F1 score of 0.96 for the Good Quality category, effectively filtering out low-quality images prior to phenotypic scoring and ensuring data integrity across analyses. Compared to manual scoring, FINS greatly reduced annotation time while maintaining interpretability via confidence visualization. The system's high-throughput screening mode enables real-time plate navigation, ambiguity detection, and prioritization of wells requiring manual review. Multi-view image aggregation improved detection of abnormalities, and all user edits are stored and incorporated into the retraining pipeline, ensuring continuous improvement. **Conclusions:** FINS represents a next-generation platform for standardized, scalable, and transparent zebrafish phenotyping. Combining deep learning automation with an interactive, interpretable interface, it enhances throughput, reduces annotator variability, and promotes reproducibility. Its modular architecture and continuous model management enable extension to new imaging modalities, phenotypic classes, and LIMS integration, supporting AI-enhanced zebrafish assays in research and regulatory workflows.

PS 4018 Targeting TLR4 and NLR3: Computational Modeling Reveals Immunotoxic Mechanisms and Nutraceutical Solutions in Farmed Catfish

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Background and Purpose: Channel catfish (*Ictalurus punctatus*), dominating U.S. freshwater aquaculture, confronts severe immunotoxic threats from persistent pollutants pentachlorophenol (PCP) and perfluorooctanoic acid (PFOA). PCP, a banned pesticide and preservative, persists via legacy contamination, inducing oxidative stress and immunosuppression. While PFOA, a bioaccumulative "forever chemical" from industrial sources, disrupts innate immunity, amplifying infection risks and undermining sustainable farming. These toxins target key inflammatory proteins: Toll-like receptor 4 (TLR4) for pathogen sensing and proinflammatory activation, and NLR family CARD domain-containing 3 (NLR3) for inflammasome regulation. **Methods:** Channel catfish TLR4 and NLR3 interactions with PCP and PFOA were quantified through computational modeling, homology, docking, and dynamics simulations. Binding affinities and conformational changes were evaluated with and without nutraceuticals (Cyanidin 3- glucoside, Melatonin and Pterostilbene) as competitive inhibitors. **Results:** Both PCP and PFOA exhibited high affinity for NLR3

and TLR4, while may result in structural perturbations associated with inflammatory dysregulation. Cyanidin 3-glucoside significantly reduced PCP affinity for NLR3, while pterostilbene attenuated PCP and PFOA binding, indicating protective modulation. Interestingly, none of the nutraceuticals had any effect on pollutants binding with TLR4 **Conclusions:** This research asserts PCP and PFOA as critical aquatic immunotoxins and specific nutraceuticals as eco-friendly tools to fortify channel catfish immunity, advancing molecular strategies for resilient aquaculture under environmental duress.

PS 4019 An Integrated Microbial Remediation and Physiologically Based Toxicokinetic (PBTk) Model of Polychlorinated Dibenzo-p-Dioxin and Dibenzofurans

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Background and Purpose: Polychlorinated dibenzo-p-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) are persistent environmental pollutants. Their long half-lives, both externally at polluted sites and internally in the human body, pose significant health risk concerns for chronic exposures to these toxic compounds. Microbial reductive dechlorination and respiration have been studied as an economical strategy for remediating PCDD/F-contaminated sites. However, it is a decades-long lengthy remediation process with risk of elevated total toxic equivalency (TEQ) at some points in time as congeners of higher toxic equivalent factor (TEF) values may be transiently produced. Should microbial remediation be implemented, health risk assessment for humans living near the contaminated sites requires an integrated approach that can predict both the evolving environmental PCDD/F congeners and the tissue burdens resulting from chronic exposures to these mixtures. Here we aim to develop a computational modeling framework that can quantitatively describe the external-to-internal PCDD/F exposure continuum. **Methods:** To this end, we have adapted and integrated two models we recently published: (i) a microbial remediation model that employs first-order kinetics to predict the dechlorination of 75 PCDD and 135 PCDF congeners, and (ii) a human physiologically based toxicokinetic (PBTk) model for mixtures of dioxin-like PCDD/F congeners that can induce liver binding proteins resulting in nonlinear tissue accumulations. The integrated model is implemented in the MATLAB-compatible freeware Octave. **Results:** The model is applied to simulate dechlorination of PCDD/F by organohalide-respiring bacteria including *Dehalococcoides mccartyi* and lifetime human exposure to evolving PCDD/F mixtures starting at concentrations representative of the Tittabawassee and Saginaw Rivers in MI, USA. The model allows us to make preliminary predictions of transformation of PCDD/F congeners and TEQs at the sites and, with the assumption of proportional intake of congeners, the blood or tissue TEQs. The model predicts that the relative contributions of individual dioxin-like congeners to site TEQ evolve over time and furthermore their lifetime contributions to tissue-level TEQs also vary due to congener-congener interactions in the liver and differential half-lives. **Conclusions:** In summary, we have developed an integrated microbial remediation and PBTk modeling framework to predict evolving tissue accumulation and TEQs of PCDD/F congeners. Upon future quantification of related uncertainty and variability, the modeling framework can be used as an *in silico* tool to help assess the health risk for people living near the contaminated Superfund sites under microbial remediation.

PS 4020 Molecular Modeling and Lung Cell Studies Uncover PFOS-Induced Ribosomal Stress and Metabolic Dysregulation

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Background and Purpose: PFOS (perfluorooctane sulfonate) is an environmental contaminant with documented links to respiratory dysfunction and inflammation, raising urgent public health concerns. **Methods:** Motivated by increasing evidence of PFOS toxicity, we first performed computational docking studies to examine its interactions with ribosomal proteins RPS6 and RPS7, and mitochondrial uncoupling protein UCP1. **Results:** Our computational modeling revealed strong binding efficiencies: PFOS exhibited binding energies of -6.6 kcal/mol for RPS7, -7.2 kcal/mol for UCP1, and similar high affinity for RPS6, indicating direct interference with cellular bioenergetics and protein synthesis. To translate these *in silico* predictions into a biological context, we next exposed A549 lung alveolar epithelial cells to PFOS and measured metabolic and transcriptional responses. Seahorse XF ATP Rate assays showed a significant increase in cellular ATP production driven largely by glycolysis, consistent with compensatory adaptation to mitochondrial stress during PFOS exposure. The qPCR analyses further demonstrated upregulation of RPS6 and RPS7, suggesting the activation of mTOR-S6K-RPS6 signaling. **Conclusions:** Together, these results provide new insights into how PFOS directly targets mitochondrial and ribosomal pathways, causing metabolic reprogramming and injury in lung epithelial cells. Our integrated computational and experimental approach underscores the potential health risks of PFOS exposure and points to molecular targets that could inform future prevention and intervention strategies.

PS 4021 Simulation of the uptake, metabolism and bioavailability of highly lipophilic constituents in petroleum fuels and oils

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Background and Purpose: Petroleum fuels are complex mixtures with a large number of chemical constituents; therefore, consideration of these mixtures requires higher throughput modeling approaches. Before considering potential interactions between the components of the mixtures, more information is needed on the characteristics of individual chemicals, as some components are well-described (e.g. benzene, toluene, ethylbenzene and xylenes, or BTEX), while other, typically more hydrophobic (log P > 4) components are data-poor. Complicating analyses, the larger constituents are often not water soluble and experimental studies typically require them to be delivered in a variety of lipid vehicles that can impact kinetic behavior. In this study, we aggregated PK data from 12 large hydrophobic constituents of fuels and oils and developed a PBPK model to help identify effects of various physicochemical and vehicle properties on the disposition of those chemicals. **Methods:** A literature search was performed to identify data on human, rat, or mouse blood concentrations of various petroleum fuel components after IV, inhalation, or oral exposure. Data from screened studies were either taken directly from tables or determined from plots using a plot digitizer. Given the models and data already available for BTEX, this set of chemicals was used to properly generate and calibrate a base multi-chemical array model. The starting structure of the model was derived from Haddad et al. (1999), which had separate blood and tissue compartments for each chemical as well as interaction terms for inhibition (or induction) of metabolism. From there, more lipophilic constituents were considered, and elements needed to capture their kinetics were added. With those elements in place, chemicals with available concentration-time data were modeled, using physiological values from Brown et al. (1997), partition coefficient estimates from the IndusChemFate model, and GI uptake and metabolism values visually optimized to the data. Blood concentrations and AUCs from simulated IV exposures were calculated based on optimized metabolism values in order to estimate bioavailability. Optimized values and bioavailability were then compared. **Results:** For non-BTEX chemicals, a total of 37 blood concentration-time courses for 12 chemicals were extracted from 15 publications. After considering the literature and models for lipophilic molecules, additional elements added on to the BTEX model included a deep fat compartment, two compartment oral uptake, and a separate lymphatic oral uptake. Comparison of observed vs. (visually-optimized) simulated concentrations for 421 data points yielded a linear regression with a slope = 1.1, $r^2 = 0.825$, and RMSE = 4.5. A total of 32 (7.6%) of simulated data was more than an order of magnitude off from the observed data, though 15 of those points were at low concentrations (<1% of maximum, typically at the end of the curve), and another 11 were from benzo[a]pyrene specifically, which showed significant variability in observed concentrations between studies. Apparent bioavailabilities ranged from 1.1-91.4% in oral dosing scenarios and 8.8-78.6% in inhalation dosing scenarios. Fluoranthene was administered orally to rats in multiple vehicles, indicating that bioavailability was slightly enhanced with a more hydrophobic vehicle (>90%, e.g. cod liver oil) vs. a more hydrophilic vehicle (85-90%, e.g. Tween 80/isotonic saline). Additionally, lymphatic uptake was relatively high (> 0.2) in 6 of 9 chemicals with oral data, though once again data from fluoranthene indicated lymphatic uptake may be vehicle dependent. **Conclusions:** This effort represents a first step towards understanding the contribution of physicochemical properties of larger constituents of oils and fuels to their overall bioavailability. Additionally, this study highlights the importance of carrier vehicle in modeling efforts as the oral uptake/bioavailability profiles may be affected by the lipophilicity of the vehicle. Being able to measure and model these trends is a key step to enable higher-throughput modeling efforts for more complex mixtures (UVCBs, like gasolines, fuels and oils).

PS 4022 Physiologically-Based Quantitative System Toxicology Thyroid Hormones Modeling Platform: Species-dependent Thyroid Hormone Changes by Test Substances in Rats and Humans

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Background and Purpose: Disruption of the thyroid hormone (TH) homeostasis by endocrine-disrupting chemicals poses a potential health concern. The extrapolation of animal data to humans is of special interest because (TH) regulation differs between species. These differences may lead to species-specific sensitivities to substances that disturb TH homeostasis, for example, through the indirect mechanism of liver enzyme induction. The presented project aims to develop a physiologically based kinetics (PBK) quantitative systems toxicology (QST) model for predicting TH levels following exposure to test substances in rats and humans. **Methods:** The model was constructed using the Open Systems Pharmacology (OSP) Suite Version 12, integrating PBK models of thyroid-active compounds with QST modules for TH regulation and quantitative adverse outcome pathway (qAOP) modules. The modular architecture allows for the incorporation of new compounds and physio-

logical processes. Model validation was performed using Quarto and the (esqlabsR) package. **Results:** The QST modules encompass pituitary synthesis of thyroid-stimulating hormone (TSH), feedback-controlled synthesis of triiodothyronine (T3) and thyroxine (T4), species-specific plasma protein binding, and active transport and clearance of TH. The platform includes PBK models for the TH disrupting chemicals methimazole (MMI), propylthiouracil (PTU), and phenobarbital (PB), each with distinct mechanisms of action, such as inhibition of thyroperoxidase (TPO) or induction of T4-specific Glucosyltransferases (UGTs). Simulations demonstrate dose-dependent changes in T3, T4, and TSH following administration of MMI or PTU in rats, as well as effects of MMI and combined MMI+PTU treatment in humans. For PB, the model predicts a decrease in T4 levels by up to -20% (observed: -6 to -60%) and an increase in TSH by up to +70% (observed: +3 to +100%) in rats, with negligible effects in humans. **Conclusions:** The developed platform identified key events of thyroid toxicity in rats, with minimal impact observed in humans. Critical key events, such as UGT induction, can be addressed by specific *in vitro* assays and the results of these *in vitro* assays can be fed into the QST model to assess the overall effect on human or rat thyroid hormone level.

PS 4023 Unifying *In Vitro* Kinetics and Open-Source Mechanistic Modeling to Accelerate Informed Decision-Making in Dermal Disposition Assessments

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Background and Purpose: Recent evaluations have compared *in silico* dermal absorption models against small dose *in vitro* permeation test (IVPT) data. Our mechanistic model showed good performance for receptor fluid kinetics but generally tended to over-predict chemical evaporation and under-predict recoveries in the epidermis and dermis. These findings highlight some key challenges, including the need for better modeling of chemical volatility/vehicle evaporation from the skin surface and chemical disposition into the skin layers. Recognizing that the rate of absorption of topically applied chemicals can vary depending on the chemical, vehicle, and amount, the overall purpose of this initiative is to deploy problem-solving tools and practical workflows to advance dermal absorption and disposition prediction capabilities. **Methods:** To enhance model reliability and generate necessary data for extrapolation to untested scenarios, an iterative workflow utilizing *in vitro* lab testing capabilities was employed. This workflow includes Vehicle Dry-down Tests to calculate evaporation rates, *in Vitro* Release Tests (IVRT), and Kinetic IVPTs. Kinetic experiments were performed to characterize the absorption and disposition of permeants with varying physicochemical properties covering a range of molecular weights (100 to 400 Da), lipophilicity (LogP -0.4 to 4), ionization states, and aqueous solubilities (1 to 500 mg/mL) (e.g., Niacinamide, Caffeine, Vanillin, Methyl Paraben, Ibuprofen) using saline vehicle and three leave-on formulations (Silicone-in-water, Oil-in-water, Oil-in-water with sunscreen protection factor). **Results:** Formulation dry-down results confirm that formulations dry down to a residual mass correlating with non-volatile ingredient amounts. Although terminal recoveries between the dislodgeable dose (skin surface + stratum corneum) and dermal delivery (viable epidermis + dermis + receptor fluid) were similar between different formulations, comparisons of area under the concentration-time curve (AUC) data for the stratum corneum, viable epidermis, and dermis revealed quantitative differences between formulations that were not evident from *in vitro* release data or terminal 24-hour recoveries. These findings suggest that different interactions between formulation components and skin may impact partitioning and/or diffusivity within specific skin layers, particularly during the early time periods (<6 hours). The derived knowledge and new experimental data were used to assess and improve the performance of a unified open-access mechanistic dermal absorption model based on visual comparisons of the predicted and observed receptor fluid and terminal recoveries from the skin surface, stratum corneum, viable epidermis, and dermis. This model incorporates complexities such as volatility, formulation effects, changes in ionization state, and is applicable to small and large dose studies using *in vitro* and *in vivo* tests. The model is being implemented into the freely available, open-source modeling platform (Open System Pharmacology Suite/PK-Sim/MoBi) to facilitate transparency and compatibility with whole-body PBPK models. Ongoing work is underway to represent metabolic clearance in the skin, which has the potential to affect bioavailability and therapeutic or toxic dose-response relationships. **Conclusions:** The established mechanistic dermal absorption model and provisional workflow provide reliable informed decision-making in the risk assessment of product ingredients applied to skin, ensuring adaptability and continued improvement by the global scientific community.

PS 4024 Interrogating Sidechain Dihedral Correlation and Essential Motion as Predictors of Multi-Kinase Specificity Using Imatinib as a Model System

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Background and Purpose: Drug promiscuity is when a compound interacts with many biological targets, inducing helpful and toxic side effects. It can expand treatments without high design, testing, and approval costs. Imatinib variably binds

kinases: strongly to Abl and Kit, moderately to Chk1 and Lck, and weakly to p38a and Src. The conserved ATP site renders binding factors unclear. All-atom classical molecular dynamics (MD) with novel post-analysis methods were used to predict imatinib specificity with these six kinases. All co-crystals show that imatinib binds the ATP site, except Chk1, which has no crystal. Triplicates were run for each bound- and apo-state system. Thermodynamic analysis yields imatinib-kinase enthalpy and dihedral configurational entropy. Force distribution analysis (FDA), principal component analysis (PCA), and sidechain dihedral correlation networks show how protein dynamics affect specificity. The study shows drug-bound MD accurately identifies these specificity factors. **Methods:** Abl, Kit, Lck, p38a, and Src co-crystals with imatinib were used. For Chk1 kinase, with no available co-crystal, structural alignment of indolylquinolinone-bound Chk1 and imatinib-bound Syk kinase was used to create a Chk1-imatinib complex. All systems used monoprotonated imatinib and retained crystal waters. Bioinformatics tools and SWISS-MODEL were used to add missing loops. A standard protocol was used to set up MD simulations using a cuboid box with 22,307 water molecules and counterions to balance charges. The enthalpy range is found with the total energy mean convergence, as bound-free-ligand+water captures the minimal and maximal triplicate values. The T-Analyst program finds the minimal and maximal dihedral entropy range in kinase binding sites and imatinib rotatable bonds, with bound-free-ligand for all triplicates. These values reveal the upper- and lower-bound Gibbs free energy with $\Delta G = \Delta H - T\Delta S$ for binding thermodynamics. GROMACS FDA shows force vector directions. α -carbon Cartesian PCA with the BKIT package shows essential motions. Sidechain dihedral correlation networks show a hidden communication network controlling protein conformational fidelity. **Results:** MD captures specificity trends by estimating upper- and lower-bound enthalpy, binding site dihedral entropy, and Gibbs free binding energy. Force vectors, α -carbon Cartesian PCA, and sidechain dihedral correlations show a hidden configurational network. Conformational data transmits across the protein, and the network fidelity corresponds with structural changes to let kinases facilitate phosphate transfer to natural substrates for signaling. The protein is more likely to adopt an open form, promote ATP competition, and undergo imatinib dissociation. The sidechain dihedral correlation network identifies distal mutants known to reduce patients' imatinib sensitivity. Higher imatinib-kinase affinity trends with a loss in sidechain dihedral correlations and weak secondary motif migration after binding, corresponding with a more restricted structure to reduce solvent and ATP competition. Lower-affinity proteins show enhanced sidechain dihedral correlation and exaggerated secondary motif motions, consistent with pocket exposure to solvent and more ATP competition. **Conclusions:** This study shows how kinase regulatory motifs adopt favorable configurations via coordinated Cartesian and dihedral motions not previously seen in literature. Predictive drug promiscuity methods were interrogated with imatinib and six kinases. The results show thermodynamics calculations, force vectors, PCA essential motions, and sidechain dihedral correlation networks may explain imatinib specificity. A hidden network shares information about the preferred kinase form after drug binding. Strong and moderate binding kinases, like Abl, Chk1, Kit, and Lck, undergo disrupted ATP competition from interrupted bound kinase breathing motions. Weaker kinases, like p38a and Src, enhance ATP competition from promoted breathing motions. This informs drug toxicity by revealing distinct structural changes corresponding with imatinib-kinase affinity. It may show distinct toxicity modes from interrupted kinase communication networks. Future work will consider the role of coordinated motions on toxin binding. Other deep learning methods to reveal further correlations will be pursued.

PS 4025 Using a Machine Learning QSAR Model to Predict the Half-Life of Organofluorine Pharmaceuticals

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Background and Purpose: Organofluorine pharmaceutical compounds are a class of small organic pharmaceutical drugs with a minimum of one fluorine atom or functional group that contains a fluorine atom. Around 20% of commercially marketed pharmaceutical drugs fall into this category, with over 300 registered globally. In 2018, 45% of the 38 small molecule pharmaceuticals approved by the FDA were organofluorines. Organofluorine pharmaceuticals can account for up to 63% of the extractable organic fluorine measured in human serum and exhibit a broad range of pharmacokinetic profiles. The objective of this study was to develop a proof-of-concept machine learning quantitative structure activity relationship (QSAR) model to predict the half-life of novel organofluorine pharmaceuticals in humans. **Methods:** "Pharmaceutical organofluorine compounds" (n=340) in the EPA CompTox Dashboard list compiled by Inoue et al. (2020) were used to train and evaluate the model. The half-lives of each compound were collected from DrugBank. To ensure complete and quality data, compounds were removed if: 1) SMILES strings were not available for the compound, 2) reported human half-lives were not available, or 3) the half-life as reported on DrugBank differed by more than 50% of the upper bound by route of administration, patient population, or dosing regimen. After curation and deduplication, 193 compounds comprised the final training dataset. Over 1400 1D and 2D physicochemical descriptors were calculated using the open source PaDEL cheminformatics software. Features below a variance threshold of 0.2 and above a globally correlated threshold of 0.8 were removed for a feature set of 281 before hyperparameter tuning. Ten-fold cross-validation and feature selection was used to identify hyperparameters for optimal model performance. A random forest regression algorithm was used and evaluated based on the R² and mean absolute error. **Results:** The model was evaluated on out-of-fold predictions following ten-fold

cross validation using the 20 most important features. Predictions of half-life in hours were within one order of magnitude of the observed half-life in over 90% of out-of-fold predictions in the organofluorine dataset. The model exhibited the highest prediction accuracy in the midrange values, while error increased towards the lower and upper ends of the range. The model's top 20 most important features included 2D descriptors from the Broto-Moreau, Moran, and Geary autocorrelation type, as well as Crippen's LogP, chi cluster, electrotopological state, and information content descriptors. **Conclusions:** Physicochemical descriptors can be used as input features in a random forest machine learning model to identify the most important physicochemical features in determining half-life and to accurately predict the half-life of organofluorine pharmaceuticals. Autocorrelation, lipophilicity, and electrotopological state descriptors emerged as key predictors of half-life. Our machine learning model demonstrated a promising application for New Approach Methods (NAMs) to accurately predict the half-life of novel organofluorine pharmaceuticals.

PS 4026 Leveraging Historical Control Data to Identify Covariates in 28-Day Repeated Dose Toxicity Studies

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Background and Purpose: In conventional vertebrate toxicology studies, control groups are essential for establishing a reference against which treatment effects are compared. Historical Control Data (HCD) aggregates control group data across multiple studies and provides a powerful framework for validating control responses [1]. It plays a critical role in the identification of treatment-related effects by identifying cases where control groups rather than treated groups deviate from the biological baseline. Additionally, HCD enables systematic investigation of external factors that may influence control responses, such as environmental conditions and study design variables. **Methods:** The current study presents a comprehensive analysis of HCD derived from 185 28-day repeated dose toxicity studies conducted at BASF between 2009 and 2023. Using a combination of regression analysis, significance testing, principal component analysis (PCA), and effect size estimation, we systematically identified covariates that impact the HCD distribution. **Results:** Variables such as study date, season, sex, starting age, and vehicle exhibited consistent and meaningful influence on control data. In contrast, factors including the presence of additional blood samples, number of animals per cage, cage enrichment, and the inclusion of urinalysis were found to have no or negligible effects on the assessed HCD. **Conclusions:** These findings strengthen the scientific foundation when integrating HCD in toxicological assessments by identifying filter criteria for more targeted and biologically meaningful HCD. They also challenge the current paradigm of limiting HCD usage to a five-year window in lieu of applying evidence-based applicability timespans. Additionally, our results provide a basis to reduce animal use in mandatory *in vivo* studies by constructing Virtual Control Groups (VCGs), which could serve as substitutes for concurrent controls in selected study designs [2]. References: 1.Kluxen, F. M. et al., Using Historical Control Data in Bioassays for Regulatory Toxicology. Regul. Toxicol. Pharmacol. 125, 105024 (2021). 2.Steger-Hartmann, T. et al., Introducing the concept of virtual control groups into preclinical toxicology animal testing. ALTEX 37(3), 343-349 (2020).

PS 4027 Leveraging Grouping Approaches to Strengthen Read-Across Decision-Making

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Background and Purpose: Confidently predicting toxicity for novel compounds is a central challenge in regulatory toxicology. Read-across, which fills data gaps by extrapolating from structurally or mechanistically similar analogues, can be performed using either a single compound or a group of analogues. Grouping approaches allow for increased confidence in the chosen potency value, which is particularly important in quantitative read-across studies considering both potency and exposure. **Methods:** Data curated from ToxValDB and Vitic were used to identify potential read-across analogues for extractables and leachables, as recommended by the ICH Q3E draft guideline. This allowed the derivation of acceptable intakes for systemic toxicity for these chemicals in the form of permitted daily exposures. *In silico* solutions developed by Lhasa Limited facilitated the formation of read-across categories, enabling the examination of a distribution of point of departure (POD) values. Analogues were assigned confidence levels based on different aspects of their similarity to the target: (i) structural (ii) mechanistic (iii) metabolic (iv) physicochemical. The potency values of the highest confidence analogues were compared to those of the entire category. **Results:** Restricting read-across categories based on similarity criteria and data reliability enables the definition of robust analogue sets, that better represent the potential toxicity of leachables. Typically, the range of POD values within a category spans two orders of magnitude. For instance, 11 of the most highly relevant analogues, identified through *in silico* methods, for 1,3-di-*tert*-butylbenzene exhibited PODs ranging from 10 to 1018 mg/kg/day. The most similar analogue to the target within the category, 1,3-di-*iso*-propylbenzene, had a POD of 150 mg/kg/day, which is located in the middle of the overall range of potency

values. In the case of tris(2,4-di-*tert*-butylphenyl)phosphate, a category of 15 relevant analogues was formed, with PODs ranging from 7.3 to 1000 mg/kg/day. In this example, the most similar analogue to the target within the category, tris(2-*iso*-propylphenyl)phosphate, had a POD of 8 mg/kg/day, at the lower end of the range of potency values. This variability underscores the need for a more comprehensive read-across strategy designed to address potential outliers. Grouping chemicals and expressing toxicity as a range, rather than a single point value, was found to better reflect the diversity of chemically similar compounds and provides a more comprehensive basis for risk assessment. **Conclusions:** Read-across can be successfully conducted using either a single analogue or a group of similar analogues, depending on the use case, specific chemical entity, and quantity of relevant toxicity data available. Expressing toxicity as a range of potency values, rather than a single point, provides a more nuanced and reliable basis for risk assessment. This approach, supported by *in silico* grouping, enhances confidence in safety decisions and reduces subjectivity, especially when the range of potency values does not overlap with the likely exposure concentration.

PS 4028 Optimizing *in Silico* Approaches to Assess the Likelihood of Nitrosylation of Cysteine Residues

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Background and Purpose: A principal mechanism of nitric oxide (NO) regulation of cellular function is the nitrosylation of cysteine (Cys) residues to alter protein function. Nitrosylation is a ubiquitous modification that regulates biological processes ranging from cell survival and growth to physiological function; and its disruption is implicated in a wide variety of pathologies. Nitrosylation can occur via a number of mechanisms and to date approximately 15,000 putative nitrosylation targets have been determined. However, there is no simple consensus sequence for the modification, which makes prediction of NO effects on cellular processes difficult. It is known that the mechanism of nitrosylation for a specific cysteine can be dictated by its microenvironment, including factors such as pKa, hydrophobicity, and surface accessibility. This suggests that the determinants of nitrosylation may be defined by 3D structure rather than merely sequence. The purpose of this study was to determine whether an AI-generated 3D model can be used to predict protein nitrosylation sites by modeling the biophysical characteristics of the sulfur moiety. **Methods:** We predicted protein microenvironments using AlphaFold (AF), a modeling software that can estimate the 3D structure of a protein. An AF-generated model was produced for the enzyme PTEN, which has ten cysteine residues and is a known nitrosylation target. Mutagenesis studies indicate that Cys83 is the primary nitrosylation site, however, Cys71, Cys124, and Cys136 have also been suggested. The variety of Cys residues in PTEN allows one to compare different microenvironments within the same structure. Computational parameters including pK_{int}, hydrophobicity, and solvent-accessible surface area (SASA) were calculated from the AF-model for each of the four cysteine residues that have been implicated in nitrosylation. These parameters were compared with potential mechanisms of nitrosylation formation namely direct nitrosylation, transnitrosation, or nitrosation. **Results:** AF modeling of PTEN revealed distinct microenvironmental signatures among the cysteine residues. Cys83 exhibited a high SASA value (89.96) and is predicted to undergo nitrosylation through transnitrosation. Cys124 displays a low pK_{int} (7.079) and is predicted to be reactive under conditions that favor nitrosation. Cys136 has a high hydrophobicity index (2.39) and is predicted to favor direct nitrosylation. These computational predictions align with previously published mutagenesis data that implicate the same residues in nitrosylation. Experimental validation using biotin-switch and LC-MS/MS analysis will be used to confirm the reaction mechanisms predicted for each of the sites. Different nitroso compounds can be used to favor the predicted reactive pathways namely S-nitrosoglutathione (GSNO) for transnitrosation, S-nitrosopropidium (SNOPPM) for direct nitrosylation, and DEANONOATE for nitrosation. In preliminary studies, 400 ng of PTEN were exposed to a 50 μM of GSNO, SNOPPM, or DEANONOATE for 45 minutes. A Biotin switch to identify nitrosylated protein was performed. Nitrosylated material was isolated using Streptavidin beads. The resultant material was examined by Western blot analysis and staining with PTEN antibody. Under these conditions, SNOPPM had the highest level of nitrosylation, while GSNO showed a low level of nitrosylation, and no SNO formation was seen with DEANONOATE. These data implicate direct nitrosylation of Cys 136 as the most efficient mechanism of NO-modification of PTEN. However, these results require further clarification by LC-MS/MS to confirm. **Conclusions:** By combining AI-predicted structural values with biochemical validation, we aim to define whether AF modeling can accurately forecast mechanism-specific nitrosylation *in vitro*. These studies will lead to a better understanding of how NO reactivity can alter cellular function.

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Background and Purpose: Increased efforts have been made to reduce animal use in human safety and efficacy studies through the adoption of new approach methodologies (NAMs) and computational-based approaches. Techniques to grow primary human airway epithelial cells *ex vivo*, specifically at air-liquid interface (ALI), have proven to be valuable for testing pharmacologic and toxicologic endpoints. However, airway region-specific differences in epithelial cell populations and technical variability in *ex vivo* culture methods introduce concerns regarding the accuracy and comparability of results generated between groups. This study sought to compare the gene expression profiles of healthy human nasal (HNEC), bronchial (HBEC), and small airway epithelial cells (SAEC) immediately following collection as well as following *ex vivo* culturing at ALI using commonly employed methodologies. **Methods:** Transcriptomic data were obtained from the All RNA-seq and ChIP-seq sample and signature search (ARCHS4) database, which aggregates and uniformly processes publicly available RNA-seq datasets from the gene expression omnibus (GEO) and sequence read archive (SRA) to enable large-scale analyses. Data from healthy HNEC, HBEC, and SAEC samples were isolated and designated as “tissue” or “ALI culture” using key word filtering and natural language processing (NLP) of metadata and linked publications. A total of 2542 samples were isolated, including 1152 HNEC, 243 HBEC, and 109 SAEC tissue samples. Dimensionality reduction was performed using Uniform Manifold Approximation and Projection (UMAP) and differential expression analysis was conducted between key groups of interest. Key gene signatures for tissue samples were determined using an elastic net model and ALI cultures were assessed for relation to their source tissue. Gene expression patterns were compared to airway cells from the LungMap CellRef 1.0 Atlas single-cell RNAseq (scRNA-seq) dataset. **Results:** UMAP plots demonstrated clear distinctions between tissue and ALI culture samples. An elastic net model trained on HNEC, HBEC, and SAEC tissue samples demonstrated strong predictive capacity resulting in a 99.2% (373/376) accuracy rate; however, ALI cultures demonstrated mixed results when predicting source tissue with 97.5% (192/197) accuracy for HNEC, 41.6% (319/767) for HBEC, and 38.6% (277/70) for SAEC samples. Differential expression analysis identified major differences between HNEC and SAEC (317 DEGs) with slight differences between HNEC and HBEC (73 DEGs) and HBEC and SAEC (82 DEGs) tissue samples. In contrast, major differences were detected between ALI culturing methods and their source tissue resulting in between 5000 and 7000 DEGs. Using an elastic net model trained on the CellRef scRNA-seq dataset, HNEC tissue samples predicted as goblet cells (94%), HBEC tissue samples demonstrated mixed ciliated/goblet cell predictions (73/27%), and SAEC tissue samples demonstrated ciliated cell predictions (96%). ALI cultured HNEC samples retained a prediction of goblet cells while HBEC and SAEC ALI cultured samples demonstrated a mix of basal, ciliated, and goblet cells. **Conclusions:** This large-scale RNA-seq analysis revealed distinct airway compartment profiles, with the greatest differences seen between nasal and small airway regions. Across all airway regions, ALI culturing induced substantial transcriptional changes, though some compartment-specific signatures persisted; HNEC largely retained goblet-like expression, while HBEC and SAEC expressed mixed basal, ciliated, and goblet-like patterns depending on *ex vivo* culture conditions.

PS 4030 Dosimetry Models of Inhaled Flavoring Compounds in ENDs Aerosols and Estimation of Tissue Concentrations in the Mouse and Human Respiratory Tracts

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Background and Purpose: Exposure to flavoring compounds in electronic nicotine delivery systems (ENDs) aerosols via inhalation raises potential health concerns. Although many flavoring compounds in ENDs products have been evaluated and affirmed as GRAS (Generally Recognized as Safe), this evaluation does not directly apply to exposure via inhalation. To better assess the health risk, physiologically based pharmacokinetic (PBPK) models have been used for interspecies extrapolation and route-to-route extrapolation. In this study, we developed PBPK models specifically for the ENDs aerosols, which are composed of vapors and suspended fine particles in the gas phase, to estimate the tissue level of flavoring compounds in the mouse and human respiratory tracts. **Methods:** We combined the PBPK model with the multiple-path particle dosimetry (MPPD) lung deposition model, because of the multiphase characteristics of the ENDs aerosol. The PBPK contained a multi-compartment respiratory tract model based on anatomical location and functional structure, comprising three regions of the respiratory tract (the upper respiratory tract [URT], the tracheobronchial region [TB], and the alveolar region) and a pulmonary compartment, which represents the gas exchange regions of the lung. Each region was further divided into a lumen for air passage, surrounded by epithelium and submucosa tissue layers, which account for the transport of absorbed chemicals across the anterior respiratory tract compartments. The structure of the model was the same for mice and humans, with species-appropriate anatomy and physiology. The MPPD model was executed for a mass median aerodynamic diameter of 0.8 μm with a geometric standard deviation of 1.4 μm for the ENDs aerosols. Tissue concentrations of nicotine and 38 select flavoring compounds in each previously

defined region were calculated and z-score normalized to compare the deposition of flavors within and between mice and humans. A correlation analysis was conducted to examine the impact of modeling parameters on the results. **Results:** The MPPD model predicted different deposition patterns in mice and humans: the aerosol deposition in the URT, the TB region, and the alveolar region was 53.3%, 2%, and 2% in mice, and 0.9%, 6%, and 15% in humans, respectively. The MPPD-PBPK model was validated using plasma nicotine concentrations from a mouse inhalation study and subsequently applied to flavoring compounds. Based on calculated concentrations, three groups of flavoring compounds were identified: four flavors with higher blood and low respiratory tissue concentrations due to very low liver clearance, 20 flavors with low blood and respiratory tissue concentrations, and 14 flavors with low blood but higher respiratory tissue concentrations. For the mouse model, tissue concentrations correlated to n-octanol:water partition coefficient, molecular weight, water solubility, and vapor pressure, with correlation coefficients of >0.67, >0.43, <0.53, and <0.19, respectively. For humans, the tissue concentration of the alveolar region weakly correlated with the n-octanol:water partition coefficient, molecular weight, water solubility, and vapor pressure, with correlation coefficients of 0.43, 0.28, -0.33, and -0.20, respectively. **Conclusions:** We developed dosimetry models for ENDs aerosols to predict tissue concentrations in the respiratory tracts of humans and mice. The results indicate species-specific deposition patterns in humans and in mice and suggest that lipophilic compounds tend to accumulate in respiratory tissues regardless of their partitioning in particle/vapor phases. The correlation analysis indicates that the behavior of flavoring compounds is more predictable based on their physicochemical properties in the mouse model than in the human model. Therefore, the use of a generic dosimetry model for human exposure may require additional caution to ensure relevance and accuracy.

PS 4031 Computational Modeling of In Vitro Antral Follicle Growth and Steroidogenesis as a New Approach Methodology in Reproductive Toxicology

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Background and Purpose: Millions of women face infertility issues, often due to the inability to produce a viable egg. Folliculogenesis, the developmental process of ovarian follicles, begins with the activation of primordial follicles and progresses through various stages until reaching the antral stage, capable of hormone production, and egg release. This complex process is regulated by a network of endocrine, paracrine, and autocrine signals, which can be disrupted by toxicants, potentially leading to infertility. We are developing *in vivo*, *in vitro*, and *in silico* models of gonadotropin-dependent ovarian follicle development to explore toxicant effects on reproduction. Our long-term goal is to use mechanistic, multiscale computational models for *in vitro* to *in vivo* extrapolation (IVIVE) of toxicant effects. Here we present a multiscale computational model of mouse antral follicle development *in vitro* that includes follicle cell proliferation and steroid hormone production. **Methods:** We have developed a spatial model simulating an *in vitro* cultured mouse follicle growing from the secondary to antral follicle stage under treatment by follicle-stimulating hormone (FSH). The model is in CompuCell3D (CC3D), which allows us to incorporate subcellular metabolism and hormone signaling. This model includes multiple cell types: two types of thecal cells (external and internal), three types of granulosa cells (outer and inner-layer mural, and cumulus), the oocyte modeled as a nucleus, cytosol and wall (zone pellucida), and a pseudo-cell type for the proteoglycan extracellular matrix of the expanding antrum. The simulation spans 8 days of follicle growth *in vitro*. Granulosa cells secrete proteoglycans into the antrum causing the antrum to expand. In addition, the granulosa and theca cells proliferate to maintain the proper layering of cells as the follicle expands. Each theca and granulosa cell has an embedded reaction kinetic network representing the steroidogenesis pathway. Theca cells include conversion of cholesterol to testosterone, and granulosa cells include conversion of testosterone to estradiol. We calibrate our model in terms of *in vitro* measured cell numbers, follicle geometry and steroid concentrations. **Results:** Our spatial model can reproduce the observed follicle growth, including relative abundances of theca and granulosa cells, the size of the antrum, and levels of secreted sex hormones in the follicular fluid, as a function of time. We find that the model is underdetermined with respect to some of the reaction kinetic parameters describing subcellular steroidogenesis. In addition, we find that additional granulosa phenotypes are needed to explain the range of behaviors that these cells exhibit. Nonetheless, we believe that our current model can make useful predictions of the effects of toxicants that interfere with the steroidogenesis pathways in theca and granulosa cells. **Conclusions:** An initial multiscale computational model of antral follicle development in CC3D reproduces key features of the developmental process, including cell proliferation and steroidogenesis. Our next goals include (1) coupling systemic follicle growth signals such as FSH and LH and (2) developing predictions of toxicant effects on steroidogenesis that lead to abnormal follicle growth. Our long term goals include coupling whole-body physiologically based kinetic models for hormone signaling between the ovary and pituitary as well as coupling toxicant exposure at the whole-body level to predictions of levels within the follicle. This latter goal is key to the development of *in silico* models needed to predict ovarian toxicity response based on whole-body exposure to toxicants.

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Background and Purpose: Humans are exposed to numerous chemicals with limited developmental neurotoxicity (DNT) data, underscoring the need for efficient strategies to identify potential developmental neurotoxicants. The NIEHS DNT Health Effect Innovation (HEI) program employs a battery of *in vitro* human and rat neural cell assays, together with embryonic zebrafish behavioral assays, to evaluate chemicals with or without existing DNT evidence. This screening battery comprises 17-endpoint associated Key Neurodevelopmental Processes (eKNDPs), such as neuronal differentiation, neurite outgrowth, network formation, etc. The NIEHS DNT HEI screening effort includes three phases: Phase 1 and Phase 2 screening are complete, while Phase 3 remains ongoing. This study focused on the development of an analytical methodology applied to Phase 1 data, with plans to extend its use to combined Phase 1 and Phase 2 datasets once available. Identifying a minimal yet informative subset of eKNDPs can streamline screening and improve resource allocation. This methodology seeks to investigate: Which eKNDPs are most critical for prioritizing known DNT compounds? **Methods:** Benchmark concentration (BMC) modeling was used to evaluate concentration-response data for assay endpoints categorized under each of the 17 eKNDPs. The 17 eKNDPs spans spanning proliferation, apoptosis, cell migration, neuronal differentiation, neurite outgrowth, neuronal maturation and synaptogenesis, oligodendrocyte differentiation, network formation, and neurobehavior. Endpoint-level results were summarized into five attributes: BMC, degree of selectivity, activity confidence, fraction of active endpoints, and selectivity binary call. Selectivity refers to the extent to which an observed DNT effect is distinct from general cytotoxicity. The five attributes were integrated using a Pareto Frontier approach to generate a level score for each chemical per eKNDP. The 17 eKNDP level scores were combined to produce an overall DNT hazard ranking. To address the research question, we assessed the ability of individual eKNDPs and all possible eKNDP combinations to enrich DNT-positive compounds at the top of the ranking. Performance was quantified using Receiver Operating Characteristic Enrichment at 10% (ROCE@10%). High-performing subsets of eKNDPs exceeding predefined ROCE@10% thresholds were further analyzed using association rule mining to identify eKNDPs that frequently co-occur in these combinations and thus represent core contributors to prioritization performance. **Results:** A total of 2,988 eKNDP combinations exceeded the top 1% ROCE@10% threshold, demonstrating strong enrichment of DNT-positive compounds among the highest-ranked chemicals. From these, 112 maximal itemsets (i.e., the largest sets of eKNDPs that frequently co-occur without being subsets of any other itemsets) were identified. Among these, 31 itemsets had ROCE@10% values above the top 1% threshold. Notably, four-eKNDP combinations were predominant within these 31 itemsets, accounting for approximately 77%. The eKNDP with the most frequent selection was LDTT Locomotor Activity @120 hpf (from larval zebrafish), followed by HCl hNP1 Proliferation@24h (from human neural progenitor cell line) and MEA Dev Network Formation@288h (from rat primary neocortex cultures). These relatively distinct and complex eKNDPs were consistently associated with higher ROCE@10% values, indicating their strong contribution to prioritizing DNT-positive compounds. **Conclusions:** Phase 1 data show that a focused subset of eKNDPs can substantially enrich DNT-positive compounds at the top of the ranking. This analytical framework will be applied to combined Phase 1 and Phase 2 datasets to validate these eKNDPs and enhance their utility for DNT prioritization and prediction.

PS 4033 Development of Machine Learning Models to Predict Blood Brain Barrier Permeability of Chemicals

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Background and Purpose: The Blood Brain Barrier (BBB) is a highly selective semi-permeable membrane that protects the brain from harmful substances circulating in the blood. By strictly controlling which molecules can enter the brain, the BBB helps protect against neurotoxicity. However, experimental determination of chemical BBB permeability remains a resource-intensive and technically challenging process. Computational approaches offer a valuable alternative to address this data gap and facilitate rapid prediction of BBB permeability for chemicals of interest. Recently, Meng et al. (*Scientific Data*, 2021, 8(1), 289) introduced the B3DB database, a comprehensive resource containing experimentally derived BBB permeability data for 7807 compounds compiled from over 50 published sources. Utilizing this dataset and different machine learning (ML) techniques, we developed predictive models for BBB permeability of chemicals. **Methods:** The B3DB data (<https://github.com/theochem/B3DB>) contained 4856 chemicals that penetrate the BBB (BBB+) and 2851 that do not (BBB-). The final, curated dataset included 2447 BBB+ and 1521 BBB-

molecules after removing salts, stereoisomers, duplicates and invalid information. Model evaluation was carried out with two splitting methods: 5-fold cross validation (CV) and structural diversity. For 5-fold CV, the data set was split into five training (80%) and test (20%) sets so that each chemical appeared in the test set at least once. For structural diversity validation, the Murcko Scaffold module from Python's RDKit package was utilized to extract molecular scaffolds from data set compounds. Molecules were split into training (80%) and test (20%) sets such that molecules with the same scaffold appeared in a single set. Classification models were developed using three sets of molecular descriptors: Molecular ACCess System structural keys, circular extended-connectivity Morgan fingerprints, and Mordred physicochemical molecular descriptors. Two ML classifiers were utilized to construct models: random forest, and XGBoost. The recently proposed graph convolutional neural network technique, implemented in the Chemprop package (<https://github.com/chemprop>), was also employed. Four statistical parameters were used to evaluate the performance of developed classification models: balanced accuracy (BACC), sensitivity (SEN), specificity (SPE) and area under the curve (AUC). **Results:** Initially, hyper-parameters for ML classifiers were optimized by performing 20 trials with different parameter sets and final models were developed using hyper-parameters from the best trials. All models had high AUC values (range: 0.85 - 0.89) and were able to correctly identify BBB+ chemicals with a sensitivity ranging from 0.86 - 0.89. However, the models had more moderate success at identifying BBB- compounds (range: 0.6 - 0.7). Such differences in model performance can be attributed to the imbalanced number of BBB+ (62%) and BBB- (38%) compounds in the modeling data set. After applying different balancing techniques, we were able to improve model performance on BBB- compounds. The best performing model was the graph convolutional neural network model with BACC = 0.79, SEN = 0.80, SPE = 0.79, and AUC = 0.88. Finally, we combined prediction of all seven balanced ML models into a consensus model that performed with a BACC = 0.81, SEN = 0.83, SPE = 0.79, and AUC = 0.89. **Conclusions:** The developed ML models provide a first step in screening a large number of chemicals for potential neurotoxicity and quickly identify chemicals for *in vitro* testing. Binary models, such as the one developed herein, provide a rapid yes/no answer to whether the predicted effect is likely to be true, which then allows the chemicals that are unlikely to cross the BBB to be deprioritized. This helps to reduce the number of necessary tests and improves study design. Regression models are being developed to estimate the extent to which chemicals cross the BBB permeability to aid in identifying chemicals with the highest potential for central nervous system toxicity. **Disclaimer:** No DoD endorsement implied.

PS 4034 Industry Perspectives on Evolving Off-Target Assessment: Insights from a Multicompany IQ DruSafe Survey

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Background and Purpose: Secondary pharmacology screening of investigational small-molecule drugs for potentially adverse off-target activities has become a well-established process and an essential stage in the characterization and development of novel pharmaceuticals. This screening helps ensure candidate molecules possess an acceptable safety profile by detecting interactions with undesirable targets. A recent publication reported that about one-third of the companies surveyed apply a core test of secondary pharmacology targets during drug lead optimization and over 90% of the companies conduct a core *in vitro* panel before drug candidate selection (Brennan et al., 2024; Schmidt et al., 2025). The paper also highlights that most organizations utilize panels exhibiting a high degree of overlap in the targets assessed, indicating that industry standards for secondary pharmacology screening are becoming increasingly harmonized. Review of data and literature by the IQ DruSafe Secondary Pharmacology Working Group helped to identify 33 additional targets for inclusion in secondary pharmacology profiling, reflecting the industry's drive towards a more comprehensive characterization of off-target risks. **Methods:** With general consensus forming around the composition of a core *in vitro* screening panel, an industry survey was conducted to identify opportunities to further expand the impact of off-target assessments of new molecules. **Results:** Sixteen companies responded to a broad range of questions which sought opinions on the potential integration of advanced computational capabilities, improving the evaluation for new or novel drug modalities, and adoption of new technologies for the assessment of off-target toxicology. The survey concluded that the greatest unmet need was an improved understanding of off-target assessments of protein degraders. Additionally, it recommended that panels for specific adverse effects such as convulsions, abuse liability, and neuropathy would be useful. Furthermore, the survey concluded that an industry-wide assessment of *in silico* capabilities and computational tools would be useful. Lastly, the group acknowledged that a broader discussion to harmonize the way secondary pharmacology results are submitted to regulatory agencies is necessary and beneficial. **Conclusions:** The effort could reveal innovative approaches that help streamline secondary pharmacology profiling and impact and further strengthen the overall efficiency of drug discovery and development processes.

PS 4035 Preclinical Safety Assessment of Targeted Protein Degraders: Current Practices and Considerations

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Background and Purpose: The preclinical safety assessment of Targeted Protein Degraders (TPDs) presents unique challenges due to their unique physicochemical characteristics and novel mode of action involving induced protein ubiquitination and degradation. This presentation provides an overview of current practices employed across the pharmaceutical industry and academia to identify and evaluate potential safety concerns associated with TPDs. **Methods:** Insights from stakeholders were gathered through workshops highlighting emerging strategies such as proteomics-based target profiling, degrader selectivity assessment, and species selection for *in vivo* studies. In addition, case studies and a stakeholder survey were used to collect detailed input on study design considerations, including the use of *in vitro* systems, analytical methodologies, *in vivo* selectivity profiling, and data interpretation. **Results:** Overall, current practices reflect a pragmatic integration of established small molecule safety principles with emerging, modality-specific approaches tailored to TPDs. Out of these strategies, a majority (~92%) of the stakeholders indicated the incorporation of proteomics (global and/or targeted) as a common practice to assess degrader selectivity, targeted evaluation of functional liabilities (on/off target assessment), and adaptive preclinical study designs informed by degradation kinetics and tissue-specific risk profiles. **Conclusions:** These efforts provide a snapshot of safety evaluation strategies currently in use and identify key challenges in translating mechanistic insights into predictive toxicology frameworks.

PS 4036 Development of a Population Pharmacokinetic Model to Optimize Florfenicol Dosing for Treating Lactating and Non-Lactating Goats

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Background and Purpose: Florfenicol is a broad-spectrum antimicrobial that is administered to goats for treating respiratory disease. However, its use in this species is extralabel in the United States, and dosing recommendations for specific physiological populations is lacking. Such gaps may lead to inappropriate drug exposure and violative residues, posing potential food safety concerns. Population pharmacokinetic (PopPK) data for goats across differing physiological states are limited. This study aimed to develop a PopPK model for florfenicol in goats and assess effects of body weight, lactation, and dosing frequency on pharmacokinetic parameters to better optimize treatment regimens for various goat populations. **Methods:** Nineteen goats, including both lactating and non-lactating animals, received two doses of florfenicol (40 mg/kg) via subcutaneous injection at a 96-h treatment interval. Plasma concentration versus time profiles were analyzed using nonlinear mixed-effects modeling in Phoenix. Model selection, variability estimation, and covariate screening were performed stepwise based on objective function reduction, diagnostic plots, and visual predictive checks. **Results:** A two-compartment model with first-order absorption and elimination best described florfenicol pharmacokinetics. The population mean estimates were: absorption rate constant (K_a) = 0.51 h⁻¹, clearance (CL) = 0.54 L/h/kg, intercompartmental clearance (CL₂) = 1.08 L/h/kg, central volume of distribution (V₁) = 20.9 L/kg, and peripheral volume (V₂) = 80.0 L/kg. Body weight did not significantly affect K_a , V₁, CL, V₂, or CL₂. Lactation was a significant covariate on CL₂, increasing intercompartmental clearance by about 16.64% compared with non-lactating animals. The inter-occasion variability (IOV) estimates for K_a , V₁, CL, V₂, and CL₂ were small (≤0.09), indicating minimal within-animal differences between the two dosing occasions. **Conclusions:** The developed PopPK model adequately captured the pharmacokinetic variability of florfenicol in goats. Lactation primarily influenced intercompartmental clearance, while dosing occasion showed minimal impact on pharmacokinetic parameters. Body weight had negligible effects. These findings provide a quantitative foundation for dose optimization and preliminary assessment of food safety in both lactating and non-lactating goats.

PS 4037 Rigorously Optimized Immunohistochemistry Methods Support Robust Assessment of Pharmacodynamic Effects in African Green Monkeys

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Background and Purpose: Immunohistochemistry (IHC) with quantitative image analysis remains an informative and cost-effective method for assessing pharmacodynamic (PD) endpoints in nonclinical species. However, variability in reagent characterization and assay verification across species limits reproducibility and biological confidence. This challenge is increasingly critical in the era of AI-assisted pathology and image analysis, where automated interpretation may conflate valid biological signals with artifacts or algorithmic hallucinations. Consequently, there is

a growing need for rigorous, multi-layered validation frameworks that can substantiate the biological and run-to-run fidelity of IHC reagents and assays. **Methods:** Our workflow integrates orthogonal molecular approaches, notably single-cell RNA sequencing (scRNA-seq) with systematic experimental optimization. Target proteins are first characterized, leveraging publicly available scRNA-seq and proteomic datasets to define expected cellular and subcellular localization patterns. This molecular context establishes a biologically grounded expectation for staining specificity and distribution. Each IHC assay is then optimized using a tissue microarray (TMA) composed of 20-30 perfused, fixed tissues from the African green monkey (AGM, *Chlorocebus sabaeus*). TMAs represent broad tissue diversity encompassing most of the FDA-recommended list for tissue cross-reactivity studies, replicate sets from distinct animals, or fixation- and phenotype-specific cohorts. After selecting appropriate TMA(s), assay development proceeds across a range of conditions designed to generate signal intensities from near extinction to saturation, enabling fine control of dynamic range and minimizing off-target staining. Both chromogenic and fluorescent modalities are optimized in parallel, facilitating adaptation to multiplexed IHC workflows. Multiple antibodies against the same target protein are assessed for commonly used markers. Following optimization, TMA slides are digitally scanned, archived, and used as long-term reference standards to assess assay stability and reproducibility. Over 120 assays were developed on AGM samples, with many also evaluated on murine TMAs. **Results:** Assay performance, optimal conditions, and comparative antibody data were compiled into a database of annotated slide decks. Antibodies that recognize epitopes associated with the same cell types (I.E., endothelial cells) or that recognize cells belonging to the same class (I.E. different classes of T-cells) were compared and grouped together in additional slide decks. Commercially available antibodies were suitable for detecting almost all proteins we attempted to detect in AGM samples. We observed that the detection of certain proteins is fixation-dependent, such as IBA1 which is nearly impossible to detect in Davidson's-fixed samples and alternative markers of macrophages and microglia were necessary. The optimal antibody and antibody concentration were also often fixation- and species-dependent. Only 1 of 3 clones of CD31 yielded an adequate signal in AGM tissues whereas a different clone was more optimal in murine tissues. PV-1 better demonstrates neovascularization in the choroid compared to other endothelial markers, whereas Claudin-5 better visualizes vessels of the blood brain barrier. Common tissues and cell types prone to non-specific signals were also made evident by this system. Of note, G cells, plasma-rich tissues, thyroid follicular cells, and acinar cells more commonly demonstrate non-specific signals. **Conclusions:** This approach provides a robust means of assessing pharmacodynamic endpoints derived from IHC and characterizes any inherent flaws of a given assay, enhances scientific confidence, and offers a harmonized standard for a high-fidelity research environment. It is a useful tool for rapidly screening antibodies in the context of different species, tissue types, and IHC methods and reagents. It reduces interobserver variability by establishing documented and easily referenced ground truths in a database. Image analysis pipelines are enhanced by facilitating more accurate annotation and enabling quality control against robust references.

PS 4038 Assessing Fibrosis: Picrosirius Red and COL1A1 Immunohistochemistry as Alternatives to Masson's Trichrome Staining

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Background and Purpose: Masson's trichrome (MT) stain, developed by Canadian pathologist Claude L. Pierre Masson in the early 20th century, remains one of the most widely applied histological methods for identifying and quantifying collagen deposition in tissues. MT and its numerous adaptations have been integral in assessing fibrosis and extracellular matrix (ECM) remodeling in a variety of disease contexts, including metabolic dysfunction-associated steatohepatitis (MASH), cardiac fibrosis, and tumor-associated stroma formation. Despite its ubiquity and low cost, MT is subject to interpretative variability and technical limitations that reduce its specificity and sensitivity, particularly when quantitative image analysis is required. **Methods:** Masson's trichrome staining was systematically compared to a modified Picrosirius Red (PSR) protocol incorporating phosphomolybdic acid and collagen type I alpha 1 (COL1A1) immunohistochemistry (IHC). The comparison was performed across a panel of 20 normal and 20 diseased tissues derived from African green monkeys (*Chlorocebus aethiops*), representing a diverse range of tissue architectures and pathologic conditions. **Results:** While MT staining reliably identified regions of dense collagen, its limitations were pronounced. Incomplete visualization of both thick and thin collagen fibers, non-specific staining of plasma and goblet cells, a broad range of blue chromatic intensities historically interpreted as "collagen," and occasional nuclear staining all contributed to challenges in both qualitative assessment and automated digital quantification. In contrast, PSR staining, particularly when enhanced with phosphomolybdic acid, produced superior fiber differentiation and more consistent contrast between collagen and surrounding parenchyma. PSR's strong birefringence under polarized light provided an additional analytical dimension, facilitating assessment of collagen maturity and organization. However, like MT, PSR remains a non-specific histochemical approach and cannot discriminate between collagen subtypes or related ECM proteins. COL1A1 IHC demonstrated the highest specificity and structural resolution among the three methods. This approach revealed extracellular matrix elements that were underrepresented or entirely absent in MT and PSR staining, including basement membranes

of hepatic and adrenal sinusoids, periadipocytic collagen, and delicate pericapillary collagen networks within the lamina propria of the small intestine. These findings were consistent with prior observations in rodent and human tissue panels from our laboratory. **Conclusions:** Overall, while MT retains value as a rapid and cost-effective stain for general fibrosis assessment, its interpretive and technical shortcomings limit its suitability for quantitative digital pathology. PSR represents an improved histochemical alternative when cost constraints preclude immunohistochemistry but may require assessment of collagen organization under polarized light. However, COL1A1 IHC provides the most biologically relevant and reproducible measurement of collagen deposition and ECM remodeling, making it the preferred method in workflows that incorporate digital image analysis or where precise mapping of collagen architecture is required. Future work will extend this comparative framework to include additional IHC reagents targeting other collagen subtypes, such as COL4A1, to better delineate the structural and functional diversity of ECM components across tissues and disease states.

PS 4039 A Sliding Scale to Evaluate Safety Margins for Drugs with a Long Half-life: Oteseconazole, A Case Study

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Background and Purpose: While there is no mandatory FDA safety requirement that explicitly states a drug must be cleared for a specific number of half-lives, the “five half-lives rule” is a widely accepted *pharmacokinetic principle*, often adopted by the FDA, and used as a general guideline for when a drug is considered to be effectively eliminated from the body or has reached steady state, as approximately 94% to 97% of the drug is cleared after 4 to 5 half-lives. The appropriateness of this concept, in context of FDA guidelines and the impact on safety margins for drugs with a long half-life will be discussed. **Methods:** Drug safety margins are typically calculated as the exposure ratio between the no observed adverse effect level (NOAEL) in animal species and the maximum recommended human dose (MRHD). This ratio is critical for assessing risk, especially in reproductive and developmental toxicity, and is outlined in S5(R3) Detection of Reproductive and Developmental Toxicity for Human Pharmaceuticals Guidance for Industry May 2021 ICH Revision 3. Safety margins and the requirement for contraception are critical considerations in clinical trials and for the use of certain pharmaceuticals, particularly when there is a potential risk of harm to a fetus. When this margin is insufficient, contraception is often required to prevent pregnancy. Patient-centered approaches now prioritize a person’s preferences and autonomy in making contraceptive decisions, but safety concerns remain a core consideration. Typically, small molecules may require the use of effective contraception for five terminal plasma half-lives. This period ensures that the systemic concentration of the drug has been sufficiently cleared. For drugs with exceedingly long half-lives, ensuring strict adherence to contraception use could prove overtly challenging and delay access to an important treatment option. This rule does not take into consideration that safety margins change over time as the drug is eliminated and a larger safety margin that is considered minimal risk could be achieved much sooner than 5 half-lives. **Results:** Oteseconazole (VIVJOA®) is approved in the USA and in China for the treatment of recurrent vaginal candidiasis, and severe vaginal candidiasis, respectively, in women who are not of reproductive potential. Oteseconazole was rationally designed to target fungal cytochrome P51 while avoiding human cytochromes thus avoiding safety concerns often associated with the azole drug class. By avoiding host cytochromes typically responsible for drug metabolism and elimination, the resulting terminal half-life is calculated to be 138 days, and pharmacokinetics are further influenced as the drug is 99.5-99.7 bound to plasma. Based on ocular abnormalities reported in rat pre- and postnatal development studies, and in accordance with the FDA Guidance S5(R3), a 3.5-fold safety margin was established for oteseconazole at the time of last dose. This is considered to be a safety margin corresponding to a threshold for “increased concern.” Pharmacokinetic analysis of plasma from women participating in Phase 3 studies indicated that at the 12-month time point from last dose (approximately 3 half-lives) a safety margin of >25-fold is established. Based on the FDA Guidance, a 25-fold safety margin is considered a “minor concern” for clinical use of a drug. **Conclusions:** The modeling supports the position that acceptable safety margins can be readily achieved for drugs with a long half-life and the “five half-lives rule” is potentially unnecessary and may limit patient access.

PS 4040 Implementation of the Modified Irwin Test as a General Functional Observation Battery for the African Green Monkey

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Background and Purpose: The use of neurobehavioural methods for determination of neurotoxic potentials of NCEs (new chemical entities) is recommended and implemented in the ICH S7A, 2000. As nonhuman primates are the closest phylogenetic relatives to human, they play an indispensable role in biomedical research due to their highly evolved behavioral repertoire which makes them valuable animal models for studies of the functional effects of potential drug therapies and neurotoxins (Sibal and Samson, 2001; Burbacher and Grant, 2000). While general functional

observation batteries (FOB) are well described for macaques (e.g. modified Irwin test; *Gauvin and Baird, 2008; Korte, 2007*), limited literature is available for safety assessments in the African green monkeys (AGMs). As these examinations are an essential part of toxicity studies in AGM’s when undergoing nonsystemic ASO, AAV and other gene therapies, or embryonic stem cell delivery in the intrathecal space or the brain, background data were required to allow adequate safety assessment. **Methods:** In two regulatory safety studies in which 50 AGMs underwent serial morning non-sedated cage side assessment of peripheral and central nervous system activities pre-dose and pre-study, excluding test article effects. At each timepoint, the recorded observations included respiration, head twitches, vocalizations, body position, jumping, auditory reflex, locomotor activity, tremor, righting reflex, sedation / excitation, twitch, ataxia, seizures, piloerection, lacrimation, aggressiveness toward handler, salivation, unusual behavior, grooming. In addition, neurological exams with non-sedated animals were conducted at designated intervals by a veterinarian and included the following tests: gait, proprioceptive positioning, placing reactions (visual and tactile), head movements, head muscle tone, eye reactions, eye symmetry, eye position, vestibular nystagmus, pupillary light reflex, nasal septum, palpebral reflex, eye tracking, jaw tone, muscle tone, perineal reflex, and withdrawal reflex. **Results:** Experimenters noted increases, decreases, and severity of the behavioral symptoms or physiological manifestations in comparison with (a) normal stock animals and (b) the control group. The standard rating system consisted of a score of 0 = normal activity of each specific outcome. Scores of 0, 1, 2, 3, or 4 grade the level of behaviors observed as normal (healthy stock animal), slight, moderate, extreme, or severe. **Conclusions:** In summary, the normalized FOB database now allows robust comparison with test article treated AGMs and provides a citable historical normal reference range.

PS 4041 Cardiovascular and Respiratory Safety Pharmacology Study of Mitragynine in Telemetered Dogs

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Background and Purpose: Opioid Use Disorder (OUD) is a chronic condition, characterized by increased opioid tolerance, compulsive and continued use of opioid despite having harmful effects and a significant risk of lethal overdose. OUD significantly diminishes quality of life, contributing to a widespread epidemic and it has been designated as “Public Health Emergency” in the United States. Buprenorphine and methadone are the first-line treatment options for OUD patients. Methadone is a long-acting, full mu-opioid receptor (MOR) agonist, available in single daily doses at highly regulated clinics. However, buprenorphine is a partial MOR agonist with a lower risk of lethal overdose and can be taken with fewer restrictions. Most of the buprenorphine patients aim to ultimately discontinue the opioid use, with the support of naltrexone (MOR antagonist) therapy. Like other opioids, buprenorphine can lead to dependence and withdrawal symptoms if stopped abruptly, in addition to the risk of serious respiratory depression with high doses. Mitragynine is a primary alkaloid derived from *Mitragyna speciosa*, commonly known as *kratom*, a tropical tree native to Southeast Asia. Its leaves have been consumed orally, either as tea or by direct chewing and used as an analgesic, a stimulant, and a mood enhancer. Mitragynine is a less potent MOR partial agonist, with lower agonist activity than buprenorphine and possesses milder withdrawal symptoms. In addition, mitragynine acts as an alpha 2 adrenergic receptor agonist. The low MOR activity makes mitragynine an appropriate bridging therapeutic for transition from long term buprenorphine to naltrexone therapy for OUD. Thus, we are developing mitragynine as an oral therapy for successful taper-off of buprenorphine in OUD patients while transitioning to naltrexone therapy to achieve opioid-free life. **Methods:** The objective of this study was to evaluate the acute effects of mitragynine following a single oral administration (0, 2.5, 7.5 and 12 mg/kg) on cardiovascular and respiratory functions in the conscious beagle dogs. Four male and four female dogs were assigned to four oral dose levels on four dosing days with assignments determined using a balanced Latin-Square crossover design, to maximize the number of dosing combinations possible (N = 4 doses/sex/group). Each dose was followed by a wash out period of at least seven days. Surgically implanted telemetry devices in the beagle dogs monitored the following parameters continuously for at least 24 hours prior to dosing and through 48 hours after dosing; body temperature (degrees Celsius), heart rate (beats per minute), diastolic, systolic and mean blood pressure (mm Hg), electrocardiogram (ECG) (in milliseconds; PR, RR, QTc and QRS intervals), and respiratory parameters [respiratory rate, tidal volume (measured), minute volume (calculated)]. Additional measurements from the study animals included moribundity/mortality, cage-side clinical observations, detailed physical examinations, body weight/body weight change, and clinical pathology parameters (clinical chemistry, hematology, and coagulation). **Results:** Administration of a single dose of mitragynine in beagle dogs did not result in any toxicologically significant findings in all the parameters evaluated. Decreases in heart rate and corresponding increases in the RR interval were observed during the study in all mitragynine-treated groups. There was an increase in systolic blood pressure in all mitragynine treated males; however, this effect was not seen in females except slight increases in the high dose group. **Conclusions:** Overall, these changes were mild and transient and were resolved usually within 24 hours of

dosing, hence considered as non-adverse. (Confidential; Supported by Contract No. HHSN2712017000161 under HEAL/NCATS program; NIDA R01 DA047855 and UG/UH3DA048353 to AS and CM).

PS 4042 Preclinical Toxicity Assessment of IS-002, a Novel Imaging Agent, in the 7-Day Toxicity Study

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Background and Purpose: IS-002 is a small molecule and near-infrared (NIR) imaging agent. This compound is under development by Intuitive Surgical (Sunnyvale, CA) as an imaging agent to aid in the visualization of prostate tumors with Firefly Fluorescence Imaging during robotic-assisted prostatectomies. The purpose of this study was to evaluate the toxicity and toxicokinetics of IS-002 in CrI:CD (SD) (Virus Antibody Free) rats following single daily intravenous administration for 7 days, with a subsequent two-week recovery period. **Methods:** The IS-002 dose levels studied were 0 (control), 0.2, 1.6, and 16 mg/kg. The test article formulation and the vehicle (0.9% Sodium Chloride Injection, USP) were administered into the tail vein once each day for seven consecutive days. CrI:CD (SD) rats were obtained from Charles River Breeding Laboratories. The following tests and measurements were used: mortality, clinical signs, physical examination, body weight, food consumption, ophthalmologic examination, clinical pathology, plasma drug level determination, pharmacokinetic analysis, and histopathology. **Results:** No adverse clinical signs were observed. There were no test article-related differences in body weight, body weight changes, or food consumption between the dose groups administered with IS-002 and the control group. Post-dose ophthalmic examination on Day 7 did not reveal any abnormalities in the eyes. There were no test article-related changes in hematology, coagulation, and urinalysis parameters at the end of the treatment period (Day 8) or after the recovery period (Day 22). On Day 8, there were substantial (males) and statistically significant (females) increases in mean creatinine in Group 4 when compared with the control group. On Day 22, in males, there were statistically significant increases in mean creatinine in Group 4 compared to the control group. There were no test article-related changes in the other clinical chemistry parameters on Day 8 or Day 22. The test article was not detected (i.e., concentration < 0.05 µg/mL) in the plasma of control group animals. In all IS-002 dose groups, peak plasma levels ($C_{max-obs}$) occurred at the 2-minute time point after single or 7-day consecutive IS-002 administration at 0.2, 1.6, or 16 mg/kg. In all IS-002 dose groups, the test article was quantifiable until the last time point (i.e., 30 hours after dosing on Days 1 and 7). The IS-002 $t_{1/2-\lambda_z}$ ranged from 7.85 to 10.74 hours, CL from 0.15 mL/min/kg to 0.23 mL/min/kg, and V_{ss} from 0.13 L/kg to 0.16 L/kg across dose, administration day, and sex cohorts. IS-002 CL, V_{ss} , V_{ss}/V_{ss} , λ_z , and $t_{1/2-\lambda_z}$ were not meaningfully influenced by dose, single-dose versus multiple-dose administration, or sex. Dose proportionality was observed for IS-002 peak and total systemic exposure between 0.2 mg/kg and 16 mg/kg intravenous doses. On Day 22, there was a statistically significant increase in the mean testes/brain weight ratio in Group 4 rats compared with the control group animals. **Conclusions:** Seven-day once daily intravenous administration of IS-002 at 16 mg/kg resulted in statistically significant increases in clinical chemistry parameters (creatinine) and relative organ weights (testes/brain weight). The no-observed-adverse-effect level (NOAEL) was established at the IS-002 dose level of 1.6 mg/kg. The results of this study were used to support the IS-002 clinical trials.

PS 4043 Pulmonary injury and resulting hypoxia as a mechanism of hemangiosarcoma induction in mice treated with amiselimod, a sphingosine-1-phosphate receptor 1 modulator

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Background and Purpose: Amiselimod is a modulator of sphingosine-1-phosphate receptor 1 (S1P1). In rodent carcinogenicity studies of amiselimod, the incidence of hemangiosarcoma (HSA) was increased in male mice, but not in female mice or in rats. Several drugs are known to induce HSA in mice, and previous research suggests that hypoxia is a key event in this process. Meanwhile, for siponimod, another S1P1 modulator, the increased incidence of HSA in mice has been linked to the induction of placental growth factor 2 (PLGF2) production in vascular endothelial cells (VEC) by a mechanism that has not yet been fully elucidated. This suggests that, in addition to hypoxia, PLGF2 induction may play an important role in the development of HSA in mice. Pulmonary lesions were observed in short- and long-term mouse toxicity studies with amiselimod, which may have caused hypoxia. Therefore, this study aimed to investigate whether low oxygen (hypoxia) is involved in amiselimod-induced HSA in mice. **Methods:** 1) Toxicogenomics: Amiselimod was administered to CrI:CD1(ICR) male mice by oral gavage at doses of 0 (vehicle) or 30 mg/kg/day once daily for up to 2 weeks. Liver and spleen were collected and processed for transcriptomic analysis at 6 and 24 hours after the first dose, and at 6 hours after the 4th and 14th doses (n=3 animals/timepoint/group). 2) Immunohistochemistry (IHC): Amiselimod was administered to CrI:CD1(ICR) mice (8 males/group) once daily by oral gavage at doses of 0 (vehicle), 30, or 100 mg/kg/day for 2 or 4 weeks. To detect tissue

hypoxia, hypoxyprobe (HP; pimonidazole) was administered via intraperitoneal injection approximately 90 minutes prior to necropsy. Liver and spleen sections were anti-pimonidazole antibody and HP-positive areas were analyzed using HALO image analysis software. To detect VEC proliferation, liver specimens were triple-immunostained with anti-Ki67 (proliferation marker), anti-Iba1 (Kupffer cell marker), and anti-CD31 (endothelial cell marker) antibodies, then analyzed by HALO image analysis software. **Results:** Toxicogenomics: The transcriptomic analysis indicated that amiselimod up-regulated the expression of macrophage activation related genes both in liver and spleen after single dosing as well as the expression of erythropoiesis and cell cycle related genes only in spleen after single dosing. IHC: In spleen, there was significant immunostaining with HP (2W,100 mg/kg), with HP positive staining observed in some animals (2W:3/16(19%),4W:2/15(13%). As for the liver, no statistically significant changes were observed in positive staining areas in any of the test conditions. VEC proliferative activity in the liver was significantly increased at 30 and 100 mg/kg/day following 4 weeks of amiselimod treatment relative to vehicle control. Collectively, these results demonstrated induction of hypoxia, macrophage activation, dysregulation of hematopoiesis, and enhanced vascular endothelial cell proliferation following amiselimod administration to mice. These findings support a mechanism similar to the hypoxia-driven pathway for mouse vascular sarcoma induction proposed by Cohen et al. Notably, there were no hypoxia-related pulmonary lesions in amiselimod chronic toxicity studies in rats or monkeys, and no changes in pulmonary function in amiselimod clinical trials. **Conclusions:** In our study, we observed that the pulmonary injury previously noted with amiselimod administration in mice was associated with hypoxic conditions, which promoted VEC proliferation. Consistent with hypoxic conditions, genes related to macrophage activation and hematopoiesis dysregulation were up-regulated. Considering these findings, the increase in HSA incidence following amiselimod exposure is likely associated with hypoxia-driven resulting VEC proliferation. Finally, considering that no pulmonary lesions were observed in chronic toxicity studies in rats and monkeys, and that no changes in pulmonary function were detected in amiselimod clinical trials, the occurrence of HSA following amiselimod administration appears to be highly specific to mice, with low extrapolability to humans.

PS 4044 CSTF2/2T CELMoD Degradors and Double Knockout Studies: On-Target Toxicological Liabilities

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Background and Purpose: Cleavage stimulation factor subunit 2 (CSTF2) and its paralog CSTF2T are involved in pre-mRNA processing via alternative polyadenylation (APA), a regulatory mechanism impacting mRNA stability and translation. Dysregulation of APA is common in cancer, including hepatocellular carcinoma (HCC), and CSTF2 regulates APA dynamics in cancer-related genes. CSTF2 overexpression correlates with poor clinical outcomes. The therapeutic hypothesis was that a CSTF2/2T CELMoD would impair proximal polyadenylation site usage of mRNA, thereby regulating oncogenic transcripts and cancer growth. **Methods:** Two potent CSTF2/2T dual degradors (BMS-A and BMS-B) demonstrating favorable ADME characteristics were synthesized, tested, and subjected to toxicity assessment at doses ranging from 30 mg/kg to 300 mg/kg for up to 7 days in humanized cereblon mouse models (NSG hCRBN and C57 hCRBN). CSTF2/2T loss-of-function was assessed in inducible double-KO (iKO) mice over 1-month and 6-month durations using varying doses of tamoxifen. **Results:** BMS-A and BMS-B evaluated in the NSG and C57 hCRBN mice revealed significant toxicities. BMS-A was administered at 100 and 300 mpk 7 days in C57 hCRBN mice and caused multiple organ toxicities. In another study, BMS A was dosed at 120 mg/kg and BMS-B was dosed at 30, 60 and 120 in NSG CRBN mice for 6-7 days. In this study BMS-A caused renal tubule degeneration and BMS-B caused both renal tubule degeneration and hepatotoxicity (elevated liver enzymes at all doses and hepatic vacuolation at 60 and 120 mg/kg). To better understand whether these toxicities were target-mediated, inducible CSTF2/2T double knock out (iKO) mice were generated and evaluated. In 1-month inducible knockout (iKO) study where target expression was excised with 5-days intraperitoneal (IP) treatment with 75mg/kg tamoxifen, mice showed good tolerability with no adverse clinical signs or body weight changes but had mild increases in AST and ALT liver enzymes. LC-MS/MS showed CSTF2 protein expression decreased significantly in spleen and kidney, while CSTF2T expression increased. A 6-month iKO mouse study was conducted with 5-days IP treatment with 150 and 200 mg/kg tamoxifen to achieve improved excision of CSTF2 and CSTF2T in peripheral tissues and in the brain. Significant and severe adverse effects including mortality in some mice were observed on study days 9 and 10. Adverse effects included 15 to 20% body weight loss, abnormal gait, half closed eyes, hunched posture, decreased activity, elevated liver enzymes and decreased glucose. Most frequent macroscopic findings included small spleen, fluid filled intestines, dark or pale caecum, male reproductive findings, and mottled, friable liver and/or enlarged gall bladder. Histopathology of liver and kidney revealed vacuolation in these tissues. Like the 1-month study, LC-MS analysis in liver revealed that CSTF2 protein levels decreased, whereas CSTF2T protein levels increased. RT-PCR using primers spanning the full length of CSTF2 and CSTF2T transcripts indicated lower CSTF2 mRNA expression, with CSTF2T showing minimal change in the liver. No evidence of mRNA truncation was detected by transcription

analysis suggesting possible translational/post-translational compensatory increase in CSTF2T protein might have occurred due to the loss of CSTF2 protein expression. **Conclusions:** Dual CSTF2/2T degradation in hCRBN mice or dual CSTF2/2T knock-down in inducible knockout mice revealed significant on-target toxicities affecting many organs including the liver and kidney and revealed an unexpected increase in CSTF2T protein, suggesting potential imbalances in expression of these proteins may lead to toxicity.

PS 4045 A Case of Cardiac Myocyte Degeneration in Cynomolgus Monkey with characteristic Concentric change of myofiber cross section: a histopathological case study from a safety assessment perspective

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Background and Purpose: Accurate evaluation of drug-induced effects in nonclinical safety studies requires a thorough understanding of background lesions and secondary changes associated with deteriorating animal conditions. In our experience, a cynomolgus monkey that exhibited chronic weight loss followed by acute deterioration and reduced activity showed distinctive myocardial degeneration characterized histologically by a concentric appearance of cross sectional myofibers. This study aims to characterize histological feature of the myocardial changes and discuss their implications for histopathological interpretation in this animal with compromised health status. **Methods:** A 9-year-old female non treated cynomolgus monkey exhibited chronic weight loss and was euthanized due to acute moribundity of decreased activity and hypothermia. Macroscopic examination and histopathologic evaluation by hematoxylin and eosin (H&E) staining were performed. Additional analyses in the heart included phosphotungstic acid hematoxylin (PTAH) as well as transmission electron microscopy. Macrophage infiltration was assessed by Iba-1 immunostaining. Comparative evaluation was conducted against an age-matched normal animal. **Results:** In histopathologic evaluation of this animal, degenerative myocardial fibers exhibiting a concentric myofibrils structure was observed without macroscopic findings in the heart. The lesion was accompanied by coagulative necrosis of the fibers. PTAH staining and ultrastructural examination revealed abnormal localization of the myofibrils, characterized by their centralized clustering around the nucleus and decreased density in the peripheral cytoplasmic area. Iba-1 immunostaining demonstrated increased macrophage infiltration surrounding the degenerated and necrotic myofibers. **Conclusions:** To our knowledge, this distinctive myocardial lesion has not been clearly documented in terms of its histological features and pathological implications, and it may represent as one form of myocardial degeneration. We considered its pathogenesis was likely contributed by various complex factors, including systemic condition and metabolic state by altering myocardial energy metabolism, oxygen supply, or cellular resilience under stress conditions. Deeper understanding of these background or secondary myocardial changes helps distinguish spontaneous or secondary degenerative processes from potential drug-induced changes, thereby improving the reliability of pathological interpretation and overall toxicologic evaluation.

PS 4046 Body weight loss without reduction in food consumption observed in cynomolgus monkeys during non-clinical toxicity studies

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Background and Purpose: Cynomolgus monkeys have been used in non-clinical toxicity studies because they are anatomically and physiologically homologous to humans compared to other mammals such as rats and minipigs. Toxicity studies involving monkeys provide valuable data for drug development regarding the potential effects of drugs on humans. Weight loss is occasionally observed in monkeys during toxicity studies and can be difficult to differentiate from drug effects. This study retrospectively analyzed data from control group monkeys without drug treatment to investigate the incidence of weight loss and its physiological and pathological characteristics. We also investigated potential improvements through enhanced animal welfare. **Methods:** Animals in the control groups of the 4-week and 13-week repeated-dose monkey toxicity studies conducted at the test facility were retrospectively analyzed. The study included data from forty-six 4-week repeated-dose toxicity studies (male: n=206, female: n=196) and thirty-three 13-week repeated-dose toxicity studies (male: n=146, female: n=136) in monkeys conducted at the test facility from 2010 to 2022. Animals were weighed before feeding. The weight change rate (%) was calculated from initial body weight of the acclimation period to final body weight during the treatment period. Data of clinical observation, food consumption, hematology, blood chemistry, organ weight, necropsy, and histopathology were also investigated. Since September 2015, measures to improve animal welfare have been implemented at the test facility such as providing enrichment items, overproviding treats, and full-scale group rearing before the start of studies. We compared weight change rate before and after the start of the enrichment program. **Results:** Among the animals in the control groups, 3 animals in the 4-week toxicity studies and 5 animals in the 13-week toxicity studies showed a weight change rate of less than

-10%, resulting in an incidence rate of 1.2%. However, these animals had adequate food consumption. The animals in the 4-week toxicity studies showed signs of stress in histopathology. Additionally, 2/3 animals in the 4-week toxicity studies had decreased blood glucose levels and 1/5 animal in the 13-week toxicity study fell into a crouching posture, suggesting hypoglycemia that was alleviated with glucose administration, indicating stress-induced metabolic abnormalities. From 2015, an enrichment program was implemented to improve animal welfare. Prior to this program, 2.4% of animals showed a weight change rate of less than -10%, which dropped to 0.25% post-implementation, suggesting the program's effectiveness in reducing stress. **Conclusions:** These results clarify the characteristics of animals that lose weight during toxicity studies and suggest that improving animal welfare can reduce the incidence rate. Reference: Takahashi, et al., Experimental Animals, in press.

PS 4047 Method Qualification for the determination of Total antibody, ADC and free payload from ADC using LC-MS/MS

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Background and Purpose: Antibody-Drug Conjugates (ADCs) are a class of targeted anticancer therapeutics composed of an antibody, a linker, and a payload/cytotoxic drug. Traditional analytical methods for ADCs predominantly rely on ELISA. However, ELISA analysis is often limited to intact ADC and total antibody, with method development being relatively slow and constrained by antibody validation requirements. In contrast, LC-MS/MS provides crucial technical support for ADC bioanalysis due to its high specificity, independence from affinity reagents, accurate quantification of key components (total drug, ADC, and free cytotoxic drug), and faster method development. Here, we present a two-in-one approach for the simultaneous measurement of total antibody and protease-cleavable cytotoxic drug, along with a separate method for detecting free cytotoxic drug. **Methods:** The process of the two-in-one approach for measuring total antibody and protease-cleavable conjugated cytotoxic drug is as follows: analytes in the test sample are captured using magnetic beads, and a portion is aliquoted for the analysis of the protease-cleavable cytotoxic drug. Papan and β -glucuronidase are added to the aliquoted sample for enzymatic cleavage. Following cleavage, proteins are precipitated using high-concentration acetonitrile and centrifuged, after which the supernatant is collected for LC-MS/MS analysis of the protease-cleavable cytotoxic drug. The remaining sample is used for total antibody analysis: it is digested with trypsin to generate signature peptide fragments of the antibody, and these signature peptides are then analyzed via LC-MS/MS as surrogates for the total antibody. The approach for measuring free cytotoxic drug: high-concentration acetonitrile is added to the sample, followed by vortexing and centrifugation; the supernatant is then injected for analysis. The LC-MS/MS system conditions are identical to those used for the protease-cleavable cytotoxic drug. **Results:** The quantitative linear range for total antibody and protease-cleavable cytotoxic drug was 0.5 - 60 μ g/mL, while that for free cytotoxic drug was 0.01 - 2 ng/mL. The method demonstrated excellent robustness and reliability during validation, and is suitable for the accurate quantification of target analytes in complex biological samples, thereby, providing a reliable tool for pharmacokinetic and biodistribution studies. **Conclusions:** This method has been successfully applied to the analysis of ADC therapeutics, consistently generating reliable and reproducible data. The results confirm its significant value for the critical quality attribute assessment and stability monitoring of complex biopharmaceuticals.

PS 4048 Antisense oligonucleotides (ASOs) for gene therapy: A retrospective analysis of toxicology and safety pharmacology study designs and class effects.

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Background and Purpose: The rapidly evolving field of gene and nucleic acid therapy has presented exciting options for the treatment of many previously untreatable or poorly managed diseases and disorders. These therapies include several different families of molecular tools, such as antisense oligonucleotides (ASOs), designed to modulate gene and protein expression. In order to bring ASO-based therapies into a clinical setting, researchers must assess their short- and long-term toxicity profiles in pre-clinical studies to establish conditions for safe clinical use. **Methods:** A retrospective analysis of ASO-specific toxicology and safety pharmacology studies conducted across multiple test sites over a span of five (5) years are presented, with a focus on study designs and class effects. Emerging industry trends are highlighted with respect to dosing regimens, species selection, study duration, and study endpoints, and provide insights and recommendations on the design of ASO therapy toxicology and safety pharmacology studies, including a commentary on biologically relevant dose ranges for these promising molecular tools. As well, ASO therapy-associated clinical signs, histopathological and clinical pathology findings were summarized to highlight class effects to consider during program design. **Results: Species and Experimental design:** Mice were most frequently used, followed by rats and non-human primates. Subcutaneous administration predominated, with intravenous

and intrathecal routes also common. Most studies employed repeat-dose regimens. Extended recovery phases (>1 to 3 months) were common, supporting long-term observation of potential effects. **Safety Pharmacology:** Only 35% of studies included safety pharmacology assessments, often limited to one parameter (e.g., ECG, respiratory, or neurobiological evaluations). ECG monitoring was frequent in NHP studies. Neurological and/or neurobehavioral evaluations were routinely included in rat and NHP studies but rarely included in mice. **Clinical Observations:** Most studies reported no significant findings. Occasional neurobehavioral signs were observed at higher doses (e.g., tremors, abnormal posture and/or decreased activity). **Respiratory and Neurological Findings:** Respiratory changes were rare (e.g., increased minute volume in ~10% of cases). Neurological findings were more common in rats and NHPs than in mice. **Clinical Pathology:** • **Hematology:** Minimal changes overall, most values remained stable; occasional increases in white blood cells (mostly lymphocytes, neutrophils and monocytes) and reticulocytes, while decreases in red blood cell mass parameters and platelets occasionally observed in some studies. • **Coagulation:** Minimal changes overall, most values remained stable; occasional increases in fibrinogen, D-dimer, and prolongation of APTT and PT observed on some studies. • **Clinical Chemistry:** Minimal changes overall, most values remained stable; occasional increases in ALT, AST, ALP, and creatinine and decreases in albumin and total protein observed in some studies. **Pathology:** Injection site reactions and histological changes in liver, kidney, spleen, and lymph nodes were most common, generally with similar sensitivity across species. No unexpected severe toxicities were identified. **Conclusions:** ASO studies demonstrate recognizable class effects with generally mild clinical, clinical pathology and pathology findings, supporting their safety profile when appropriately dosed. Opportunities may exist to improve study designs by incorporating more comprehensive safety pharmacology evaluations into toxicology studies, therefore reducing the overall number of animals used.

PS 4049 Establishing Delivery Routes and Volumetric Compartment Measurements for Nonclinical Pediatric Antisense Oligonucleotides Assessment using Repeated Administration in Intrathecal Port Catheterized 3- and 12-Month Old African Green Monkeys

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Background and Purpose: Antisense oligonucleotide (ASO) drugs are a rapidly expanding drug class with proven success in significantly altering the course of heritable diseases. When targeting inherited neurodevelopmental disorders, early diagnosis and therapeutic intervention are critical. To appropriately model IT delivery of ASOs to the brain in infant and juvenile St. Kitts African green monkeys (AGM; *Chlorocebus sabeus*), this study optimized the surgically implanted ported IT catheter selection and evaluated long-term port patency for repeated IT deliveries using contrast-enhanced MRI. **Methods:** To determine a safe volume limitations for dosing infants and juveniles, baseline T2-CSF DRIVE MRI sequences were acquired and volumetric measurements of the CSF were made in both spinal cord and brain. Following healing of ported IT catheter surgery in infants and juveniles, patency was assessed via contrast MRI at 2 weeks, 1.5 months, and 3 months post-implantation. Infants and juveniles showed no signs of pain or distress following surgery and demonstrated full mobility within hours of the surgery. Additionally, we evaluated the post-surgery healing capacity and improved methods to enhance infant-mother bonding while observing for maternal rejection behaviors. **Results:** We found cleaning/masking surgical-related smells (e.g., aseptic substances and ECG electrode adhesives), leaving low-profile sutures with no skinfolds, and only permitting home cage reunions when both infant and mother are fully awake reliably resulted in no maternal rejections. Infants had variable port patency issues based on port and catheter selection. At 2 weeks following implantation, all infants showed delayed contrast movement—taking nearly 1-1.5 hours to reach the brain. By 1.5 and 3 months, only the low-profile port with 2 French catheter had successful patency in infants with contrast reaching the brain within 15-20 minutes of dosing in most subjects. Juveniles with low-profile port with 3 French catheter remained patent across all timepoints with contrast taking nearly 40-60 minutes to reach the brain at 2 weeks following implantation and improving to within 15-20 minutes at 1.5 and 3 months in most subjects. All ported IT catheters and delivered contrast and flush volumes were well tolerated in subjects after multiple IT administrations with no adverse event findings. **Conclusions:** We identified reliable low-profile port heads and appropriately sized catheters for infant and juvenile AGMs to support IND-enabling nonclinical research studies and successfully demonstrated they could safely undergo ported IT catheter surgery and maintain patency for up to 3 months.

PS 4050 Impact of Target Sequence and Pretreatment Conditions on ddPCR-Based Quantification of AAV Dosing Formulations

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Background and Purpose: In non-clinical safety studies of AAV vector products, accurate quantification of vector genome titers in dosing formulations is critical for dose setting and safety evaluation. Droplet digital PCR (ddPCR), which enables absolute quantification, has been widely adopted as a standard method for confirming the

dosing formulation concentrations, and the inverted terminal repeat (ITR) sequence is commonly used as the target for this assay due to its versatility. However, recent findings have revealed that the ITR sequence has structural challenges, such as stable hairpin secondary structures and mutations or deletions occurring during replication, which can hinder primer binding and amplification efficiency. As one approach to addressing these structural challenges, we focused on pretreatment methods, which are considered to cause variation in quantitation among testing facilities. To identify issues specific to the ITR sequence, we used the GFP sequence—a structurally simple and stable gene of interest (GOI) used as a control—to examine how the structural complexity of ITR influences ddPCR quantification accuracy and to assess to what extent these issues can be mitigated. **Methods:** First, singleplex ddPCR assays targeting ITR and GFP were conducted using an AAV2-CAG-GFP preparation. Quantification performance was assessed by evaluating dilution linearity and reproducibility. AAV dosing formulations were prepared at each concentration, followed by DNase treatment to remove unencapsidated DNA from samples. Samples were then lysed at 95°C for 10 minutes to disrupt the viral capsids, release genomic DNA, inactivate DNase, and prepare the test samples for the ddPCR assay. Next, to investigate how the structural complexity of the ITR affects measurement outputs under different pretreatment conditions, we focused on the effects of Proteinase K and heat treatment. The former was intended to facilitate capsid disruption and improve accessibility to the ITR; the latter aimed to completely disrupt the capsid and help resolve higher-order structures within the ITR. As an additional condition for ITR, we added HpaII, a restriction enzyme, to the master mix to reduce secondary structures and assessed its impact on quantification results. Measurement outputs obtained under each condition were converted to vg/mL and compared. **Results:** We first evaluated the effect of the target sequences, ITR and GFP, on the variability, accuracy, precision, and reproducibility of the measurement outputs. Both targets provided reliable and reproducible quantification under the tested conditions. We then examined the effect of pretreatment conditions on measurement outcomes. Proteinase K treatment alone did not affect the measured outputs. Without restriction enzyme treatment, the ITR target showed higher measured outputs than the GFP target, regardless of heat treatment. Since the ITR sequence is located at both ends of the viral genome, restriction enzyme treatment was expected to produce twice as many detectable copies as untreated samples. However, the observed increase was limited to 1.65-fold, suggesting that the complexity and incompleteness of ITR higher-order structures may influence the measured output. When restriction enzyme treatment was applied, the measured concentration (vg/mL) of the ITR target was lower than that of the GFP target after 10 minutes of heat treatment. However, extending the heat treatment to 30 minutes resulted in an increase in the measured outputs. **Conclusions:** These findings suggest that repeated measurements using consistent pretreatment methods yield good reproducibility. However, when ITR is used as the target, variations in pretreatment conditions caused fluctuations in measured outputs, indicating that the measurement may not accurately reflect the actual concentration of the dosing solution. Furthermore, the complexity and incompleteness of ITR secondary structures may significantly affect quantification accuracy. Therefore, careful optimization of pretreatment conditions is essential to overcome structural challenges and ensure accurate, consistent, and scientifically robust quantification when using ITR.

PS 4051 Evaluation of Intravenous Oncolytic Virus Delivery in a Hamster Syngeneic Pancreatic Cancer Model

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Background and Purpose: With advances in understanding cancer-driving mechanisms, personalized and targeted therapies have become increasingly important. Oncolytic viruses (OVs) represent a promising class of therapeutics that enable selective delivery of vaccinia virus to tumors via intravenous administration. Armed vaccinia viruses can convert tumors into localized “anti-tumor drug factories,” secreting cytokines and chemokines that promote tumor cell destruction while minimizing systemic exposure and long-term adverse effects. This study evaluated the potential toxicity of an oncolytic vaccinia virus following a single intravenous dose in LVG golden hamsters bearing Hap-T1 syngeneic pancreatic tumors. **Methods:** • Tumor model: Hap-T1 cells (5×10⁷ cells/mL) were subcutaneously inoculated into the flank. • Doses: 1×10⁶, 1×10⁷, and 1×10⁸ PFU/animal (i.v.). • Observation period: 21 days. • Parameters: Clinical signs, body weight, food intake, tumor growth, clinical chemistry, organ weight, histopathology, TK, biodistribution, neutralizing antibodies, and cytokines. **Results:** • Survival/Tolerability: All animals survived to termination. Mild decreases in body weight and food intake at ≥1×10⁶ PFU on Day 8. Minimal adrenal cortical necrosis/inflammation (non-adverse) at higher doses. No abnormal cytokine changes. • Tumor inhibition: On Day 22, inhibition reached 33%, 65%, and 81% in males and 27%, 72%, and 76% in females at ascending doses (all p<0.05 or p<0.001). • Cytokines: Elevated human CXCL-9 and IL-12p70 in a dose-dependent manner. TK/Immunogenicity/Biodistribution: Dose-dependent exposure declining over time; 100% neutralizing antibody positivity by Day 22; virus mainly detected in adrenal glands and ovaries. **Conclusions:** A single intravenous dose of the oncolytic vaccinia virus demonstrated a NOAEL of 1×10⁷ PFU/animal in LVG golden hamsters. The virus mediated tumor suppression through direct oncolysis, IL-12p70- driven

immune activation, and CXCL-9- dependent effector cell recruitment, establishing a local, self-sustaining anti-tumor loop while avoiding systemic cytokine toxicity. These findings indicate a favorable safety profile and durable anti-tumor efficacy.

PS 4052 Risk Assessment of Germline Transmission for Gene Therapy Drugs

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Background and Purpose: This study aimed to evaluate the potential germline transmission risk of a gene therapy drug by assessing gene editing efficiency in C57BL/6J mice semen and F1 offspring tissues. Males received a single tail vein injection with a 10-week recovery period to measure gene editing efficiency in semen. Females received a single tail vein injection and were mated with untreated wild-type males. Gene editing efficiency in the tail tip tissues of F1 offspring at postnatal day 7 (PND7) was measured. The results provide a scientific basis for clinical research. **Methods:** Twenty 9-10-week-old male C57BL/6J mice received a single intravenous injection of the test article at 10 mL/kg. Semen was collected from the epididymis, and DNA was extracted for gene editing efficiency measurement after a 10-week observation period. Twenty 10-week-old female C57BL/6J mice were mated with untreated males one week after administration. The day of successful mating was defined as gestational day 0 (GD0), and the day of birth was postnatal day 0 (PND0). Tail tips of all F1 offspring were collected at PND7, and tissue DNA was extracted for gene editing efficiency measurement. **Results:** In male mice, the mean gene editing efficiency in semen samples collected 10 weeks post-dosing (after one complete spermatogenesis cycle) was less than 0.03%. In a concurrent toxicity study, the mean gene editing efficiency in the epididymis at the same dose was below 0.40% (comparable to the vehicle control group). In comparison, it exceeded 50% in the target organ (liver). These results indicate that the test article did not effectively enter the spermatogenic microenvironment, and no gene editing occurred in sperm. In female mice, the gene editing efficiency in the tail tip tissues of F1 offspring was below 0.05%, significantly lower than the mean editing efficiency in the target organ (liver) of female mice in the toxicity study (exceeding 50%). The mean editing efficiency in the ovary was below 0.10% (comparable to the vehicle control group). The editing efficiency in offspring tail tips (<0.05%) was far below the level required for stably heritable levels (literature reports indicate that germline editing must exceed 1% to be biologically significant) and consistent with ovarian background levels (0.03%). This suggests that the test article did not affect germ cells or early embryonic genomes. **Conclusions:** This study evaluated the gene editing efficiency in male semen and female oocytes (assessed via F1 offspring tissues) to investigate the potential germline transmission risk of the gene therapy drug. The results showed no significant gene editing signals in male semen or F1 offspring tail tip tissues. Compared with vehicle control groups and toxicity study data on reproductive organs, the editing efficiencies were at background levels. Under the current study conditions, the test article shows no apparent risk of germline transmission.

PS 4053 Application and Case Sharing of Disease Model Animals in Toxicology Studies of Gene-Edited Cell Therapies

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Background and Purpose: Disease model animals can be used in toxicological studies of gene-edited cell therapies to evaluate biodistribution, immunotoxicity, tumorigenicity, treatment-related side effects, and gene editing safety. Through tumor models, immunodeficient animals models, and cardiovascular or neurological disease models, etc., the clinical pathological environment can be simulated to study risks such as cell migration, homing, abnormal proliferation, and immunogenicity, providing critical data for preclinical safety assessment. This study uses NPG tumor-bearing mice as an example to illustrate the application of disease model animals in the toxicity study of UCAR-T cells. The UCAR-T Cell Injection is clinically intended for the treatment of B-cell acute lymphoblastic leukemia (B-ALL). NPG tumor-bearing mice were intravenously injected with UCAR-T cells once and observed for 5-6 weeks to evaluate the nature, severity, and reversibility of potential toxic effects, identify target organs or tissues, and conduct accompanying safety pharmacology and cell persistence studies to provide reference for clinical application. **Methods:** A human acute B-cell lymphoblastic leukemia (NALM6 cell) model was established in NPG mice. Based on body weight or tumor burden (*in vivo* imaging), mice were randomly divided into five toxicity test groups (n=40/group): non-tumor-bearing vehicle control, tumor-bearing vehicle control, T-cell control, low-dose UCAR-T (1×10^7 CAR-T cells/animal), and high-dose UCAR-T (3×10^7 CAR-T cells/animal). Three toxicokinetic satellite groups (n=16/group) were also included: non-tumor-bearing vehicle control, low-dose UCAR-T, and high-dose UCAR-T. Animals were equally distributed by sex and dosed once, with the dosing day defined as Day 1 (D1). During the study, the general status of mice was observed daily, and body weight and food consumption were measured weekly. Functional observational battery (FOB) tests were performed on subsets of animals before dosing and at 2 h, 48 h, D8, D15, and D28 after dosing. Scheduled necropsies were conducted on D8 and at the end of the observation period (which was terminated early due to high tumor-related mortality: on D30 [females]/

D34 [males] for the low-dose group and on D37 for all other groups) for ophthalmological examination, hematology, clinical biochemistry, immunotoxicity indicators (cytokines, T-lymphocyte subsets), organ weights, gross pathology, and histopathology. Whole blood was collected for cell persistence (TK) analysis on D8 and D37 for the non-tumor-bearing vehicle control and high-dose groups, and for the low-dose group on D8 and D30 (females only), with males sampled only on D8. **Results:** In this study, no deaths/morbidity or severe toxic reactions directly related to UCAR-T were observed. Tumor-related deaths/morbidity were observed in tumor-bearing animals. UCAR-T significantly inhibited tumor proliferation and extended mean and median survival times, accompanied by increases in peripheral blood lymphocytes (LYMPH), CAR⁺ cells, and IFN- γ related to the pharmacological antitumor effect. At the end of the observation period, progressive tumor burden-related abnormalities were observed in clinical pathology parameters, including AST, ALT, WBC, MONO, and LYMPH. No obvious test article-related changes were found in safety pharmacology (central nervous system), gross pathology, or histopathology. Cell persistence results showed detectable UCAR-T cells only in the high-dose group on D8, with no detection at other time points. **Conclusions:** For approximately 6 weeks after a single intravenous injection of UCAR-T (1×10^7 CAR-T cells/animal or 3×10^7 CAR-T cells/animal) to NPG tumor-bearing mice, no deaths or other serious toxic reactions related to UCAR-T were observed in either sex. The severely toxic dose in 10% of animals (STD₁₀) was 3×10^7 CAR-T cells/animal for both sexes.

PS 4054 Toxicologic Profiles of Lipid Nanoparticle (LNP) used as Delivery Systems for Nucleic Acid Therapeutics in Cynomolgus Monkeys: Insights from Cross-Study Analysis of Various LNP Formulations

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Background and Purpose: Lipid nanoparticles (LNPs) are widely used as delivery vehicles for nucleic acid therapeutics. While extensive research has addressed LNP-mRNA vaccine safety in clinical settings, LNP-only toxicological responses remain insufficiently characterized (in preclinical evaluations) in nonhuman primates (NHPs). The review by Wang et al. (2024, Vaccines) summarized that LNP toxicity involves several mechanisms, including inflammation, immune responses, and off-target accumulation in organs such as the liver, lungs, and spleen, and highlighted the lack of LNP-specific human and NHP data. To address this translational gap, we conducted a cross-study characterization of lipid-based carriers without a therapeutic payload or empty LNP-induced toxicity in cynomolgus monkeys to establish a nonclinical vehicle baseline to compare with LNP-encapsulated nucleic acid products. **Methods:** We utilized Certara's SEND Explorer (Warehouse version) to integrate multiple LNP preclinical studies (2022-2025) into a cross-study toxicity profile, which enabled the detection of reproducible organ/system findings, quantification of incidence/severity trends, and data-driven ranking of LNP reactivity. Data was obtained from >20 single- and repeat-dose toxicity studies involving empty LNP formulations and selected representative formulations differing in ionizable lipid chemistry and dosing regimen (up to 8 mg/kg; IV or IM). Clinical pathology data consisting of hematology and serum chemistry results (including acute-phase proteins: C-reactive protein [CRP], fibrinogen, and albumin) were reviewed in conjunction with microscopic findings across major organs (liver, spleen, lymphoid tissues, and bone marrow). Clinical and macroscopic observations were also reviewed. **Results:** Transient and reversible pronounced increases in liver transaminase activities (i.e., ALT, AST) and acute phase protein concentrations (e.g., CRP) were observed approximately 2 to 5 days post-administration across these studies. Notable microscopic findings included single-cell necrosis of the hepatocytes, inflammatory cell infiltration, hypertrophy of the hepatocytes and Kupffer cells in the liver, and extramedullary hematopoiesis in the spleen and lymph nodes. Similar results were discovered in repeated toxicity studies of patisiran (siRNA-LNP), single-cell necrosis of hepatocytes was commonly observed with associated hepatic enzyme increase, and this toxicity was considered to be caused by LNP itself rather than mRNA-LNP (EMA/CHMP Assessment Report, 2018). In some of the investigative studies, necropsies were performed more than a week after administration, which may indicate that hepatocellular injury (accompanied by elevated liver enzymes) that occurred a few days after administration was in the process of recovery or had resolved at the time of necropsy. The clinical observations remained unremarkable. These reproducible patterns align with mechanistic evidence from the literature (Wang et al., 2024), showing that ionizable lipids may activate and further induce hepatic and/or immune responses. **Conclusions:** These findings establish a vehicle-specific baseline that distinguishes payload-independent effects and supports standardization of nonclinical LNP safety evaluation through historical-control integration. This dataset provides a foundation for future benchmarking of LNP-based therapeutics.

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Background and Purpose: Lipid nanoparticles (LNPs) serve as carrier vehicles that protect RNA from rapid degradation and facilitate intracellular delivery and endosomal escape, thereby significantly advancing mRNA-based vaccine development and related therapies for cancer and rare diseases. The LNP platform promotes robust humoral immune responses, indicating adjuvant activity. However, animals receiving LNP-mRNA vaccines often exhibit side effects with various tissue and organ reactions. These adjuvant effects and reported side effects may arise from the inflammatory properties of LNPs. In preclinical safety studies, such side effects can obscure the toxicological impact of the test substance and lead to misinterpretation. Based on these observations, this study aims to identify specific parameters and methods for evaluating LNP-associated side effects in preclinical safety studies involving NHPs. **Methods:** To investigate LNP-associated side effects in preclinical safety evaluations performed in NHPs, data from 19 preclinical toxicity studies conducted over the past five years which evaluated the safety of LNP-containing substances, were analyzed. A series of parameters were assessed, including animal information, clinical signs, body weight, and related laboratory indices such as clinical pathology, immunocyte phenotyping, cytokines, and tissue responses. **Results:** Data analysis revealed that LNP-associated safety studies in NHPs were primarily conducted at dose levels of 1 to 2000 µg/kg in various dosing routes, using animals aged 1.1 to 5.3 years. Clinical signs were not age-related but were dose-dependent and varied significantly based on LNP dose levels. Most clinical signs were related to the dosing site, including erythema and/or swelling, with incidences ranging from 20.3% to 46.5%. These signs typically emerged on the second day after inoculation, resolved within 1 to 4 days, but might lead to ulceration and/or mass formation later. Some animals exhibited reduced body weight gain (up to 11.2%) attributable to irritation/inflammation at dosing site induced by LNPs. Concurrent with inflammation, several clinical pathology changes were commonly observed. These included mild reductions in red blood cell (RBC)-related parameters—RBC count, hemoglobin (HGB), hematocrit (HCT), and mean corpuscular hemoglobin concentration (MCHC) ranging from 1.3% to 47.6%, as well as increased white blood cell (WBC) counts and decreased lymphocyte counts (Lymph), ranging approximately from 21.3% to 138.2%. The rise in WBC was predominantly characterized by an increase in neutrophils. Coagulation changes were also frequently noted, with elevated APTT and fibrinogen (FIB) levels ranging from 17.3% to 155.1%. Additionally, clinical chemistry changes included decreases of albumin (13.2% to 32.7%) and albumin-to-globulin ratio (A/G, 21.5% to 48.2%), alongside increased globulin (6.1% to 37.2%) were observed in LNP-treated animals. Cytokine levels, analyzed as indicators of tissue response, showed changes correlated with LNP-induced tissue reactions. These consisted primarily of elevated IL-6 levels (53% to 4034%) observed 1 to 6 hours post-dosing, as well as increased C-reactive protein (CRP) levels (47% to 704%), without changes in lymphocyte phenotyping or serum complement, regardless of dosing route or dose level. Histopathological findings associated included inflammatory reactions and/or muscle degeneration/necrosis at the dosing site, reduced lymphocyte cellularity in thymus, hepatocyte vacuolation and/or increased Ito cells in liver, hemorrhage, mineralization, and/or inflammatory cell infiltration in the adrenal gland, as well as atrophy in the mammary and prostate glands. The incidence of these findings ranged from 8.3% to 72.3%. These clinical and laboratory changes in NHPs should be distinguished from the toxicological effects of test materials containing LNP during preclinical toxicity studies. **Conclusions:** The results of this review offer valuable reference for preclinical toxicity studies of drug candidates utilizing LNPs in NHPs. Although NHPs have proven to be a reliable animal model for predicting human toxicological impacts and physiological effects of biologics, the side effects associated with the LNP delivery platform may mask the actual toxicity of the test substance and complicate data interpretation in preclinical safety studies.

PS 4056 Non-Clinical Safety Evaluation of Exosomes derived from Human Adipose Tissue-Derived Mesenchymal Stem/Stromal Cells

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Background and Purpose: Extracellular Vesicles (EV), often also called exosomes, can shuttle multiple molecules between cells, and are being developed as functional nanomedicines for human use. Exosomes are nano-sized extracellular vesicles that are transferred to target cells for cell-to-cell communication. Although MSC-derived exosomes are suggested as novel cell-free therapeutics for various human diseases, evaluation studies for the safety and toxicity of MSC-derived exosomes are limited. The purpose of our study was to evaluate the toxicological profile, including general toxicity, genotoxicity, and safety pharmacology tests using exosomes derived from human adipose tissue-derived MSCs (ASC-EXO) in accordance with the OECD guidelines and the principles of Good Laboratory Practice. **Methods:** To evaluate the toxicological profile of ASC-EXO, we carried out general toxicity test including the 4 weeks repeated-dose toxicity and genotoxicity including Ames test and *in vitro* and *in vivo* micronucleus tests. In addition, the core battery of safety pharmacol-

ogy tests, including assessments of the central nervous system, respiratory system, and cardiovascular system (*in vitro* hERG assay and *in vivo* telemetry system) were performed under GLP regulation. **Results:** No toxicological changes in clinical signs, body weights, water and food consumption, urinalysis, hematology, clinical biochemistry, gross findings, or histopathological examinations during the repeated-dose toxicity, including the 4-week dosing periods. In addition, ASC-EXO was classified as non-mutagenic and non-clastogenic in the genotoxicity tests. Additionally, no animal toxic effects were observed, including no changes in body weight or clinical signs in the *in vitro* and *in vivo* micronucleus tests. In safety pharmacology studies, ASC-EXO did not show any pharmacologically adverse effects in the modified Irwin's test for the central nervous system, whole-body plethysmography for the respiratory system, and hERG assay and telemetry system for the cardiovascular system. **Conclusions:** Our findings are the first to suggest that ASC-Exosome safe for use as a treatment, with no adverse effects observed in general toxicological testing including genotoxicities as non-mutagenic and non-clastogenic and in safety pharmacology testing. It shows potential application as a therapeutic agent, cosmetic ingredient, or for other biological uses.

PS 4057 Predicted Liver Safety of GlyphAgo, a Lymphatic Targeted Prodrug of Agomelatine That Avoids First-Pass Hepatic Metabolism

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Background and Purpose: Agomelatine (AGM), a melatonin 1 & 2 receptor agonist and serotonin 2C receptor antagonist, has demonstrated clear clinical efficacy and best-in-class tolerability in generalized anxiety disorder and major depressive disorder. However, AGM undergoes extensive first-pass hepatic metabolism, resulting in low bioavailability, variable exposure, and occasional drug-induced liver injury (DILI), generally manifesting as dose-dependent elevations in alanine aminotransferase (ALT) or aspartate aminotransferase. Therefore, liver monitoring is frequently required in regions where it has received regulatory approval, a requirement cited as a significant barrier to clinical use. The culprit mechanism is not definitively known, but serum transaminase elevations are dose dependent. GlyphAgo™ (SPT-320 or Glyph™ Agomelatine) is an oral prodrug of AGM being developed using the Glyph platform, a prodrug technology that shifts absorption toward intestinal lymphatics, avoiding first-pass hepatic metabolism. GlyphAgo has the potential to achieve therapeutically efficacious exposure levels at a lower dose, which may reduce liver exposure and reduce/eliminate the need for liver enzyme monitoring. This abstract reports the results of DILSym, a quantitative systems toxicology model, to predict DILI reduction for GlyphAgo relative to AGM. **Methods:** DILSym combines compound exposure at the site of action (i.e., liver) with concentration-dependent hepatocyte toxicity and inter-individual variability to predict liver injury. A physiologically based pharmacokinetic (PBPK) model of AGM exposure was applied to predict liver concentrations of AGM and selected metabolites following oral administration of approved doses. A CYP1A2-derived AGM metabolite was optimized to induce hepatocyte cell death, empirically reproducing dose dependent ALT elevations ≤10% at approved doses of AGM in a simulated population (SimPops). The AGM PBPK model was adapted to represent GlyphAgo, where lymphatic distribution bypassing first-pass metabolism was represented by intravenous administration. Simulations were conducted in SimPops where approved 25 and 50 mg once daily (QD) dosing of AGM were set as the standard. GlyphAgo dosing included doses achieving comparable AGM plasma concentrations to oral AGM and were simulated to predict GlyphAgo DILI in SimPops. **Results:** The AGM PBPK model produced output comparable to published data on exposure dynamics of AGM and two key metabolites, with C_{max} and AUC values generally falling within 0.5x-2x of reported values. In DILSym, a CYP1A2-derived metabolite was optimized to induce hepatocyte death, yielding 1.4% and 9.5% ALT elevations in SimPops following treatment with approved 25 or 50 mg QD AGM dosing. While 9.5% is significantly higher than the observed 50 mg ALT elevation rate of 2.4%, this was allowed to provide an additional safety factor for estimated GlyphAgo ALT elevation rates. Together, these modeling efforts were consistent with the known exposure and hepatotoxicity data for AGM. The AGM PBPK model was then modified to represent GlyphAgo using AGM equivalent doses. In DILSym, dosing of GlyphAgo yielded no ALT elevations in SimPops, supporting the hypothesis that partial bypass of first-pass metabolism by the prodrug reduced DILI risk while maintaining clinically relevant exposure to AGM. In SimPops, GlyphAgo did not precipitate ALT elevations until the dose was significantly escalated beyond the approved AGM dose, suggesting the potential to deliver more active drug with minimal hepatic consequences. **Conclusions:** By leveraging absorption through the intestinal lymphatics with the Glyph platform, GlyphAgo may be able to reduce DILI risk by avoiding first-pass hepatic metabolism. These results support the phase 1 proof-of-concept clinical trial of GlyphAgo in healthy volunteers evaluating the safety, tolerability, and pharmacokinetics compared to AGM. Further, the Glyph platform has the potential to be widely applied to other therapeutic molecules that have clinical limitations from high first-pass hepatic metabolism leading to low oral bioavailability and side effects like elevated liver enzymes or hepatotoxicity.

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Background and Purpose: Unexpected toxicity remains a major contributor to drug development failure, accounting for roughly 30% of discovery-stage attrition [1] and millions of adverse drug reaction-related medical visits annually [2]. Early evaluation of off-target pharmacology is a key strategy for identifying potential safety risks before clinical development. Most pharmaceutical organizations employ *in vitro* pharmacological profiling to detect off-target activity during early discovery [3]; however, screening data alone are often insufficient to quantify clinical safety risk [4]. To address this limitation, the Safety Margin Tool within PharmaPendium was developed to integrate data on clinical adverse drug reactions, therapeutic free exposure, and *in vitro* target affinities from well-established safety pharmacology panels [5-7]. This framework enables systematic estimation of safety margins that link target engagement with clinical exposure, supporting quantitative risk assessment and interpretation of mechanistic safety findings, particularly in the context of polypharmacy. [1] Bai C. et al., *Adv. Sci.*, 2025; 12(16): 2413405. [2] Howard I. et al., *Sci. Rep.*, 2022; 12(1): 2622. [3] Valentin J.-P. et al., *J. Pharmacol. Toxicol. Methods*, 2018; 93: 7-14. [4] Urban L. et al., *Drug Discov. Today*, 2016; 21(8): 1232-1242. [5] Brennan R. et al., *Nat. Rev. Drug Discov.*, 2024; 23: 525-545. [6] Bowes J. et al., *Nat. Rev. Drug Discov.*, 2012; 11(12): 909-922. [7] Lynch J.J. 3rd et al., *J. Pharmacol. Toxicol. Methods*, 2017; 87: 108-126. **Methods:** A systematic workflow was implemented to estimate safety margins across key off-target classes, including G protein-coupled receptors (GPCRs), kinases, ion channels, nuclear receptors, enzymes, and transporters. *In vitro* affinity data (IC₅₀, EC₅₀, Ki) were compiled and standardized from published studies and patent literature for drugs with established pharmacological mechanisms. Corresponding pharmacokinetic data (C_{max}, AUC, unbound fraction) were retrieved primarily from regulatory submissions and literature sources to reflect free therapeutic exposure. Safety margins were calculated as the ratio between *in vitro* potency and observed clinical exposure. A permutation-based uncertainty analysis incorporating affinity and exposure variability was applied to derive drug-specific and target-level safety margins. Statistical approaches were compared to evaluate conservative versus relaxed methods for quantifying uncertainty and classifying potential safety risks. **Results:** Application of the workflow resulted in the estimation of 58 target-level safety margins across well-characterized off-target panels. Refinement of the analytical approach enabled two complementary risk assessment modes: (1) a conservative approach designed to minimize false negatives by prioritizing sensitivity to potential safety risks, and (2) a relaxed approach that reduces false positives by accounting for variability and therapeutic context. The conservative mode supports early-stage risk identification, while the relaxed mode minimizes unnecessary exclusion of viable candidates. Comparative analysis across molecular targets highlighted trends linking specific target classes with distinct adverse drug reaction profiles, offering a framework for interpreting potential mechanistic contributors to observed toxicities. **Conclusions:** The Safety Margin Tool provides a systematic and data-driven approach for estimating safety margins that integrate *in vitro* pharmacology with clinical exposure data. This methodology supports early identification of potential off-target risks, improves the interpretation of observed adverse drug reactions, and enhances mechanistic understanding of target liability. Incorporating safety margin analysis into discovery and development workflows can strengthen evidence-based decision-making and contribute to more consistent, transparent safety evaluations, particularly in complex therapeutic settings involving polypharmacy.

PS 4059 *In Vitro* Cytotoxicity and Genotoxicity Assessment of Pyridinium and Quinuclidinium Oximes using a 3D Human Liver Spheroid Model

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Background and Purpose: Organophosphate (OP) poisoning continues to pose a significant global health threat, and oximes remain the primary therapeutic agents due to their capacity to reactivate acetylcholinesterase. Although the clinically established oxime HI-6 exhibits strong reactivation potential, its limited spectrum of efficacy highlights the need for improved alternatives. This study aimed to evaluate and compare the safety profiles of bis-pyridinium HI-6 and two newly developed oximes, pyridinium GM508 and quinuclidinium Q5 using a physiologically relevant 3D human liver spheroid model. **Methods:** Human HepG2 spheroids were exposed for 24 hours to HI-6 at 800 µM and increasing concentrations of GM508 and Q5 ranging from 6.25 to 50 µM. Cytotoxicity was determined using the tetrazolium-based (MTS) assay, while DNA strand breaks were assessed by the alkaline comet assay on single-cell suspensions obtained after enzymatic disaggregation of spheroids. Flow cytometry was employed to simultaneously evaluate cell cycle distribution, proliferation (Ki67), DNA damage (γH2AX), and mitotic activity (phosphorylated histone H3). Specific positive controls, 15% DMSO for viability and etoposide (17 µM or 1.7

µM) for genotoxicity and flow cytometry were included in each assay. **Results:** All oximes demonstrated low cytotoxicity at the tested concentrations with cell viability comparable to untreated controls. DNA strand break analysis using the alkaline comet assay showed no significant increase in DNA damage in any treatment group. Flow cytometric evaluation revealed no alterations in cell cycle phase distribution and preserved proliferative activity, as indicated by Ki67 staining. In addition, there was no induction of γH2AX or phosphorylated histone H3 signals, confirming the absence of DNA double-strand breaks or mitotic disruption. **Conclusions:** The novel oximes GM508 and Q5 exhibited favorable safety profiles in the 3D human liver spheroid model. All compounds showed minimal cytotoxic and no detectable genotoxic effects, with preserved cell viability, proliferative capacity, and genomic stability. These findings provide strong support for their continued evaluation as promising candidates for next-generation antidotes in organophosphate poisoning. **Funding:** This research was funded by the Slovenian Research Agency ARIS [P1-0245, J1-2465, Z1-3191], bilateral projects [BI-HR/18-19-003; BI-HR/20-21-019], European Regional Development Fund project KK.01.1.1.02.0007 (Rec-IMI), European Union-Next Generation EU 533-03-23-0006 (BioMolTox) and Croatian Science Foundation (grant UIP-2017-05-7260 (CellToxTargets)).

PS 4060 Age accelerates memory deficits in offspring with perinatal electronic cigarette exposure

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Background and Purpose: Electronic cigarette (Ecig) use has shown to elicit harmful effects on multiple body systems, including changes in behavior and memory. Hippocampus deterioration is associated with memory impairment in mice, in which long term memory is interrupted when mice are performing memory tasks. With normal aging, hippocampus dependence on memory is also reduced. Novel object recognition (NOR) testing is a hippocampal-dependent memory based task in rodents that evaluates the ability to detect changes in a familiar environment. Aging has been shown to impair NOR, but changes related to Ecig exposure and advancing age in offspring with *in utero* exposure are not yet known. We tested the hypothesis that memory defects would be worse with increasing age in mice with history of *in utero* Ecig exposure. **Methods:** We studied offspring from time-mated pregnant C57BL/6J mice with exposure to either Air (control, n=14 dams) or Ecig aerosol (using 50:50 VG:PG, no flavor or nicotine; n=13/dams) from gestational day 7 to 21. The NOR test was performed at ~6-months (n=29 male, n=29 female), and again, at ~12-months (n=27 male, n=27 female) to assess memory function in offspring. On the first day of NOR testing, mice were familiarized with the environment (i.e. box) without any objects. The second day mice were given 10 minutes with two of the same objects in the box. Four hours later, mice behavior was evaluated for 5 minutes with a novel (N) object in place of one of the familiar (F) objects in the box. Data analysis we performed using Any-maze Software (v. 7.5, Wood Dale, IL) and conducted after the completion of testing. Hippocampus size in 12 month old offspring was lower in Ecig compared to control offspring (Ecig 1.73±0.07 mm³ vs Air 2.35±0.12 mm³, p<0.05). **Results:** At 6-months, offspring with maternal Ecig exposure had 2.85 times fewer investigations of N (vs F) object compared to controls (i.e. Ecig F=7.2±1.6 vs N=11.6±1.4 vs control F=3.9±1.0 vs N=10.7±1.8; mean ±SE p<0.05). Also, the % total time for Ecig offspring with the N object (vs F object) was 2.1 times lower compared to controls (i.e. Ecig F=36.7±4.8% vs N=63.3±4.8%, p<0.05; and control F=28.1±4.2% vs N=71.9±4.1%; p<0.05). At 12-months, neither control nor Ecig offspring show significant preference for N vs F objects when evaluating the number of investigations or % total time with the N object. Hippocampus size in 12 month old offspring was lower in Ecig compared to control offspring (Ecig 1.73±0.07 mm³ vs Air 2.35±0.12 mm³, p<0.05). **Conclusions:** Offspring with a history of perinatal exposure to Ecig show earlier declines in hippocampal-dependent memory tasks compared to controls, and have reduced hippocampus size with advanced age, suggesting an accelerated loss for the ability to consolidate (or form long-term) memory associated with *in utero* Ecig exposure. **Funding Support:** NIH R56 ES035719-01, P20GM121322, U54GM104942, WV-INBRE NIH Grant P20GM103434 and WVU Stroke CoBRE P20 GM109098.

PS 4061 C-reactive protein levels during pregnancy and their association with tobacco product exposure- evidence from the NHANES dataset

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Background and Purpose: C-reactive protein (CRP) is a commonly used marker of low-grade systemic inflammation. It is one of the most sensitive, though non-specific, indicators of inflammation. CRP levels in blood increase as a part of normal physiologic change of pregnancy in addition to both infectious and noninfectious environmental substances, including tobacco smoke. During critical periods such as pregnancy, CRP-linked low-grade inflammation has been associated with adverse events such as vascular dysfunction, preterm birth, preeclampsia, and increased

long-term health risks for both mothers and their offspring. The goal of the current study is to assess whether CRP levels are modified by both active and passive tobacco product exposures during pregnancy, using the U.S. National Health and Nutrition Examination Survey (NHANES) dataset. This work aims to improve the understanding of how low-grade chronic inflammation in pregnancies can be influenced by tobacco product exposures. **Methods:** We aimed to answer the question: Is there a relationship between CRP in maternal serum and tobacco product exposure? We hypothesized that there would be a positive association between CRP levels and tobacco product exposures. This cross-sectional research study of 9,015 participants utilized data from the 2021-2023 NHANES database. Participants were grouped by pregnancy status and tobacco exposure: (a) pregnant and exposed to cigarettes, (b) pregnant and exposed to e-cigarettes, (c) non-pregnant and exposed, and (d) non-pregnant and unexposed. Measurements of high-sensitivity C-reactive protein (hs-CRP), in mg/L, were obtained from the serum of the NHANES participants with 0.15 mg/L as the lower limit of detection. Pregnancy status was verified when there was a positive urine pregnancy test result for a participant [Icon 25 hCG test kit (Beckman Coulter)]. Tobacco product exposure data were obtained from positive answers to cigarette use, household smokers, and recent tobacco use questionnaires. We used Fisher's exact tests, Wilcoxon rank sum test, and/or Pearson's Chi-squared test to assess any potential associations, with significance set at $p < 0.05$. **Results:** Over the 2021 - 2023 period, 41 of the total 9,015 participants had evidence of positive pregnancy test results. Pregnant women who were exposed to either cigarettes or e-cigarettes based on the tobacco product use and household smoker variables, had sample sizes of ten and one, respectively. Overall, mean ($\pm$ standard deviation) CRP levels among the 9,015 participants in this cross-sectional study were 3.6 ± 7.4 mg/L. Pregnant women who were exposed to tobacco products had higher mean CRP levels (5.2 ± 5.4 mg/L, $n=10$) compared to those who were not pregnant and had not been exposed to tobacco products (3.6 ± 7.4 mg/L, $n=6500$), and this was statistically significant ($p=0.036$). Median concentrations (Q1, Q3) for the pregnant and exposed women and nonpregnant and unexposed women were 3.7 (2.6, 5.7) and 1.6 (0.7, 3.9) mg/L, respectively. While there was no statistically significant difference between pregnant and unexposed women ($n=31$) compared to pregnant and exposed women ($n=10$), the minimum and maximum values of CRP were higher for the latter group (0.2, 17.9 mg/L) vs (0.7, 19.6 mg/L). An assessment of volatile organic compounds (VOCs) and trihalomethanes in whole blood revealed that only one VOC, ethyl acetate, was significantly higher ($p < 0.001$) in the blood of participants who were confirmed pregnant (0.9 ± 2.8 ng/mL, $n=14$) compared to nonpregnant participants (0.3 ± 2.1 ng/mL, $n=2783$). However, this effect was not observed when tobacco product exposure was considered due to small sample sizes for the available blood sample data. **Conclusions:** This cross-sectional study utilized data from NHANES to provide evidence of potential associations between tobacco product exposure during pregnancy and increased levels of CRP. Future work will assess specific populations and include more individuals from the continuous NHANES dataset. Our findings suggest that tobacco exposure during pregnancy may contribute to systemic inflammation, underscoring the need for targeted interventions to reduce maternal and fetal health risks.

PS 4062 Perinatal E-cigarette Exposure Disrupts Lung Function and Germ Cell Development in Offspring

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Background and Purpose: Recent reports indicate that about 7% of women have used e-cigarettes during their pregnancy, exposing the fetus to harmful toxicants during critical developmental periods. We have previously shown that perinatal nicotine exposure in rodents led to a transgenerational asthma phenotype until the F3 generation. Here, we tested the hypothesis that perinatal e-cigarette exposure alters lung and germ cell transcriptomes in F1 offspring. **Methods:** Pregnant Sprague Dawley rats were exposed daily to e-cigarette vapor from embryonic day 6 (E6) to postnatal day 21 (PND21), achieving serum cotinine levels comparable to those of habitual human smokers. At PND21, F1 pups were subjected to pulmonary function testing and lungs were collected for RNA sequencing. To assess potential germline mechanisms underlying multigenerational effects, we also collected testes and ovaries from F1 pups for single-nucleus RNA + ATAC sequencing. **Results:** F1 offspring exposed to perinatal e-cigarette vapor exhibited increased total airway resistance and decreased lung compliance ($N=6-10$; $p < 0.05$), consistent with an asthma-like phenotype. RNA sequencing in e-cigarette treated lungs ($N=4$) revealed that male pups (461 total DEGs, $FC > 1.5$, $\text{padj} < 0.05$) had a higher number of differentially expressed genes (DEGs) than females (84 total DEGs) compared to controls. Notably, differentially expressed genes were associated with cilia assembly and organization in both sexes. Single nucleus RNA sequencing of F1 pup ovaries and testes ($N=3$) captured all major cell types. E-cigarette exposed testes displayed markedly more DEGs ($q < 0.05$) than ovaries, with early spermatogenic populations (spermatogonia and spermatocytes) showing the highest DEG burden compared with later-stage spermatids. **Conclusions:** Perinatal e-cigarette exposure in rats is associated with sex specific responses in the lungs and gonads of F1 pups. Male lungs displayed a much larger transcriptional response than female lungs, with shared enrichment in cilia-related pathways. In the germline, early-stage male germ cells were particularly sensitive to exposure, exhibiting robust transcriptomic alterations. These data provide molecular evidence that perinatal vaping perturbs both pulmonary develop-

ment and germ cell development, supporting potential mechanisms for multigenerational transmission of vaping-related disease risk. **Grant Support:** HL151769, T32 ES015457 (NIH); T32IP5044, T32IR5048, T32IR5365, T291R0737, T34IR8406 (Tobacco Related Disease Research Program); and EDUC4-12837 (CIRM)

PS 4063 A preliminary investigation of the maternal and perinatal health effects of electronic cigarette use during pregnancy

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Background and Purpose: Electronic cigarette (e-cigarette) use during pregnancy has been increasing in recent years, prompting growing concern about its potential impact on both maternal and fetal health. Many pregnant women may be susceptible to initiating or continuing e-cigarette use (vaping) because e-cigarettes are available in a wide variety of appealing flavors that may appeal to their pregnancy-related cravings, and because these products are often marketed or perceived as safer alternatives to traditional cigarettes. However, the unique hormonal, metabolic, and physiological changes that occur during pregnancy may heighten women's sensitivity to nicotine and the numerous chemical constituents found in e-cigarette aerosols and increase the risk of adverse health outcomes. Despite this scientific research examining the prevalence, patterns of use, and health effects associated with vaping remains limited. **Methods:** This study investigates the prevalence and health effects of vaping during pregnancy in a cohort of patients receiving prenatal care at the University of Alabama at Birmingham (UAB). Participants are recruited from prenatal clinics to complete a smartphone-based survey on their tobacco use. Upon completion of the survey participants are given the option to provide their contact information to be contacted about participation in the in-person part of this study. Those who provide their contact information are invited to fill out the consent form for this study before being invited to participate. In Aim 1 participants ($n=100$) are invited to perform an exhaled carbon monoxide test and urinary cotinine test to biologically validate their self-reported tobacco. In Aim 2, two groups of pregnant vapers ($n=40$) and pregnant non-users ($n=40$) will be invited to provide blood and urine samples which will then be analyzed for biomarkers of exposure and inflammation and compared between the groups to evaluate whether vaping results in increased exposures and inflammation. In Aim 3, a subset of 20 participants from the vaper and non-user groups in Aim 2 will be invited to provide umbilical cord blood samples which will then be analyzed for exposure and inflammatory biomarkers to assess whether vaping-related chemicals can reach fetal circulation. **Results:** To date, 597 participants have participated in our survey and of those 487 have answered our questions about their tobacco use. Among those, 25 (5.1%) reported that they are exclusively vaping during their pregnancy and an additional 4 reported vaping along with using other tobacco products. The mean age of the overall sample population is 29.24 years old with a standard deviation of 6.46 years. The sample is predominantly White (50.2%) followed by Black (38.01%) and mixed race (4.27%) while vapers were disproportionately White (60%). Additionally, 7.39% of the sample is Hispanic. Among those who reported vaping during their pregnancy most reported vaping daily, using disposable e-cigarettes/vapes, using flavored e-liquids, and that they switched from using cigarettes to vaping. Then Vapers were split between taking 1-10 puffs per day and taking more than 50 puffs per day on a typical day of use. Notably, vapers had significantly lower educational attainment compared to participants reporting no tobacco use ($p = 0.0012$). Finally, of the 23 participants whose tobacco use has been biologically validated so far, 21 matched their self-reported use, while 2 who denied tobacco use tested positive for urinary cotinine and negative for exhaled carbon monoxide use which is indicative of vaping or other smokeless tobacco use. **Conclusions:** These preliminary findings underscore the importance of continuing to investigate the effects of vaping during pregnancy, particularly given its growing prevalence and the unique vulnerabilities of pregnant individuals and their developing fetuses. Our ongoing biomarker analyses will directly measure maternal exposure to nicotine and other chemicals in e-cigarette aerosols and assess whether this results in increased inflammation compared to non-users the extent to which these substances cross the placenta and reach fetal circulation. Ultimately, the evidence generated from this study will be essential for guiding healthcare providers, informing clinical counseling, and shaping public health recommendations and policy aimed at reducing harm and supporting healthier pregnancies.

PS 4064 Secondhand E-cigarette exposure induces sex and region-specific lung collagen deposition

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Background and Purpose: Electronic cigarettes are offered as a "safer" alternative to traditional combustible cigarettes, a perception that has driven their widespread use, and consequently, increased secondhand aerosols and vapors exposure. These aerosols contain particulate matter, volatile organic compounds, polycyclic aromatic hydrocarbons, and metals that remain airborne. Along with nicotine, carrier solvents like propylene glycol and glycerin, and metals released from atomizer components, contribute to airway irritation and impaired lung function, raising concern for chronic

respiratory injury in bystanders. In tissue fibrosis, dysregulated proliferation of fibroblasts leads to abnormal collagen buildup in lung tissue, making collagen a key indicator of fibrotic progression. Research has shown inflammatory and oxidative responses to aerosolized organic compounds and metals, both of which are associated with fibrotic lung injury. This study aimed to assess lung collagen deposition in female and male mice after subchronic and chronic exposure to secondhand e-cigarette vapor, with and without the metallic atomizer component, as a marker of fibrotic injury linked to early development of pulmonary fibrosis. **Methods:** Wildtype C57BL/6J mice (7 weeks old; n=5/sex per group) were exposed nose-only to secondhand e-cigarette vapor (eCig) for 2 hours/day, 4 days/week, for either 3 (subchronic) or 6 months (chronic). Exposure groups included purified air (control), eCig generated with a metallic atomizer, or vaporized metal-free e-liquid (E-liq). Aerosol was produced using a MOD device equipped with a nickel-chromium coil (3-s puff every 30s) at an average particle concentration of $15 \pm 3 \text{ mg/m}^3$. Following exposure, the left lung lobes were fixed, sectioned, and stained with Masson's Trichrome to visualize collagen. Whole-lung and region-specific (perivascular, peribronchial, alveolar) images were captured using automated bright-field microscopy, and collagen area was quantified with ImageJ software. **Results:** After 3 months of exposure, male mice in both the eCig and E-liq groups showed greater lung collagen deposition compared to controls, whereas females showed increases only in the eCig group. At 6 months, eCig-exposed males exhibited significantly higher collagen levels than both controls and E-liq males. In contrast, females in the eCig and E-liq groups displayed higher collagen after 3 months than after 6 months, suggesting a transient fibrotic response. Region-specific analysis revealed that the perivascular region was the primary site of collagen deposition in females across both exposure conditions, whereas males exhibited more diffuse patterns. **Conclusions:** Our results suggest that exposure to secondhand e-cigarette vapor with nicotine induces sex-specific lung collagen deposition, a hallmark of fibrotic injury. Differences observed in the extent and localization of collagen deposition between males and females, particularly within the perivascular, alveolar, and peribronchial regions, suggest a sex-specific susceptibility to fibrotic remodeling. Notably, increased collagen deposition was also observed in animals exposed to vaporized e-liquid, indicating that organic constituents and their thermal byproducts, independent of metal emissions, can promote fibrotic changes. These findings underscore the need to evaluate both e-liquid formulations and heating components when assessing e-cigarette use health risks. Future studies should investigate the sex-specific molecular mechanisms underlying fibrotic responses to organic versus metal components to inform targeted prevention and regulation.

PS 4065 Sex-Specific Transcriptomic Responses to Acute and Subchronic E-cigarette Exposure

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Background and Purpose: Electronic cigarette (e-cigarette) use has rapidly risen in popularity, particularly among youth and young adult populations. Despite widespread use, there is currently limited longitudinal data on health outcomes related to vaping. The purpose of this study was to compare the responses of mice to acute and subchronic e-cigarette exposures. **Methods:** For this study, we exposed male and female C57BL/6 mice (n = 6-8, 6-8 weeks old) to e-cigarette aerosols containing humectants (30% propylene glycol/70% glycerin) with and without free-base nicotine (6 mg/mL) produced from third-generation box mods for 1 hour a day for 1 week or 3 months using the whole-body InExpose system. Following the final exposure, RNA was isolated from lungs and bulk RNAseq was conducted. Differentially expressed gene (DEG) analysis was performed using EnrichR with false discovery rate correction. Pathway enrichment analysis was performed using PathfindR. **Results:** We found the most genes (n=30) to be differentially expressed in mice exposed to e-cigarette aerosol containing nicotine for 1 week. At 3 months, there were no significant differences in either exposure group. However, stratification of the exposed mice by sex demonstrated a differential response. At 1 week, DEGs were driven by female mice with no significant differences in male mice for either exposure. At 3 months, there was only 1 significant DEG in female mice exposed to humectants only whereas male mice exhibited DEGs in response to e-cigarette aerosols with (n = 14) and without nicotine (n = 10). The top enriched pathways for female mice were associated with cilium movement and response to xenobiotic stress. For male mice, the top pathways were cell-substrate adhesion, chemotaxis, and endothelium development. **Conclusions:** Our data indicate that male and female mice have divergent responses to nicotine-containing and nicotine-free e-cigarette aerosols from third-generation devices. Further, humectants alone may be drivers of these transcriptomic changes and potential cellular dysfunction with chronic exposure. Future work is focused on identifying the impacts at the cellular and molecular level, as these changes may be insightful for the potential consequences of chronic daily e-cigarette use.

PS 4066 Sex differences in regional brain volume changes in adult offspring with *in utero* electronic cigarette exposure

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Background and Purpose: Electronic cigarette (Ecig) use has increased rapidly and has been promoted as a safer alternative to smoking to pregnant women. Yet, Ecig use is known to increase oxidative stress and the long-term neurobiological effects to the maternal-fetal dyad remain poorly understood. Given that the brain is particularly vulnerable to perinatal environmental exposure, the use of magnetic resonance imaging (MRI) provides a non-invasive approach to assess structural alterations in during the course of life in progeny. The purpose of this study was to evaluate potential changes in brain volumes in offspring with history of maternal use of Ecigs during pregnancy. We tested the hypothesis that *in utero* Ecig exposure would produce significant changes in either whole brain and/or regional brain volumes compared to controls (i.e. air only exposure). **Methods:** We used 1T Bruker MRI system to examine whole brain volume and regional brain volumes (i.e. forebrain, hindbrain, olfactory and ventricles) in adult offspring at both 9- and 12-months of age with history of either *in utero* exposure to Ecig or Air. Time-mated C57BL/6J female mice were exposed from gestational day 7-21 to either Air (control, n=14 dams) or Ecig aerosol using 50:50 VG:PG, no flavor or nicotine (n=13 dams). MRI parameters for 9-month old male and female offspring were T₂-weighted, 160μm sagittal view. MRI parameters for 12-month old male and female offspring were T₁-weighted, 220μm sagittal and axial view. Regional brain volumes are reported normalized to whole brain volume (e.g. regional/WBV). **Results:** Overall ANOVA analysis revealed significant decrease in olfactory size with both exposure and time effects in Ecig offspring compared to air controls. However, whole and regional brain volumes effects were also found to have sex-specific effects. In males, with both 9- and 12-month-old offspring with history of *in utero* Ecig exposure, had an average of 3.0% smaller whole brain volume (WBV, p=0.003) compared to controls; as well as 2.7% decrease in WBV with advancing age (9- vs 12-month old, p=0.011) compared to controls (at 1.7%). Significant time-based changes were also seen with brain parenchymal fraction (BPF, p<0.001). And, WBV normalized forebrain (p=0.006) and hindbrain (p=0.0051) volumes show significant interaction effect for exposure x time. No significant changes were observed in WBV normalized olfactory bulb volumes. Absolute ventricular volume was lower with age in males (p<.001), but not with exposure. In females, WBV and normalized forebrain volume did not differ by exposure or time effects. Normalized hindbrain volume (p=0.022) and BPF (p<0.001) exhibited significant time (but not exposure) effects. WBV normalized olfactory bulb volume showed smaller time-based change in Ecig offspring compared to controls (4.6% vs 13.3%, respectively; p<0.001). Absolute ventricular volume was also lower with age in females (p<.001), but not with exposure. **Conclusions:** These findings are consistent with emerging evidence of adverse neurobiological health in offspring which experience *in utero* exposure to harmful or potentially harmful constituents (HPHCs) from Ecigs. Future studies need to provide longitudinal MRI assessment and behavior testing in offspring throughout its life course. Funding Support: NIH R56 ES035719-01, P20GM121322, U54GM104942 and WV-INBRE NIH Grant P20GM103434.

PS 4067 Investigating the Sex-Specific Impacts of E-Cigarettes on Humoral Immunity *In Vitro*

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Background and Purpose: E-cigarettes, initially designed to aid the cessation of traditional cigarettes, have become increasingly popular among young adults. Despite this notable increase in use, the health effects of e-cigarette exposure remain uncertain, especially regarding the immune system. B lymphocytes represent a key part of adaptive immunity because of their ability to generate specific long-term memory to pathogens. B lymphocytes originate in the bone marrow from a common lymphoid progenitor, and then develop from pro-B cells to mature B cells that can migrate to secondary lymphoid organs and secrete antibodies. Preliminary findings indicate that a two-week *in vivo* e-cigarette exposure decreases B lymphocyte populations in the bone marrow, with significant effects only seen in females. We aim to develop a model to interrogate these findings *in vitro*. **Methods:** Primary murine bone marrow from female and male C57BL/6 was isolated and utilized in Whitlock-Witte cultures for 7 days. These cultures were then treated with extracts from e-cigarettes or their component parts: propylene glycol and vegetable glycerin (PG/VG), ethanol, nicotine salts, tobacco-flavouring, STLTH extract, and Vuse extract. Cultures were treated for three days. B lymphocytes were analyzed using spectral flow cytometry to investigate cell death along with the Hardy fractions, representing the B cell populations during the different stages of B cell development. **Results:** Three-day *in vitro* treatment reduced the frequency of B220+ stained B lymphocytes, without a change in viability. Cultures from male mice were more sensitive than cultures from female mice. Cultures responded similarly to extracts from e-cigarette components. **Conclusions:** Our results suggest that B cell differentiation could be impaired following e-cigarette exposure while cell viability is preserved. Additionally, these preliminary findings suggest that the male primary B cell populations seem to be more sensitive to e-cigarette exposure *in vitro*. This suggests that primary B cell cultures are suitable *in vitro* models to investigate the molecular mechanisms of action of e-cigarettes.

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Background and Purpose: Cardiovascular disease (CVD) is the global leading cause of mortality for both sexes, but there is a feminine advantage in both lower prevalence and later onset. Yet despite this increased protection, females experience a greater acceleration in CVD risk around the menopausal transition compared to age matched males. Additionally, sex differences in CV risk factors reveal disproportionate vulnerability among women. Comorbidities such as diabetes and hypertension confer twice the risk of heart failure in women compared to men with the same conditions. A similar pattern is observed for the leading modifiable risk factor for myocardial infarction (MI): cigarette smoking. Although women smoke at lower rates and generally exhibit lower baseline CVD incidence, female smokers experience a 25% higher risk of developing coronary artery disease than male smokers and a 3x higher risk for MI. Yet, despite this clear mechanistic difference, preclinical CVD research in animal models is overwhelmingly biased, over 70% of studies solely use male mice. Furthermore, <1% of CVD preclinical studies use a human-like menopausal phenotype. Accounting for menopause is crucial when evaluating the cardiovascular impact of environmental stressors that span a significant portion of life. To address these limitations and create a translationally relevant model, we superimposed cigarette smoke exposure on a preclinical model of menopause. **Methods:** We replicated the human transition from perimenopause to menopause in female C57BL/6 mice by administering 4-vinylcyclohexene diepoxide (VCD), a selectively ovotoxic molecule that induces follicular atresia, resulting in a gradual decline and cessation of non-androgenic endocrine production. Following persistent diestrus, mice were exposed to cigarette smoke or air for 1 hour/day, 5 days/week, for 20 weeks. Our study thus included 4 groups, labelled as: VCD-Cig, VCD-Air, Estrous-Cig, and Estrous-Air. Functional changes to the descending thoracic aorta were quantified via vasoactive biaxial testing using phenylephrine for vasoconstriction and acetylcholine (ACh) for vasodilation. Vasodilation is largely mediated by the production of nitric oxide (NO) by endothelial nitric oxide synthase (eNOS), which then diffuses to vascular smooth muscle cells, triggering relaxation. A second ACh challenge following eNOS inhibition by L-NAME determined the contribution of eNOS to endothelial function. The difference between the two challenges acted as a proxy for NO bioavailability. Passive inflation-extension testing was used to determine changes in biaxial stiffness, stress, and cyclic distensibility. **Results:** The menopausal aorta phenotype exhibited two main features, increased contractility and impaired vasodilation in response to adrenergic and cholinergic stimuli, respectively, both of which were exacerbated by cigarette smoking. VCD and cigarette smoking increased contractility by 2.1x and 1.3x respectively, but VCD-Cig aortas showed 3x contractility compared to Estrous-Air controls. Endothelial function was significantly impaired by oxidative stress, VCD-Cig aortas only recovering to 57% relaxation compared to VCD-Air (59%), Estrous-Cig (72%), Estrous-Air (98%). VCD-Cig aortas exhibited the greatest increase in tissue stiffness, which in turn elevated pressure-induced circumferential wall stress, indicating that vascular cells may be unable to sustain normal physiological loading conditions. **Conclusions:** Overall, these results confirm that the menopausal transition presents a turning point for endothelial function and further suggests that the menopausal endocrine shift primes the vasculature for dysfunction when combined with exogenous sources of oxidative stress. Endothelial dysfunction is the primum movens of CVD, triggering a cascade of events resulting in hypertension, arterial stiffening, and heart disease. Ultimately, accounting for menopausal transition and menopause is necessary for translational relevance when studying aging-related disease in preclinical models. By excluding these effects, specific risks facing half of the aging populations may be overlooked. Utilizing menopause models in environmental exposure studies will advance understanding of the mechanisms that underly sex-dependent disease and risk disparities.

PS 4069 Electronic Cigarette Use During Pregnancy Impairs Uteroplacental Structure and Function

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Background and Purpose: Electronic cigarette (e-cig) use has increased among adults and pregnant individuals, with 1-15% reporting use during pregnancy. Although marketed as "safe", e-cigs contain nicotine, propylene glycol, vegetable glycerin, heavy metals, and flavorings that may adversely affect the uterine environment and compromise maternal and fetal health. We previously found that gestational e-cig exposure reduces maternal diastolic function, uterine artery blood flow, fetal heart rate, fetal growth, and placental efficiency, yet the underlying mechanisms remain unclear. We hypothesize e-cig use during pregnancy impairs placental development and function by reducing trophoblast differentiation and regulated cell death. Defining how e-cig aerosols alter trophoblast biology is essential for understanding their impact on uteroplacental structure and function for maternal and fetal health outcome prediction. **Methods:** Pregnant FVB/J mice were exposed to e-cig aerosols without nicotine (50:50 propylene glycol and vegetable glycerin, PV), or with 2% nicotine (PVN) for 5 days per week. At E17.5, dams underwent cesarean collection of fetal, placental and uterine artery tissues for fixation, H&E staining, or immunohistochemistry for morphometric analysis. **Results:** At E17.5, PV placentas showed reduced trophoblast giant cell density in the junctional zone ($P<0.05$, one-way

ANOVA), increased CD335/NKp46+ immune cell density in the decidua ($P<0.05$, one-way ANOVA) and decreased cleaved caspase-3 staining within the decidua ($P<0.05$, one-way ANOVA) compared to filtered air (FA) controls. Uterine arteries from exposed dams displayed reduced lumen area ($P<0.05$, one-way ANOVA). CD31 and α -SMA staining were unchanged between groups ($P>0.05$), indicating preserved endothelial and smooth muscle layers despite lumen narrowing. **Conclusions:** Gestational e-cig exposure, even in the absence of nicotine, disrupts trophoblast differentiation, regulated cell death, and uteroplacental vascular structure, contributing to impaired placental development and reduced placental efficiency. Together, these findings challenge the perception of e-cig use during pregnancy as "harmless" and highlight the need to reevaluate their safety for maternal and fetal health.

PS 4070 Combined Toxicity of E-Cigarette Flavoring Chemicals and Metals in Human Lung Epithelial Cells

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Background and Purpose: Menthol- and tobacco-flavored electronic cigarettes (e-cigarettes) are widely marketed as safer alternatives to combustible cigarettes. E-cigarette aerosols contain both flavoring chemicals, such as aldehydes, and metals, including nickel. The combined toxicity of these chemical-metal mixtures remains poorly understood. We hypothesized that e-cigarette-derived chemicals and metals exert synergistic toxicity on lung epithelial cells. **Methods:** Human bronchial epithelial cells (BEAS-2B) were exposed to common flavoring chemicals (vanillin, ethyl vanillin, p-anisaldehyde, and WS23) at 10 μ M-1000 μ M to assess dose-responses, individually and in mixtures (1:1-1:49), with or without nickel oxide (10 - 50 μ g/cm²). Twenty-four hours post-treatment, cytotoxicity was determined by MTT assay and AO/PI assays, and the elicited inflammatory response was assessed by quantifying IL-6 and IL-8 cytokine levels using ELISA in conditioned media. **Results:** Aldehydes induced differential toxicity thresholds (LOAELs: vanillin, 200 μ M; ethyl vanillin, 300 μ M; p-anisaldehyde, 100 μ M; WS23, 50 μ M). Mixture toxicity of vanillin and WS23 induced synergistic cytotoxicity, whereas ethyl vanillin and p-anisaldehyde showed antagonism. Ethyl vanillin and nickel oxide induced significant cytotoxicity and augmented IL-6 and IL-8 responses compared to either component alone. **Conclusions:** E-cigarette flavoring aldehydes interact with each other and with metal constituents to drive augmented epithelial injury and inflammation. Our data underscore the importance of mixture toxicity analysis in evaluating e-cigarette safety and suggest that regulatory policies should account for combined chemical-metal exposures rather than single-agent effects. This study was supported by the National Institutes of Health R00ES033835.

PS 4071 Intracellular cannabidiol induces translational repression and stress granules through eIF2alpha phosphorylation in human pulmonary cells

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Background and Purpose: Cannabidiol (CBD) is a compound extracted from Cannabis. Because CBD does not cause hallucinations, it is used for health purposes, and so on. Stress granules (SGs) are phase-separated and membrane-less intracellular organelles composed of proteins and RNA and play a cytoprotective role. The assembly of SGs is formed transiently in response to various stress stimuli to ensure cell survival. However, the stress responses including formation of SGs against inhaled CBD have not been extensively studied. In this study, we examined the effects of CBD on SG formation and translational repression using pulmonary cells to assess the toxicity of inhaled CBD. **Methods:** To examine time-dependent cellular uptake of CBD, the cells were exposed to 25 μ M CBD for 0-6 hrs. The cellular CBD concentrations were analyzed by LC-MS/MS. GFP-tagged G3BP1 expressing CHO-K1 cells (G3BP1-CHO) and human pulmonary cell lines, A549 and BEAS-2B (Beas), were exposed to 0-50 μ M CBD for 1 hr. The SG formation in G3BP1-CHO cells was observed using fluorescent microscope. A549 and Beas cells were immunostained with anti-G3BP1 and a fluorescence-tagged secondary antibodies to observe SG formation. A549 and Beas cells were lysed in RIPA buffer, and phosphorylated eIF2 α and total eIF2 α levels were examined by western blotting. To investigate whether reactive oxygen species (ROS) are involved in SG formation, CHO-K1 cells expressing Antioxidant Response Element (ARE)-Luciferase were exposed to 0-5 μ M CBD, and a reporter gene assay was performed. Translational repression was examined by puromycin-based SUNSET assay in CBD-exposed A549 and Beas cells. **Results:** LC-MS/MS analysis revealed that intracellular CBD rapidly increased after CBD exposure and accumulated in a time-dependent manner. In response to the intracellular CBD following exposure to 25-50 μ M CBD, G3BP1-CHO, A549 and Beas cells formed discrete SG assemblies after exposure to CBD. The levels of phosphorylation of eIF2 α increased by CBD in both A549 and Beas cells, suggesting that CBD induced canonical SG formation. In reporter gene assay, CBD significantly increased ARE activity. Puromycin-labeled newly synthesized proteins were robustly reduced in CBD-exposed A549 and Beas cells. **Conclusions:** These results suggest that pulmonary cells generate SGs in response to exposure to CBD via a canonical pathway to reduce the adverse effects. Furthermore, ROS might contribute to this process.

PS 4072 **Induction of Cytotoxicity in A549 Human Lung Carcinoma Cells by Illicit Flavored Disposable E-cigarette Products in the US Market**

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Background and Purpose: To date, the U.S. Food and Drug Administration (FDA) has authorized only tobacco and menthol flavored electronic nicotine delivery systems (ENDS). Despite this, illicit flavored disposable ENDS have become widely accessible in the United States (US). Recent data show that illicit flavored disposables represent approximately 80% of the US ENDS market. With the prevalent use of illicit flavored ENDS devices in the United States, consumers and regulatory bodies require a better understanding of the potential hazards associated with the use of these products. The purpose of this study was to screen a variety of e-liquids extracted from illicit ENDS available in the US market and assess the ability of those products to induce cytotoxicity in A549 cells. **Methods:** To assess the cytotoxicity of illicit product e-liquids we utilized the Neutral Red Uptake "NRU" assay with A549 cells, a human lung carcinoma cell line. The NRU assay is a robust assay which measures the ability of healthy cells to take up the dye neutral red. All NRU testing was conducted following OECD 129 and ICCVAM 07-4519 guidance modified to perform the test in A549 cells. Unflavored 1.5% nicotine and 5% nicotine strength e-liquids were prepared with equimolar benzoic acid and used as negative controls. As part of a broader market study to examine the chemical composition of e-liquids from 74 illicit ENDS products, 16 products were selected for further *in vitro* toxicology testing. E-liquids were diluted 1:1 with DMSO and then serially diluted into assay media to treat cells. All products were evaluated with 8 separate concentrations. Final DMSO concentration did not exceed 0.5% (v/v). Products that caused $\geq 30\%$ decrease in cell viability were considered cytotoxic (ISO 10993-5). Osmolality and pH were measured for assay media, vehicle control (VC), and the top two concentrations of test article. Product testing was separated into 2 waves with 5 to 10 products tested per wave. In this study, 16 disposable e-vapor products from the United States market were selected for cytotoxicity testing. **Results:** Sodium lauryl sulfate positive controls performed consistently with results from previously published interlaboratory studies (Coresta 2024) for A549. No impacts on pH or osmolality were observed with inclusion of test article as compared to VC. We found that all illicit flavored disposable e-liquids tested caused cytotoxicity in A549 after 48-hour treatments. Cell viabilities ranged from 67.1% to 10.8%. In comparison, negative control e-liquids did not elicit cytotoxicity in A549. Future studies will explore which aspect(s) of illicit products may drive cytotoxicity and expand to other prevalent cytotoxicity models such as Balb/c 3T3 clone a31. **Conclusions:** All illicit disposable ENDS products assessed in this study failed the NRU assay as described while our negative control e-liquids, 1.5% and 5% nicotine strength unflavored e-liquids, were found to be not cytotoxic. These findings highlight the need for better understanding of what factors drive cytotoxicity in these illicit flavored products and the importance of rigorous regulatory review of ENDS products.

PS 4073 **Enhanced Cytotoxic and Oxidative Stress Responses Induced by 6-Methyl and 4-Methyl Nicotine in Human Bronchial Epithelial Cells**

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Background and Purpose: 6-Methylnicotine (6MN) is an emerging synthetic nicotine analog identified in e-cigarette and oral pouch products, often marketed outside current FDA regulatory frameworks. 4-Methylnicotine (4MN), a positional isomer and synthesis byproduct, not currently used in commercial products, was included to explore structure-activity relationships. Systematic toxicological data for both analogs are lacking. This study compared the cytotoxicity and Oxidative stress-induced effects of 6MN and 4MN with nicotine in human bronchial epithelial cells (BEAS-2B). **Methods:** BEAS-2B cells were exposed to serial concentrations of NIC, 6MN, or 4MN for 24 hours. Assessments included cell viability (CKK-8), membrane integrity (LDH release), apoptosis (Caspase-3/7), proliferation (EdU assay), DNA damage (γ H2A.X foci), mitochondrial membrane potential (MMP), reactive oxygen species (ROS), glutathione (GSH) content, and expression of Nrf2-mediated antioxidant pathway genes. **Results:** Both 6MN and 4MN exhibited significantly greater cytotoxicity than nicotine, as reflected by lower IC₅₀ values and substantially higher LDH release. The methylated analogs also induced stronger anti-proliferative and genotoxic effects, along with more pronounced mitochondrial dysfunction, indicated by reduced MMP. Furthermore, 6MN and 4MN provoked a heightened oxidative stress response, characterized by increased ROS and GSH levels, and upregulation of Nrf2 target genes, suggesting an activated adaptive antioxidant response. **Conclusions:** 6MN and 4MN induce more severe cytotoxicity and oxidative stress in human bronchial epithelial cells than nicotine. These findings indicate that pyridine ring methylation enhances the toxic potential of nicotine, highlighting the need for comprehensive toxicological evaluation and regulatory scrutiny of these emerging synthetic analogs.

PS 4074 **Electronic Cigarette Aerosols Redirect Human Airway Epithelial Fate Toward Squamous Metaplasia with Partial Reversal After Exposure Cessation**

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Background and Purpose: Electronic cigarette (EC) aerosols contain chemicals capable of altering airway epithelial physiology, yet their toxicological effects remain inadequately characterized. Squamous metaplasia (SM) is a well-recognized consequence of smoking, but its induction by electronic cigarette (EC) aerosols has not been evaluated. SM involves replacement of the normal pseudostratified epithelium with squamous cells. This remodeling compromises mucociliary clearance, disrupts epithelial homeostasis, and increases the risk of chronic lung disease, such as squamous cells carcinoma; however, SM is recognized to reverse when the underlying cause is removed. This study used a 3D human tracheobronchial epithelial tissue (hTET) model derived from airway basal stem cells to determine whether EC aerosols induce SM, characterize dose- and time-dependent responses, assess donor-specific susceptibility, and evaluate the potential for reversibility following exposure cessation. **Methods:** Airway basal stem cells from deceased human donors were expanded and differentiated at the air-liquid interface for 28 days to generate mucociliary hTETs. To first establish a temporal and dose-dependent exposure protocol, hTETs were exposed daily to aerosols from the NJOY ACE EC for three weeks at 1 puff/day or 5 puffs/day, with clean air as a control. Morphometric endpoints and markers of mucin (MUC5AC), cilia (acetylated α -tubulin), and squamous differentiation (involucrin) were quantified after week 1, 2, and 3 using immunolabeling, image-based analyses, and Western blotting. After the exposure protocol was defined, the same exposure was applied to aerosols generated from NJOY ACE and two popular brand devices (Vuse Alto and JUUL) using hTETs derived from six human donors. Following the 3-week exposure period, tissues were subjected to an additional two-week aerosol-free recovery period. SM severity (mild, moderate, severe) and reversibility were quantified by a min-max rescaling approach integrating all measured endpoints. **Results:** NJOY ACE exposure demonstrated that MUC5AC increased through week 1, peaked at week 2, and declined by week 3 under 5-puffs conditions. Early SM indicators, including ciliary loss and involucrin elevation, emerged by week 1 and amplified with increasing puff number. By week 2, tissues exhibited pronounced ciliary loss, reduced epithelial thickness, horizontal nuclear reorientation, and elevated involucrin. By week 3, squamous metaplasia phenotype was evident in both dose groups. Quantitative analyses demonstrated involucrin levels plateaued by week 3, at which point the 1-puff group reached concentrations comparable to those observed in the 5-puff group. Applying the established exposure protocol to multiple EC products and donors demonstrated both device- and donor-dependent variability. Vuse Alto induced the most severe remodeling, followed by JUUL and NJOY ACE. All donors exposed to Vuse Alto developed moderate-to-severe SM, whereas JUUL and NJOY ACE produced a wider range of responses, with four donors showing severe remodeling, one moderate, and one minimal. Recovery depended strongly on the initial severity of remodeling, with mildly affected tissues showing near-complete reversal and severely remodeled tissues exhibiting only partial restoration evidenced by limited ciliogenesis, reduced involucrin, and incomplete epithelial reorganization. Full mucociliary recovery was not observed. **Conclusions:** These findings demonstrate that EC aerosols from widely used products can reprogram human airway epithelium toward a squamous fate in a dose- and time-dependent manner, and that severe remodeling shows limited capacity for repair. The substantial variability across devices and donors emphasizes the complexity of EC-associated airway injury and reveals the limitations of relying on traditional toxicology screening assays. Together, these observations highlight the need for more product assessment and strengthened surveillance as EC device designs and formulations continue to evolve.

PS 4075 **Macrophages Synergistically Enhance the Airway Epithelial Cell Response to E-cigarette Exposure**

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Background and Purpose: The US Centers for Disease Control and Prevention estimates that approximately 6.5% of adults in the United States are current electronic cigarette (e-cigarette) users. E-cigarettes are drug delivery devices that produce aerosols by heating and aerosolizing e-liquids containing primarily propylene glycol, glycerol, nicotine, and flavorants. Clinical reports indicate detrimental health impacts of e-cigarettes in adult users, yet there is still a common perception that e-cigarettes are safer to use than conventional tobacco cigarettes. *In vivo* investigations using rodents have shown increased oxidative stress and inflammation in the lungs following aerosol exposure to e-cigarettes. However, *in vitro* studies using lung epithelial cell lines and primary cultures have reported inconsistent outcomes using comparable modalities for exposure to e-cigarettes. Because the alveolar macrophage is a significant cellular contributor to mucosal immunity in the lung, we hypothesized that macrophage and lung epithelial cells may function synergistically when exposed to e-cigarettes. To test this hypothesis, we used established human cell lines for macrophages and lung epithelial cells to investigate whether cocultures modulate the inflammatory response to e-cigarettes, using the neutrophilic chemokine IL-8 as

an outcome measure. **Methods:** The human promyelocytic leukemia HL-60 cell line was used to model a macrophage phenotype. The human bronchial epithelial cell line BEAS-2B and human adenocarcinoma alveolar epithelial cell line A549 were used to model conducting airway and alveolar epithelium, respectively. HL-60 cells were differentiated into macrophages by culturing with phorbol 12-myristate 13-acetate for 48 hours. BEAS-2B or A549 cells were directly cultured on the basal membrane surface of Transwell inserts until confluent. Differentiated HL-60, BEAS-2B and A549 cells were separately treated with 3% tobacco-flavored JUUL diluted in media, 3% propylene glycol and glycerin vehicle (PGVG) diluted in media, or media control for 6 hours. After treatment, all cells were washed with phosphate buffered saline, then combined as cocultures for 24 hours. Media from the basolateral Transwell compartment (apical surface of epithelium) were collected and evaluated for IL-8 protein using ELISA. **Results:** In monocultures of HL60, BEAS-2B and A549 cells, 3% JUUL exposure did not result in a significant increase in IL-8 secretion relative to unexposed controls. JUUL-exposed BEAS-2B cells significantly decreased IL-8 secretion when cocultured with JUUL-exposed HL-60 cells, relative to unexposed control HL-60 cells. In contrast, JUUL-exposed A549 cells showed a significant increase in IL-8 secretion when cocultured with HL-60 cells, independent of prior JUUL exposure. Additionally, there was a modest, but significant increase in IL-8 secretion when comparing JUUL-exposed A549 cells with JUUL-exposed HL-60 cells, relative to control HL-60 cells. To assess for synergistic interactions, we compared expected IL-8 concentrations derived from monocultures relative to coculture outcomes for all exposure groups. For both BEAS-2B and A549 cells, there was a significant difference between expected and actual IL-8 concentration resulting from cocultures, primarily in conjunction with JUUL treatment. In BEAS-2B cocultures, we observed 2-fold increase in IL-8 secretion with HL-60 cells relative to expected concentrations, despite the attenuated response with JUUL treatment relative to controls. In A549 cocultures, we also found an approximate 2-fold increase in IL-8 secretion relative to expected concentrations. **Conclusions:** Taken together, our findings support a synergistic relationship between macrophages and lung epithelium that modulates the innate immune response following exposure to e-cigarette liquids. Differential outcomes between conducting airway and alveolar epithelium may reflect regionally distinct inflammatory pathways. Future studies will incorporate *in vitro* aerosol exposures to validate e-cigarette liquid findings, as well as the use of primary alveolar macrophage and airway epithelial cell cultures.

PS 4076 Tobacco Harm Reduction: *In Vitro* Toxicological Assessment of Next Generation Tobacco and Nicotine Products versus Combustible Cigarettes and Smokeless Tobacco

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Background and Purpose: Next generation tobacco and nicotine (NGP) products, which include Electronic Nicotine Delivery Systems (ENDS), Heated Tobacco Products (HP), and Nicotine Pouch (NP) products, provide alternative, potentially less harmful, nicotine products to adult smokers and nicotine consumers wanting to transition from combustible and other tobacco containing products. NGPs must undergo the premarket tobacco product application (PMTA) process and obtain a marketing granted order (MGO) from FDA Center of Tobacco Products (CTP) to become authorized for marketing. Currently, FDA-CTP PMTA submissions require comprehensive listings of product ingredients and components, harmful and potentially harmful constituents (HPHC), product stability and information on potential health risks including risk assessments, *in vitro* toxicological, and clinical testing to establish a Weight of Evidence (WoE) to determine if the marketing of an NGP is appropriate for the protection of the public health (APPH). **Methods:** This assessment will examine the *in vitro* toxicological testing of representative ENDS, HP and NP products (all menthol or mint flavor varieties) through a series of three standard genotoxicity and cytotoxicity assays and compared to combustible cigarettes (CC) and traditional smokeless tobacco (ST). The three standard regulatory toxicology assays utilized include the Ames (mutagenicity), *in vitro* micronucleus (iVMN: genotoxicity), and neutral red uptake (NRU: cytotoxicity) following respective OECD guidelines. Test samples from CC, HP, and ENDS included pad-collected total particulate matter (TPM: CC & HP) or aerosol collected material (ACM: ENDS), all combined with their respective gas vapor phase (GVP) preparations as well as combustible cigarette whole smoke (WS) and whole aerosol (WA) from ENDS and HP (NRU). NP products with either tobacco-derived (TDN) or synthetic (SYN) nicotine and ST (snus) were tested using complete artificial saliva (CAS) extracts in all three assays. The Ames assay utilized a recommended battery of five *Salmonella* tester strains (TA98, TA100, TA102, TA1535 & TA1537) with and without metabolic activation ($\pm$ S9). The three standard exposure schedules were performed in the iVMN, with an additional long-term exposure schedule (24-hr, -S9, with 24-hr recovery) previously shown to increase sensitivity of these test items in the iVMN assay. Cytotoxicity of CC, ENDS and HP was determined by exposing mammalian cells at the air-liquid interface (ALI) to freshly generated WS (CC) or WA (ENDS, HP) while NP and ST samples utilized CAS extracts. For all assays, nicotine content of the test samples was determined to allow testing to be based on nicotine equivalents and allow comparison across NGP categories. **Results:** CC TPM+GVP was genotoxic in both the Ames (TA98; $\pm$ S9: TA100; $\pm$ S9 TA1537; +S9) and iVMN (all exposure schedules) assays while ENDS and NP tested negative in both the Ames (all strains, $\pm$ S9) and iVMN (all exposure schedules). The HP was non-mutagenic in the Ames but genotoxic in the iVMN (short-term, -S9; long-term with recovery), albeit less toxic when comparing the slopes of the CC

and HP response curves ($p < 0.0001$) at $\sim 10\times$ higher delivered nicotine equivalent concentrations compared to CC. CC WS was cytotoxic ($IC_{50} \sim 2.0 - 2.4 \mu\text{g}$ nicotine equivalents) while an IC_{50} could not be determined for ENDS up to maximum delivered dose ($\sim 3 \text{ mg}$ nicotine equivalents). An IC_{50} was determined for HP WA ($\sim 70 \mu\text{g}$ nicotine equivalents) that was statistically significantly higher (less cytotoxic) than CC WS ($p < 0.0001$). CAS extracts of NP (TDN and SYN) and ST (snus) were found to be non-mutagenic (all strains, $\pm$ S9), non-genotoxic (all exposure schedules) and non-cytotoxic (NRU) at the tested doses. **Conclusions:** Overall, the results from this series of studies found ENDS and NP to be non-genotoxic and non-cytotoxic and HP with decreased genotoxicity and cytotoxicity when compared to CC and provides important data to the WoE necessary to determine if an NGP is APPH. Additionally, the *in vitro* data supports the possible role these NGPs have in the tobacco harm reduction paradigm.

PS 4077 Development of the combined oxidative stress and cytotoxicity model in MucilAir tissues for the exposure of cigarette smoke and Heated Tobacco Product aerosols

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Background and Purpose: Oxidative stress plays an important role in the progression of some diseases, such as cardiovascular disease and various inflammatory conditions such as chronic obstructive pulmonary disease (COPD). Several clinical markers of oxidative stress exist including Nrf2 transcription factor and 8-isoprostane (8-IP), a stable downstream marker following Nrf2 activation. Cigarette smoking induces oxidative stress in the lungs of smokers due to the abundance of chemical toxicants in cigarette smoke. Heated Tobacco Products (HTP) are postulated as a potentially reduced risk product compared to cigarettes, and HTP aerosols show lower biological activity in *in vitro* and clinical studies as compared to cigarette smoke. We exposed the 3D lung model, MucilAir, to cigarette smoke and a HTP aerosol to measure 8-IP as a biomarker for oxidative stress and combine this with a measure of cytotoxicity using MTT reduction. Doses were carefully selected to ensure an appropriate number of doses within the non-cytotoxic range ($>70\%$ cytotoxicity) for 8-IP measurements. **Methods:** Eight 1R6F cigarettes were smoked under ISO 20778 (55mL puff volume, taken over 2 seconds, every 30 seconds, with vents 100% blocked) on a Vitrocell® VC 10® and MucilAir tissues were exposed to the whole resulting aerosol for approximately 30 minutes. Seven diluting airflows ranging from 10L/min to 0.5 L/min were used. An HTP aerosol was generated using the Vitrocell VC 1/7 under ISO 5501-1 (55mL puff volume, taken over 2 seconds, every 30 seconds, with vents unblocked). 24 sticks were puffed over 120 minutes, using 7 diluting airflows ranging from 4L/min to 0 L/min. Positive controls Phorbol 12-myristate 13-acetate (PMA), ionomycin and tert-butyl hydroperoxide (tBHP) were included. Following exposure, a sample of the culture media was collected from the exposure module media and the recovery media, and analysed for 8-IP using ELISA kits. Cytotoxicity was assessed using the MTT reduction assay. Nicotine was also measured in a blank Transwell for each dose of aerosol delivered as a dosimetry metric. **Results:** PMA, ionomycin and t-BHP induced 8-IP responses that were typically >10 fold and >3 -fold the vehicle control, respectively. 1R6F smoke induced dose related increases in 8-IP release that typically exceeded ~ 3 fold compared to the concurrent air control at non-cytotoxic doses. In addition, dose related decreases in tissue survival was observed at aerosol dilutions ranging between 4 and 0.5 L/min, and IC_{50} values could be derived. The HTP aerosol also induced dose related increases in 8-isoprostane that typically reached 2 to 3-fold the concurrent air controls at non-cytotoxic concentrations, and dose related decreases in tissue survival were observed at diluting airflows between 2 and 0 L/min. These responses were only achieved when a more concentrated aerosol and longer exposure duration was used. To account for the differences in the exposure duration and aerosol concentration between the 1R6F and HTP exposures, the deposited nicotine was used as a dose descriptor. At the lowest diluting airflows (highest aerosol concentration), 1R6F deposited a mean nicotine concentration of $25.3 \mu\text{g/mL}$, whereas the HTP deposited $150.7 \mu\text{g/mL}$. There is a clear differentiation between cigarettes and HTPs for both 8-IP release and cytotoxicity, with 1R6F clearly inducing higher levels at significantly lower levels of delivered nicotine compared to HTPs. **Conclusions:** A combined model to determine both 8-IP release as a surrogate for oxidative stress and cytotoxicity in the same *in vitro* tissue culture has been developed. The positive controls induced significant increases in 8-IP release at different levels of induction, demonstrating the potential of the assay to detect low and high responses to tested aerosols. Exposure to whole aerosol from 1R6F and HTP showed dose related increases in 8-IP release at non-cytotoxic doses, and complete cytotoxicity as measured by MTT reduction. However, 1R6F induced higher levels of 8-IP, and significantly longer exposure duration and more concentrated aerosol concentrations were required to induce responses from the HTP, adding to the weight of evidence that HTPs offer potentially reduced risks as compared to traditional cigarettes and can thereby contribute to tobacco harm reduction.

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Background and Purpose: It was recently shown that e-cigarette (EC) use during pregnancy increases the risk of cardiovascular disease (CVD) in the offspring. While EC usage has been on the rise among pregnant women, this is not the only means by which innocent fetuses get exposed. Indeed, there is evidence that ECs are also a source of thirdhand exposure (THEC), which is formed from toxicants "produced" by vapor that accumulates on materials including carpets, clothing, furniture etc. In this connection, we have recently shown that *in utero* thirdhand smoke increases the risk of thrombosis. However, whether *in utero* THEC exposure poses any health effects on the offspring remains unknown. Our objective was to characterize the impact of maternal THEC on platelet functional responses and thrombotic CVD, and gain mechanistic insight into platelet transcriptomic changes in the exposed adult offspring that could mediate thrombogenicity triggered by maternal THEC exposure. **Methods:** Mice were housed with EC-exposed or clean air (CA) material for one week prior to mating and throughout the pregnancy to mimic real-life exposure scenarios. To assess the phenotype, platelet functional assays were performed. Blood was collected from twenty mice in each exposure group, and platelets were depleted of leukocytes and red blood cells. Additionally, RNA sequencing was utilized to identify differentially expressed platelet expression profiles between maternal THEC and CA exposed mice. All data was collected on the adult offspring at 10-12 weeks of age, and in a sex dependent manner. **Results:** Our results indicate that maternal THEC exposed mice exhibit shortened bleeding and occlusion times in comparison to clean air. Platelet function assays revealed platelet aggregation and dense & alpha granule secretion were potentiated in the maternal THEC exposed mice upon agonist stimulation. Additionally, integrin activation and phosphatidylserine exposure, separate platelet functional responses, were also enhanced. Notably, no detectable differences were observed in platelet number, and maternal THEC exposure did not impact the weight of the offspring. Furthermore, our platelet transcriptomic data highlighted 257 dysregulated pathways in females primarily associated with platelet activation and aggregation, including activated P2Y purinergic receptor, thrombin, and GP6 signaling pathways. In contrast, males had 175 dysregulated pathways mainly associated with inflammation and oxidative stress, suggesting an indirect effect on platelet function, including but not limited to NIK noncanonical NF-KB signaling and KEAP1-NFE2L2 pathway. Moreover, eNOS signaling was dysregulated in both males and females suggesting that the integrity of the vascular wall was compromised as a result of THEC exposure. Differentially expressed (DE) platelet patterns displayed 121 and 17 clustered genes in males and females, respectively. Integrated analysis of DE genes and dysregulated pathways designated several interactions to be associated with platelet signaling pathways, inflammation and oxidative stress, or impaired vascular wall integrity. Notably, there were some sex differences observed in specific aspects of the phenotype, which is consistent with the transcriptomic data. **Conclusions:** It is evidenced by our data, and demonstrated for the first time, that the observed prothrombotic phenotype is a multifactorial process involving a combination of direct and indirect platelet function alterations that are programmed during fetal development, which highlights it as an underestimated risk factor for CVD in the offspring. These findings could inform cessation efforts and guide policy development to prevent humans from being subjected to any known and unknown health risks associated with THEC; especially in vulnerable populations such as pregnant females.

PS 4079 Serum Surfactant Protein-D is Not Elevated in E-Cigarette Users:
Divergent Alveolar Responses Compared to Cigarette Smoking or
Too Early To Tell?

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Background and Purpose: Alveolar type II (ATII) cells synthesize surfactant protein D (SP-D) and secrete this into the alveolar lumen. In the setting of lung injury, SP-D is translocated into the circulatory system, resulting in increased serum SP-D. These processes seem to be driven by ATII cell stress and alveolar leakage as opposed to increased synthesis systemically. While elevations in serum SP-D have been documented in combustible cigarette smokers, the effect of e-cigarette vaping on the release of SP-D into the circulation has not been studied. Here we utilized our cohort of young, healthy, chronic e-cigarette vapers to determine whether 1. circulating SP-D is elevated in the setting of vaping, and 2. serum SP-D concentrations correlate with recent vaping, using self-reported time since last vape as well as a cotinine concentration, a serum biomarker of nicotine exposure. **Methods:** A cross-sectional observational study was performed in healthy young adults aged 18-35 years. Participants were grouped based on their history of inhalant use: non-smoking non-vaping controls (n=23), nicotine vapers (n=12), and all other inhalant users (cannabis vapers, cannabis smokers, tobacco smokers and mixed inhalant users; n=73). Blood was drawn from all subjects, serum isolated, and SP-D quantified via ELISA (R&D

Systems). Data were analyzed using ANOVA, Welch's t-tests, and linear regression. *SFTPD* gene expression was measured in human lung epithelial cells (H441), after exposure to Air, Nicotine control, Vehicle control and JUULTM Mint, Mango, Menthol and Virginia Tobacco flavored aerosols *in vitro*, via droplet digital polymerase chain reaction (ddPCR). **Results:** Serum SP-D concentrations did not vary between nicotine vapers (60.6 ng/mL) and controls (51.0 ng/mL; Welch's t (18.6) = 0.61, p=0.55, $\eta^2=0.02$). Likewise, when all inhalant users were compared to controls, no difference was observed (62.0 vs. 51.0 ng/mL, respectively; t(92.8)= 0.78, p=0.44, $\eta^2=0.006$). Inhalant users did have greater variance in serum SP-D levels compared to controls (F=6.82, p<0.0001). In nicotine vapers, serum SP-D did not vary by self-reported time since last vape (ANOVA: F = 1.24, p=0.30, R²=0.05) or by their serum cotinine level (linear regression: F(1,70)=0.0029, p=0.96, R²<0.001; β = -0.00014 [95% CI -0.0055 to 0.0052]). Expression of *SFTPD* in human epithelial cells exposed *in vitro* did not vary between different JUULTM flavored aerosols and air controls 24 hours after exposure (ANOVA: F = 0.30, p=0.922, R²=0.11). **Conclusions:** No association between elevated serum SP-D levels and chronic e-cigarette vaping was detected in human subjects, nor was a correlation to nicotine exposure identified. No change in expression of *SFTPD* in human epithelial cells exposed *in vitro* was found between JUULTM flavored aerosols and air control. These findings suggest that e-cigarette vaping does not cause the same acute ATII cell injury seen in patients using combustible cigarettes, which drives increased SP-D levels in the circulation. Alternatively, serum SP-D might not be related to chemical exposure but rather to lung diseases such as COPD, which develop over time. This may suggest that the effect of vaping on alveolar damage is minimal or that systemic SP-D only rises after decades of chronic exposure. Unfortunately in this study we were unable to include subjects who only smoke combustible tobacco, because it is a rare pattern of inhalant use in this age group. Future studies will follow subjects longitudinally to assess for trends in serum SP-D over time, as has been previously described in cigarette smoking.

PS 4080 Characterization of breath biomarkers in vapers, smokers, and non-users via a novel breath biopsy method

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Background and Purpose: E-cigarette use (vaping) continues to rise across the world, particularly among young adults. While e-cigarettes are considered less harmful than combustible cigarettes, heating of e-liquids generates volatile organic compounds (VOCs) and other toxicants with potential respiratory, cardiovascular, and immune-related effects. A key challenge in vaping-toxicity research has been the difficulty of distinguishing vaping-associated biomarkers from those of smoking and other tobacco product use, with current assessment methods being mostly invasive. Breath biopsy offers a non-invasive approach to assess both exogenous exposures and endogenous biomarkers linked to inhaled toxicants. Therefore, this pilot study aimed to explore the potential of 'breath biopsy' - a non-invasive and highly sensitive technique - to identify unique biomarkers associated with vaping. **Methods:** We conducted a single-site, cross-sectional observational study of 24 healthy males (aged 21-35) that included cigarette smokers (n=8), exclusive e-cigarette users (n=8), and those who did not use any tobacco products (n=8). Breath samples were collected from all groups, with an additional abstinence condition in vapers: samples were taken at baseline and after 24 hours without vaping. Owlstone Medical's (UK) ReCIVA breath sampler with their breath biopsy platform was used to capture and analyze exhaled VOCs. **Results:** Vapers and smokers exhibited clearly distinct VOC biomarker clusters, underscoring the ability of breath biopsy to identify and differentiate inhalation exposures from e-cigarette and combustible cigarette aerosols. Overall, VOC biomarkers associated with health risks were elevated in smokers and vapers compared to controls. A 24-hour abstinence period in vapers demonstrated decreased VOC levels, suggesting that some vaping-related biomarkers are transient and that the method is sensitive to recent use. Further analysis of VOCs in the breath demonstrated that the gut microbiome and nutrient uptake can be negatively impacted in vapers and smokers. Despite the modest sample size, the tightly controlled design and exclusive user groups lent robustness to the findings. **Conclusions:** This exploratory study demonstrates the feasibility of using breath biopsy to distinguish between smokers and vapers based on VOC profiles. VOC biomarkers may provide early insights into physiological changes from vaping, including selenium depletion and microbiome alterations. These findings support larger longitudinal studies to better define vaping-related health risks.

PS 4081 Stability of Combustible Cigarettes Gas Vapour Phase Extracts For In Vitro Testing

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Background and Purpose: The *in vitro* testing of cigarettes often involves the fractionating of the aerosol produced by the product, by using automated smoking robots. The particulate matter is captured on Cambridge filter pads (Total Particulate Matter; TPM), and the gas-vapour phase (GVP) that passes through the pad is collected in an impinger solution. The impingers often contain aqueous or organic solvents that act

as a gas wash to trap volatile and semi-volatile substances, such as carbonyls. The TPM and GVP are often combined into one test article and tested *in vitro*. TPM has been well characterised and much is known about its long term stability. However, less is known about the GVP stability. Because of its assumed instability due to the presence of volatile and semi-volatile compounds, scientific literature often states that GVP should be used within 1 hour, or at least on the same day of generation. Furthermore, no studies have investigated the potential for long-term storage of the GVP for the same period and conditions as the TPM. This study was designed to address four key questions: Is PBS or ethanol more efficient at trapping carbonyls; at what point does the GVP become saturated; at what timepoint does the GVP begin to deteriorate on the day of generation; and what is the long-term stability of GVP under frozen conditions? **Methods:** 1R6F cigarettes were smoked on a Korber RM200 smoking robot, and the TPM trapped on Cambridge filter pads. The GVP that passed through the pad was trapped in two impingers in series into a fixed volume of either PBS or ethanol - the second impinger was used to quantify any blow off from the first impinger. The following methods were then applied to address each question: An incremental number of cigarettes were smoked to determine the point of saturation for both solvents. A fixed number of cigarettes was then puffed and the GVP captured in impingers containing PBS. Immediately after the completion of the smoking run, the GVP was then aliquoted into glass vials with minimal headspace and refrigerated. 14 timepoints spanning immediately after aliquotting and up to 6 hours storage were analysed for carbonyls. Aliquots of GVP were stored at -80°C and analysed at specified timepoints for up to 7 months storage. **Results:** The data demonstrated that no saturation of carbonyls occurred, when up to 12 cigarettes were puffed into PBS or ethanol. A linear trend in increasing levels of carbonyls was observed with the increasing number of cigarettes. The secondary impinger to determine any blow off that occurred from the initial trapping there was approximately 20-30% capture when compared to the initial values for PBS. For ethanol, similar values were seen at lower number of cigarettes; however at higher concentrations this increased to approximately 50%, suggesting that PBS was more efficient at trapping carbonyls compared to ethanol. Both the PBS and ethanol samples were stable for up to 6-hour storage, when kept under refrigerated conditions with minimal headspace. The long-term stability phase demonstrated that the GVP was stable for up to 4 months storage; however after this timepoint, some deterioration of some carbonyls were observed. **Conclusions:** This study has demonstrated that PBS has a higher efficiency for trapping carbonyls than ethanol and no saturation was observed when up to 12 cigarettes were smoked. When the GVP was stored refrigerated for up to 6 hours, no deterioration in any carbonyls were observed and the long-term stability demonstrated that GVP can be stored for up to 4 months without a significant loss of carbonyls.

PS 4082 Preliminary investigation of the chemistry and respiratory toxicology of vaping

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Background and Purpose: Vape liquids, typically composed of propylene glycol (PG), vegetable glycerine (VG), nicotine, and ethanol, undergo heating and aerosolisation during use, generating additional toxic by-products. Understanding the health risks of vaping requires a dual approach: chemical characterisation of aerosols and biological assessment of their effects on lung tissue. Lung-on-a-chip technology offers a biomimetic model bridging *in vitro* studies and clinical relevance. The newly released Emulate Chip-A1, optimised for aerosol studies, motivated our investigation into vaping-related respiratory risks. Here, we present 2D cell culture data and outline an experimental framework for aerosol analysis and chip-based testing. **Methods:** Human alveolar epithelial cells (NCI-H441), cultured in 96- and 24-well plates, were exposed to various vape liquid components, PG/VG, nicotine (3-36 mg/ml) and ethanol (1-30% v/v) for 24 hours. Cell supernatants were collected and analysed for evidence of cytotoxicity (lactate dehydrogenase, LDH) and cytokines (IL-8 and IL-10). **Results:** Vape aerosols are highly diluted in the airways, exposing alveoli to ~1% of the original vape liquid. To model this, NCI-H441 cells were treated with varying PG and VG dilutions. At 10%, PG induced greater cytotoxicity than VG, and nicotine increased this effect dose-dependently, while ethanol showed no toxicity. Thus, 10% solutions were used for cytotoxicity assays and 1% for non-cytotoxic inflammatory responses. At 1%, PG and nicotine significantly elevated IL-8 without affecting IL-10, ethanol remained non-toxic. We adapted a vaping machine to generate aerosols under controlled puffing regimes for chemical analysis and exposure within a lung-on-a-chip system. **Conclusions:** Preliminary data suggest that vape liquids may impair immune responses in alveolar epithelial cells by increasing pro-inflammatory IL-8 while not affecting anti-inflammatory IL-10. Although ethanol alone had no effect in 2D cell culture, its volatility and -OH group could alter aerosol composition and chemical interactions during heating. We will next use our vaping machine to collect whole aerosols for analysis of PG, VG, nicotine, ethanol, carbonyls, and metal content, and for controlled exposure of human cells in the 3D lung-on-a-chip system. This approach will extend our 2D culture findings, modelling aerosol exposure in a more physiologically relevant system. A key focus will be measuring the impact of increasing power wattage, which raises aerosolisation temperature and may enhance the formation of harmful compounds. As the first group in New Zealand applying lung-on-a-chip technology to vaping research, we aim to provide mechanistic insights into the respiratory effects of vape aerosols. Our findings will have regulatory relevance and may support evidence-based harm reduction strategies and public health policy.

PS 4083 Developing a multi-task quantitative structure-activity relationship (QSAR) model for the prediction of e-cigarette emissions toxicity

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Background and Purpose: Electronic cigarette (e-cigarette) products and emissions contain a complex mixture of active ingredients, solvents, flavors, thermal degradation products, and contaminants. Despite recent efforts, evaluating the toxicity of each organic constituent through experimental methods alone is infeasible due to the large volume and variability of compounds, posing a significant challenge for effective hazard assessment. To address this gap, we developed interpretable deep neural network (DNN)-based quantitative structure-activity relationship (QSAR) models to predict e-cigarette toxicity for multiple endpoints: eye irritation, skin irritation and sensitization, carcinogenicity, and genotoxicity. **Methods:** The dataset of over 980 diverse e-cigarette chemicals was curated from existing literature and toxicological databases (e.g., CompTox Chemicals Dashboard) and included labeled data for each endpoint ("1": Toxic, "0": Non-Toxic). A total of seven DNN-QSAR models were developed using PyTorch: five single-task models to independently predict related endpoints, and 2 multi-task models to simultaneously predict related endpoints. For the multi-task models, eye irritation, skin irritation, and skin sensitization were grouped into one model, and carcinogenicity and genotoxicity were grouped together into a second model. All models used nested cross validation with Bayesian optimization to optimize hyperparameter tuning and reduce the risk of overfitting. The best-performing model was tested on a holdout set for final evaluation, and metrics were computed across endpoints. Finally, applicability domain analysis was conducted to define the chemical space reliably covered by the model and SHapley Additive explanation (SHAP) to identify important chemical features that most significantly impacted model performance. **Results:** The optimized DNN-QSAR models demonstrated good predictive performance for all endpoints (all AUC ≥ 0.66, all Accuracy ≥ 61%). Compared to the best performing single-task models, certain endpoints, such as eye irritation, saw substantial performance improvement (AUC +13 %, Accuracy +20%), while others, like carcinogenicity saw reduced performance (AUC -13 %, Accuracy -5%). Feature importance analysis revealed that nitrogen-containing groups (e.g., amines and amides) were influential predictors of eye and skin toxicity, whereas cancer-related endpoints were highly dependent on the electrostatic and electronegative properties of compounds. These results highlight the importance of carefully selecting tasks that share high degrees of structural feature overlap, sample sizes, and class distributions for efficient multi-task models. **Conclusions:** This study presents novel single- and multi-task QSAR models capable of predicting important toxicity endpoints for e-cigarette emission constituents that currently lack detailed toxicity data. These models can help address current data gaps and support more comprehensive, high-throughput hazard evaluations of e-cigarette products, potentially informing regulatory decision-making and supporting safer product formulation. Future work will focus on expanding the model's chemical coverage and validating predictions with experimental data to further strengthen its application in toxicity risk assessment.

PS 4084 Emissions characterization and modelled dosimetry estimates from ultrasonic Ecig and IQOS devices

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Background and Purpose: E-cigarette (Ecig) that vaporize liquid has been undergoing advancements to attract users. SURGE, a modern device, aerosolizes e-liquid via ultrasonic vibrations (~3 million hits/sec) to generate emissions. IQOS is a heated tobacco product (HTP) that warms a tobacco-laced cigarette without burning it to generate its emissions. Particle and chemical constituents in these emissions may be different based on the composition of the materials (e.g., e-liquid vs tobacco leaves), puff topography (i.e., volume, heating power-wattages), or the differences in underlying heating technology. Comparison of particles and chemicals between technologically varying vape devices are still poorly characterized and their toxic potentials on health outcomes after long-term exposures remain great concerns. The purpose of the study was to: 1) characterize and compare particles and chemicals emitted from Ecig (i.e., JoyeTech), IQOS, and SURGE at two puff volumes, and 2) evaluate dosimetric estimates for lung depositions among primary users. **Methods:** Emissions were generated by an automated syringe set up at 55- and 100-mL puff volumes within 5 sec duration and 30 sec intervals from JoyeTech at 9W, IQOS, and Surge. Total particle mass and mass-based particle size distribution (PSD) were determined off-line using a cascade impactor, MiniMOUDI™. PSD was reported using median aerodynamic diameters (MMAD) ± geometric standard deviation (GSD). Estimate for modelled dosimetric depositions in head, tracheobronchial (TB), and alveolar regions was calculated using multiple path particle dosimetry model (MPPD v 3.04). Real-time measurements of speciated chemicals were considered using Fourier Transform Infrared spectroscopy (FTIR). For brevity, measurements for total of 13 volatile carbonyl compounds (VCC) and 31 other toxicant chemicals (OTC) are presented in parts per millions (PPM). Data acquisition (for at-least three trials) was conducted for particle and chemical assays using JMP® Pro 16.0.0. Average and standard deviations (SD) were considered for total mass (mg/puff) and MMAD (µm) aerosol generated with 55-, and 100- mL puff volumes. Significant differences (p <

0.05) between MMADs, and total aerosol masses were determined using an ANOVA and Tukey's test for multiple comparisons. Average of total and maximum (in PPM) of VCC and OTC are presented for both puff volumes. **Results:** Higher aerosol mass (mg/puff) were emitted at 100- than 55- mL puff volume: JoyeTech (10.19 vs 8.39) > Surge (4.73 vs 4.38) > IQOS (0.79 vs 0.59). Further, higher puff volume 100 mL vs 55 mL emitted smaller size of particles: MMAD of IQOS (0.53 μ m vs 0.60 μ m) that were also resulted into significantly different ($p < 0.05$) than JoyeTech (1.25 μ m vs 1.55 μ m) and Surge (0.96 μ m vs 1.25 μ m). In general, all emissions resulted in polydisperse PSD with GSD ranged 1.4-1.6. Modelled dosimetry estimated similar regional respiratory deposition in head (~39%), TB (~21%), and alveolar (~40%) for all the emissions. Real-time measurements with FTIR resulted into higher VCC and OTC for 100- than 55-mL for Surge, IQOS, and Joyetech. Maximum values of VCC for 55 mL vs 100 mL constituted Pentenal (PPM: 59 vs 70) and Acetaldehyde (PPM: 132 vs 172), respectively for Surge and IQOS. Glycerol (PPM: 200.71-1780.49) was the maximum out of all the OTCs for both Surge and IQOS at 55- and 100-mL. JoyeTech also emitted VCC: Isobutyraldehyde (PPM: 72 vs 52) and OTC: Nitrobenzene (PPM: 2197 vs 2116) at 100 mL vs 55 mL puff volume. According to IARC, Acetaldehyde and Nitrobenzene are group 2B: possibly carcinogenic to humans. **Conclusions:** We conclude that: 1) statistical difference in MMADs do not imply practically for regional respiratory deposits, 2) real-time speciation of chemicals reveals previously unreported chemicals, 3) maximum concentrations of chemicals outweigh traditionally reported averages that could undervalue exposure assessment and thereby biological impacts, and 4) though quantitatively lesser, cumulative burden of smaller sized particles and chemicals from Surge and IQOS devices do not suggest safer emissions compared to other Ecig devices. Although, characterization of Ecig emissions is a complex dynamic, it important to evaluate PSDs with as less fortification as possible for accurate evaluation of their deposition dose and thereby, to understanding biological insults. Future study will include animal exposure to better address inferences of such emissions.

PS 4085 Substantially Equivalent and Equally Addictive: The Case of Cool Non-menthol Cigarettes in California

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Background and Purpose: In 2022, California banned flavors in tobacco products, including menthol in cigarettes. In response, a big tobacco company (RJ Reynolds) introduced "non-menthol" cigarettes in the state after securing a substantially equivalent (SE) report from the FDA, stating that these new cigarettes do not raise different questions of public health. We interpreted this SE designation as the new cigarettes are equally addictive and toxic as menthol cigarettes, and wanted to verify this chemically. **Methods:** We purchased non-menthol cigarettes in California (Camel and Newport), brand-matched unflavored regular and menthol cigarettes in Ohio and Georgia, cigarettes from another manufacturer with brand names emphasizing cooling sensations in Ohio (Marlboro), and filtered little cigars for comparison. We tested for nicotine, menthol, and synthetic cooling agents (Wilkinson Sword (WS)-3 and WS-23) in the cigarette rod and mainstream smoke, generated using smoking machines with total particulate matter (TPM) collected on filters and measured gravimetrically. We extracted the four chemicals using liquid-liquid extraction and quantified them on gas chromatography-mass spectrometry (GC-MS). We assessed the transfer of the chemicals from the rod to the smoke and compared the cooling strength across products. **Results:** The only cooling agent detected in non-menthol Newport and Camel cigarettes was WS-3 (0.88-1.93 mg/cigarette). Like menthol in the mentholated counterparts (0.59-1.40 mg/cigarette), the level of WS-3 was 10% of that of nicotine (9.3-15.52 mg/cigarette). All cigarettes emitted the same level of TPM (~33 mg/cigarette). Although the transfer of menthol was higher for Newport menthol (22-27%) compared to non-menthol cigarettes (13-16%), the calculated cooling strength was the same. The newly introduced Marlboro brands had much higher menthol (0.93-3.78 mg/cigarette) and were comparable to filtered little cigars (5.19-6.71 mg/cigar). **Conclusions:** Non-menthol cigarettes introduced in California have the same cooling strength as the banned menthol cigarettes. Our work confirms the SE interpretation that these cigarettes are equally harmful to public health as menthol cigarettes and have the potential to undermine local, state, or federal bans on menthol cigarettes.

PS 4086 Applicability of USP-1664, ICH-Q3E and ISO 10993-17 Standards to the Toxicological Risk Assessment of Leachables from Illicit ENDS Product: A Case Study

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Background and Purpose: To date, the U.S. Food and Drug Administration (FDA) has authorized only tobacco and menthol flavored electronic nicotine delivery systems (ENDS). Despite this, illicit flavored disposable ENDS have become widely accessible in the United States. Contemporary disposable vapes are often illicit flavored disposable ENDS that are made of larger tank leading to high puff counts. Tank and containers of e-cigarettes and vaping products can be made from various plastic

materials. A deconstructing study of contemporary disposable vapes has identified various polymeric plastic materials such as poly(n-butyl methacrylate) (PBMA), polycarbonate (PC), polydimethylsiloxane (PDMS), polyethylene (PE) etc. Polyvinyl chloride (PVC) can be used in combination with these polymers (e.g., PBMA, PC, PDMS, PE etc.) to create new plastic materials with enhanced properties, such as rigidity, flexibility, thermal stability, durability and cost-effectiveness. The FDA's final Premarket Tobacco Product Application rule for ENDS indicates that the container closure system could, intentionally or unintentionally, leach ingredients from the packaging into the product. Leachable substances are chemical components that can migrate from a contact surface of a product into the surrounding media or its contents or process stream under intended use conditions. A case study was developed using pharmaceutical grade PVC in a hypothetical ENDS product and toxicologically evaluating reported leachable compounds using three different standards: USP-1664; ICH-Q3E and ISO 10993-17. **Methods:** Leachable levels of 24 different chemicals in pharmaceutical products stored in PVC containers was extrapolated to a hypothetical ENDS product that uses a PVC tank. ElfBar was chosen as a hypothetical ENDS product because it is an illicit flavored ENDS product. In addition, study findings published (2024) in the "The Journal of Medical Internet Research" concluded that there was an increasing trend in Google searches for Elf Bar, which indicated that there was a rising interest in this brand in the United States. The leachable chemicals were evaluated for their potential risks based on the use and exposure scenario of the ElfBar illicit E-Cigarette (tank volume of 13 mL, e-liquid density of 1.16 g/mL, consumption of 0.06 pod per day). Toxicological risk assessment was carried out by estimating the daily consumer exposure level of leachable chemicals to the selected toxicity thresholds established by three different standards: USP-1664 (Qualification Threshold-QT & Safety Concern Threshold-SCT); ICH-Q3E (Safety Concern Threshold-SCT); and ISO 10993-17 (Toxicological Screening Limit-TSL). **Results:** Margin of exposure assessment of leachable chemicals showed one chemical exceeded the SCT of 0.15 μ g/day and was considered to pose a toxicity risk, requiring further assessment for both carcinogenic and genotoxic effects. All the 24 leachable chemicals were below the QT of 5 μ g/day and the TSL of 600 μ g/day and was considered to have negligible toxicological risk for both systemic and local toxicity. **Conclusions:** This case study of a hypothetical ENDS product illustrated use of different standards (USP-1664 -QT & SCT, ICH-Q3E - SCT), and ISO 10993-17 - TSL) for toxicological analysis of leachable compounds from pharmaceutical grade PVC. It also highlighted that even if pharmaceutical grade PVC is used in ENDS device construction, chemicals may potentially leach into the product, which would require further toxicological evaluation.

PS 4087 Pesticide exposure and placental circadian phase using transcriptomics

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Background and Purpose: Circadian timing is integral to placental physiology and fetal development. Environmental toxicants may act as chronodisruptors, yet human evidence of the impact of prenatal exposures on genes that demonstrate circadian rhythmicity in the placenta is limited. In a cohort of Thai farmworking women, we tested whether prenatal exposure to mixtures of organophosphate (OP) and pyrethroid (PY) pesticides, or a combined OP-PY mixture (MIX), were associated with differences in the amplitude or phase of peak expression of circadian rhythmic genes in the placenta. **Methods:** Placental transcriptomes were obtained by RNA-seq from the SAWASDEE cohort (n=252). Gene-level rhythmicity was modeled with DiscoRhythm and LimoRhyde, yielding per-gene amplitudes and times of peak expression (0-24 h). These were applied to a z-scored expression matrix to compute two sample-level circadian indices: (1) acrophase (ϕ), representing the average peak timing across rhythmic genes, and (2) amplitude ($\hat{A}$), the mean strength of rhythmic expression. These indices were tested for association with total-pregnancy and trimester-specific exposures — OP, PY, and MIX—using quantile g-computation (q=4), applying the same covariate adjustment across all models (gestational age, maternal age, BMI, infant sex, and estimated placental cell-type proportions). Sensitivity analyses tested subsets of daytime and vaginal deliveries. **Results:** PY exposure was associated with a delayed shift in the average circadian phase (ϕ) of rhythmic placental genes, with the strongest trimester-specific effect in the third trimester (q = 0.066). PY was also modestly associated with increased rhythm strength ($\hat{A}$) in daytime deliveries (q = 0.057). Metabolite-specific associations showed that 3-phenoxybenzoic acid (3-PBA, a PY metabolite) and total dialkyl phosphate (DAP, OP markers) were linked to delayed phase and reduced amplitude. In contrast, diethylphosphate (DEP), dimethylphosphate (DMP), and trans-DCCA (tDCCA) showed the opposite pattern, contributing to earlier phase and increased amplitude. Decomposition of the sample-level circadian phase (ϕ) into its sine and cosine components revealed a net phase displacement of approximately 4.6 hours (magnitude of the phase shift $|\phi| = 0.074$, p = 0.345), primarily driven by the sine component ($\phi_{\sin}$). This component captures vertical variation in rhythmic phase positioning and was most influenced by 3-PBA and DEP/DMP. In contrast, total DAP attenuated both phase components, leading to a muted shift. **Conclusions:** Prenatal exposure to pesticide mixtures—particularly PY in late gestation—is associated with gene-level altered circadian phase and rhythm

strength in the placenta. Metabolite-specific effects show opposing influences on circadian timing and amplitude, suggesting complex, component-specific mechanisms of disruption. These findings highlight the placenta as a sensitive target of circadian misalignment following environmental exposures and underscore the need for replication and mechanistic studies.

PS 4088 Phenotypically Anchored Transcriptomics Across Diverse Agrichemicals Reveals Conserved Pathways and Unique Gene Expression Signatures in Zebrafish

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Background and Purpose: Agrichemicals such as herbicides, fungicides, insecticides, and biocides represent some of the most structurally and biologically diverse chemical classes in wide-scale use. This diversity, coupled with overlapping adverse outcomes, make them powerful tools for developing and testing new approaches to hazard assessment. Although many agrichemicals are represented in high-throughput *in vitro* programs such as the U.S. EPA's ToxCast program, systematic *in vivo* evaluation using transcriptomic profiling—a method that can reveal both chemical-specific signatures and shared toxicity pathways—remains limited. **Methods:** We evaluated a structurally diverse subset of agrichemicals from the U.S. EPA's ToxCast Phase I and II libraries by statically exposing early life stage zebrafish from 6 hours post fertilization (hpf) to 120 hpf at concentrations ranging from 0.25–100 μ M. Morphological outcomes were assessed at 120 hpf across 10 endpoints, including yolk sac edema, craniofacial malformations, and axis abnormalities. Chemicals that produced robust concentration-response relationships were selected for transcriptomic profiling. For transcriptomic analysis, zebrafish were statically exposed to each chemical and sampled at 48 hpf, prior to the onset of morphological effects observed at 120 hpf. **Results:** Differential expression analysis identified between 0 and 4,538 differentially expressed genes (DEGs) per chemical, with no clear correlation to morphological severity. Both DEG and co-expression network analyses revealed chemical-specific expression patterns that converged on shared biological pathways, including neurodevelopment and cytoskeletal organization. Key regulatory genes such as *mylpfa* and *krt4* were identified within co-expression modules, suggesting their potential role in conserved toxicity mechanisms. Semantic similarity analysis of enriched gene ontology (GO) terms, when compared to existing datasets, highlighted gaps in the annotation of neurodevelopmental processes, indicating that some *in vivo* effects may not be fully captured by current curated resources. **Conclusions:** This study highlights the value of integrating transcriptomic profiling with assessments of adverse morphological outcomes in a vertebrate model. Rather than relying on transcriptomic profiling alone, we demonstrate how its use as a complement to traditional morphological endpoints can capture early molecular disruptions, thereby providing deeper insight into the mechanisms of toxicity. Agrichemicals offer a uniquely challenging and informative chemical space for assessing the robustness of transcriptomics to predict and contextualize toxicological outcomes across diverse chemical structures and biological targets. By integrating these molecular responses in a vertebrate model, we outline a broadly applicable framework for advancing transcriptomics as a core component of next-generation hazard assessment.

PS 4089 Exploring the Toxicological Effects of Commonly Sold Pesticides on Non-Target Organisms and Human Health

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Background and Purpose: Global pesticide usage has increased by nearly 94% since 1990, reaching about 3.7 million metric tons annually. The World Health Organization reports nearly one million unintentional pesticide poisonings each year, resulting in approximately 20,000 deaths. In the United States, more than one billion pounds of pesticides are applied annually. Although pesticides are designed to protect crops, their persistence and bio-accumulative nature pose serious risks to non-target species—such as pollinators, birds, aquatic life, and humans. Chronic exposure has been linked to oxidative stress, neurodegeneration, and diseases including cancer, Parkinson's, and Alzheimer's. This study investigates the types of pesticides commonly sold by major retail outlets and identifies their active chemical constituents to assess their potential toxicological hazards. We hypothesize that while not all ingredients are acutely toxic, several may cause significant harm to non-targeted organisms and human health through environmental contamination and bioaccumulation. **Methods:** A survey of major retail stores (e.g., Walmart, Home Depot, Lowe's, Dollar Tree, Dollar General and Tractor Supply) was conducted to document widely sold pesticide formulations and their active ingredients. Laboratory analyses using *Drosophila melanogaster* were performed to examine the neurotoxic and oxidative effects of selected compounds such as paraquat, glyphosate, and atrazine. Use GraphPad Prism 5 software to analyze the data wherever necessary. **Results:** Survey data indicated that Roundup, Spectracide, and Ortho were among the most frequently purchased products. Behavioral and molecular assays in *Drosophila* revealed that exposure to these formulations induced oxidative stress, dopaminergic neuronal loss, and circadian rhythm disruption. Some compounds also affected cell-cycle and cellular integrity. **Conclusions:** Findings indicate that certain active ingredients in commonly marketed pesticides may contribute to oxidative damage and neurodegenerative outcomes. These findings highlight the urgent need to strengthen regula-

tory measures, consumer awareness, and further molecular investigations into their mechanisms of toxicity. Ongoing studies will employ genomic and proteomic analyses in *Drosophila* to identify gene and protein targets affected by pesticide exposure, enhancing understanding of their molecular toxicity pathways.

PS 4090 Toxicological Assessment of BCS-BT38895: A Novel Aniline Metabolite of Diflufenican

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Background and Purpose: Diflufenican is a selective herbicide of the phenoxy-nicotinamide class, registered in Europe and several countries in Asia, Africa, and South America, and recently accepted for registration in Canada and the USA for pre-emergent use on soybean and corn. During soybean field trials supporting the North American registration, a malonic acid conjugate of the known 2,4-difluoroaniline metabolite of diflufenican, named BCS-BT38895, was detected above the limit of quantitation in 4 out of 22 trials. This metabolite has not been observed in any plant, rat or livestock metabolism studies. In agreement with the U.S. Environmental Protection Agency (EPA) and the Canadian Pest Management Regulatory Agency (PMRA), a hazard characterization was conducted through an *in silico* analysis, a literature review, and a suite of toxicity studies. **Methods:** SciFinder (CAS) was used to search Cplus and MEDLINE databases using the CAS number for each molecule, with no time restrictions. Each identified study was downloaded and reviewed in a rapid assessment analysis. Studies with supplemental or supportive information for a human health risk assessment were reviewed in detail. Toxicity studies included an acute oral toxicity study in rats (tested up to 2,000 mg/kg bodyweight), *in vitro* mutagenicity assays (Ames test, hypoxanthine phosphoribosyl transferase (HPRT) mutation test, and a micronucleus test), and a 28-day repeat-dose dietary study in Wistar rats. In this study, three dose groups (6 males and 6 females each) received BCS-BT38895 at 0 (control), 50, 300, or 1500 ppm (resulting in overall achieved dosages of 4.68, 28.2 and 139 mg/kg/day in males and 3.97, 25.0 and 124 mg/kg/day in females, respectively). Observations included clinical signs, sensory reactivity, motor activity, hematology (including methemoglobin and Heinz body analysis), blood chemistry, organ weights, and histopathology. **Results:** The *in silico* analysis identified structural alerts for methemoglobinemia, hepatotoxicity, and nephrotoxicity, but none for carcinogenicity. The literature review did not identify any studies or publications relevant to a human health risk assessment. The acute oral toxicity study established an LD₅₀ > 2000 mg/kg bodyweight, indicating low acute toxicity. The genotoxicity assays showed no gene mutations or micronuclei, confirming non-mutagenic potential. In the 28-day repeat-dose study, BCS-BT38895 induced classic aniline toxicity hallmarks: methemoglobinemia, spleen congestion, and increased spleen weight at 300 and 1500 ppm; however, no Heinz bodies were observed in any dose level. Findings at 50 ppm were minor and considered non-adverse. The NOAEL was therefore 3.97 mg/kg/day. **Conclusions:** BCS-BT38895, found exclusively in soybean seeds, exhibited no acute toxicity and was non-mutagenic *in vitro*. No literature findings were supportive for use in a human health risk assessment. The 28-day study showed treatment was well-tolerated, with methemoglobinemia and spleen effects observed only at higher doses. A robust NOAEL was therefore established at the low dose. According to the US EPA Human Health Risk Assessment, anticipated exposure (5×10^{-6} mg/kg/day) is orders of magnitude below the chronic population adjusted dose of 4×10^{-3} mg/kg/day from the 28-day oral rat study NOAEL and a 1000x uncertainty factor applied. Given its specificity to soybean seed, low exposure potential, and a well-understood mechanism of toxicity for aniline, further long-term *in vivo* studies are unwarranted and inconsistent with animal use reduction principles.

PS 4091 The limitations of alternative approaches for predicting irritation and sensitization hazards of solid agrochemical formulations

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Background and Purpose: Pesticides are one of the most heavily regulated and toxicologically evaluated classes of molecules on the market. In addition to the extensive testing required by global regulatory agencies on the pesticidal active ingredient, each new formulated product must be evaluated for acute mammalian toxicity endpoints in a data package colloquially referred to as the "Acute Tox 6-Pack". According to a public statement in 2016, the USEPA reviews 500 pesticide product registrations every year. With an average of ~50 animals per acute tox package, this level of review potentially represents average annual testing on ~ 25,000 animals for new products submissions. As a result, efforts across both industry and regulatory agencies have been made to reduce animal testing by adhering to the 3Rs of responsible animal research. Consequently, there is interest in utilization of alternatives for the evaluation of irritation and sensitization potential, yet the *in vivo* irritation and sensitization studies remain the gold standard. The purpose of this project was to retroactively evaluate predictive accuracy of alternative approaches for evaluation of irritation and sensitization potential for solid agrochemical formulations. **Methods:** Solid formulations with alternative assessments for eye, skin, and sensitization potential (i.e., GHS calculation and/or *in vitro* methods) were selected from

the Corteva products (current and historical). Appropriate *in vivo* studies were those performed for regulatory submissions either under OECD/OPPTS guidelines for skin, eye, and sensitization potential. Appropriate *in vitro* studies were those performed for regulatory submissions or for internal screening either under OECD 492 (EpiOcular)/OECD 437(BCOP) (or similar) or OECD 439/431 (EpiSkin™) (or similar) guidelines for eye and skin irritation, and OECD 442 respectively. GHS Calculations for hazard predictions were also included for all formulations. Evaluation of predictivity was determined by confusion matrix and calculation of sensitivity, specificity, and false positive/negative rates. **Results:** Thirteen formulations were determined as having the appropriate data available for evaluation or irritation, though not all formulations had both irritation and corrosion tests available. Of those formulations there were 8 herbicides, 3 insecticides, and 2 fungicides representing Water dispersible granules (WG, 8), wettable powder (WP, 2), granule (GR, 2), and bait formulation (1) types. All the formulations were considered “non-irritating” according to *in vivo* studies. The *In vitro* skin irritation study (OECD 439) correctly predicted 9/10 formulations as non-irritating. The Eye irritation study (OECD 492) correctly predicted only 3/9 formulations as non-irritating and while all three of the eye corrosion assays (OECD 437) correctly identified the tested formulations as non-irritating, the limited number of OECD 437 studies available (N=3) inhibits further interpretation from the corrosion assay. For sensitization, 12 formulations had *in vivo* data, and all were negative. *In vitro* predictions varied, with DPRA had *in vitro* data available. Of those four formulations, Keratinosens (KE2) accurately predicted non-sensitizer for all 4, while GARD had 3 false positives, and DPRA could only be run for 1 formulation due to solubility limitations. In all cases. For GHS Calculations, lack of skin irritation hazard was accurately captured 90% of the time while eye irritation and skin sensitization was over predicted 67% of the time. **Conclusions:** While useful in the context of evaluation for liquid formulations, there is a tendency to over predict hazard potential with solid formulations. These results suggest that negative predictions for irritation of solid formulations are likely representative of the true lack of irritancy potential and could be used for regulatory classification in the absence of animal testing. Further, these results suggest that current ‘default’ testing procedures may not be appropriate for solid formulations and encourage refinement of approaches more suitable for testing solid formulations. However, robustness of these interpretations are limited by the small data set which can be expanded through industry collaboration.

PS 4092 Modulation of Organophosphate Metabolism and Toxicity via NRF2 Signaling

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Background and Purpose: Human exposure to organophosphate (OP) pesticides such as chlorpyrifos (CPF) occurs in agricultural, occupational and military settings worldwide. OP pesticides, such as CPF, can lead to cholinergic crisis via acetylcholinesterase (AChE) inhibition and accumulation of the neurotransmitter acetylcholine. Downstream effects of OP intoxication include seizures and production of reactive oxygen species (ROS), likely due to glutamate (Glu) excitotoxicity. Furthermore, there is evidence that CPF exposure correlates with additional adverse outcomes, including depression and neurodegenerative diseases. Molecular mechanisms for such conditions are elusive and may involve targets other than AChE. Metabolism of CPF is critical to cholinergic toxicity as it can yield either bioactivation to the toxic chlorpyrifos-oxon (CPO) or detoxication via glutathione (GSH)/glutathione-S-transferase (GST; dechlorination) or hydrolysis of CPO via the esterases, paraoxonase 1 (PON1) and carboxylesterase 1 and 2 (CES), with CES serving as a “sink” to protect AChE. Interestingly, several of the enzymes critical to the metabolism of CPF are under the control of the transcription factor NRF2, including: GSH/GST, PON1 and CES. Activation of NRF2 is known to induce expression of numerous antioxidant enzymes to mitigate aberrant ROS production. The goal of our work is to explore the role of NRF2 modulation in mitigating risk of toxicity and adverse effects to CPF/OP agents and the therapeutic potential to target this pathway to mitigate CPF/OP toxicity. **Methods:** qPCR, Esterase Activity Assays, CellROX imaging, MTT assays **Results:** Preliminary work found that pretreatment of PC12 cells with potent activators of NRF2 provides protection against acute CPF/CPO treatment at sub-micromolar concentrations. In addition, we have shown that NRF2 activators induce the expression of both CES and PON1 in HepG2 cells (liver). We found that NRF2 activation increases esterase activity in HepG2 cells. Protection against the organophosphates CPF and Paraoxon in HepG2 cells was also observed. **Conclusions:** These data indicate the potential for NRF2 as a target to mitigate short- and long-term OP/CPF toxicity, and that through activation of liver NRF2, soluble serum esterases could provide systemic protection against OP poisoning.

PS 4093 Reconsidering the use of repeat dose dermal toxicity study for agrochemical risk assessment

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Background and Purpose: As part of standard toxicology packages for regulatory submissions of agrochemicals in North America, repeat dose dermal toxicity (RDDT) studies (28/21-day exposure) are required to assess dermal exposure. However,

often RDDT studies are not ultimately used as points of departure (PODs) for final risk assessments. To evaluate RDDT utility, we conducted a retrospective analysis of publicly available Human Health Risk Assessment (HHRA) documents from United States Environmental Protection Agency (US EPA) and Health Canada Pest Management Regulatory Agency (PMRA) for multiple agrochemical active ingredients registered in North America. **Methods:** In this study, 52 HHRA documents from the US EPA and 41 publicly available pesticide registration documents from PMRA were evaluated. Critical information was manually extracted in standardized format to generate a robust dataset. For each active ingredient molecule, we collected pesticidal class, dermal POD and derivation study, and dermal absorption factor (DAF). To represent risk assessment without RDDT, surrogate dermal PODs were selected and dermal equivalent doses (DEDs) calculated by dividing surrogate POD by DAF. DEDs were compared against the limit dose; when DEDs were at or near the limit dose, no dermal risk was concluded, aligning with US EPA practice. **Results:** Our analysis indicates that US EPA often cites RDDT studies 30/52 (58%) for dermal risk assessment. However, only 6/30 were used to set dermal PODs and which were based on systemic effects. For the remaining 24/30 the conclusion was no dermal risk (as NOAEL at or above the limit dose). For these 30 molecules, 22/30, had DEDs at or above the limit dose. Oral studies were used to conduct dermal risk assessments on 22/52 (42%) molecules. In 19/22 this was due to developmental, reproductive, or functional effects not assessed in dermal studies. Overall, the US EPA could have completed the dermal risk assessment for 41/52 (79%) molecules without a RDDT study. In contrast, PMRA relies more often on oral studies to set dermal POD: 27/41 (66%). The majority (23/27) fell outside the scope of RDDT experimental design due to endpoints associated with susceptibility of the young or developmental effects. For the remaining molecules 14/41 (34%) RDDT studies were cited for dermal risk assessment: 8/14 the dermal PODs were based on systemic effects; 3/14 no dermal risk concluded; 3/14 the dermal POD was set at the limit dose. For those 14 molecules using RDDT studies, 8/14, had DEDs at or above the limit dose. Overall, PMRA dermal risk assessment for 31/41 (78%) molecules could have been conducted without a RDDT study **Conclusions:** Regardless of region in North America, RDDT studies were generally not useful for risk assessment in >78% of cases and could have been waived. Primary waiver rationales include: (1) RDDT studies did not assess critical toxic effects observed in oral studies (young susceptibility, developmental effects, neurotoxicity), or (2) calculated DED at or near the limit dose. Based on these results, we developed a decision tree to guide RDDT necessity for risk assessment that could align with both PMRA and US EPA processes. This work demonstrates limited RDDT utility and suggests a practical decision tree could refine North American dermal toxicity testing and risk assessment.

PS 4094 90-day dog study for crop protection active substance toxicity testing program

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Background and Purpose: Crop protection active substances are among the most extensively evaluated chemical substances for toxicological effects. For a new crop protection active substance registration, repeated dose oral toxicity studies in both rodent and non-rodent species are required as part of a standard battery of tests to support human health risk assessment in many geographies. Dogs historically have been recommended as the non-rodent species and have been widely accepted by global regulatory agencies. The 90-day guideline study for non-rodent species (i.e., OECD No. 409) requires a minimum of 32 dogs with additional dogs needed for range finding (phase I) and 28 day studies (phase II). The phase I and II studies provide data for dose-setting and target organ identification for a subsequent 90-day study (phase III). Thus, a typical new crop protection active substance requires approximately 48-58 dogs for the completion of the testing program. There are concerted directives from regulatory agencies and efforts from the agrochemical sector to reduce, replace, or refine vertebrate animal testing for animal welfare. To reduce the number of dogs used in a toxicity testing program and meet the data requirements for a crop protection active substance, we use scientifically driven approaches such as: 1) Using the same animals for both a preliminary dose range-finding (Phase I), followed by a wash out period, and the 28 day repeat dosing (Phase II) study; 2) Using the minimum number of dogs/sex/group and not using a control group in the Phase I and Phase II studies; and 3) when the toxicity profile is favorable, using the limit dose approach with testing only at 1000 mg/kg in the Phase III 90-day study. **Methods:** In our approach, a crop protection active substance was used to evaluate toxicity and determine the toxicokinetics when administered orally via capsules to Beagles once daily for 90 days with preliminary dose range finding (Phase I) and repeat dosing (Phase II) studies conducted prior to the 90-day study. During the Phase I study, two dogs/sex were dosed at 250 mg/kg (Group 1) on Days 1 through 3. A second group of two dogs/sex were dosed with 500 mg/kg on Days 8 through 10. Following a wash out period of 12 days, Group 1 dogs were dosed again on Days 15 through 17 with 750 mg/kg and Group 2 dogs dosed with 1000 mg/kg on Days 22 through 24. Dose levels for Phase I were adjusted depending on study findings following the previous dose. A washout period of at least 2 weeks occurred between the completion of Phase I and the start of the subsequent Phase II 28-day repeat dose study. During Phase II doses of 500 mg/kg (Group 1, n=2 dogs/sex) and 1000 mg/kg (Group 2, n=2 dogs/sex) were administered by oral capsule daily for 28 consecutive days, excluding the day of necropsy. All animals survived to their scheduled necropsy. No adverse test-article related changes were noted in clinical observations, body weight,

food consumption, clinical pathology, gross necropsy evaluation, or microscopic histopathology observations. Based on these results, the no observed adverse effect level of crop protection active substance administered via capsule was 1000 mg/kg/day in male and female Beagle dogs. Based upon the findings in Phase I and II studies, the Phase III 90-day study utilized only the limit dose level of 1000 mg/kg and a control group, thus further reducing the number of animals used with four dogs/sex/group. In this study, all animals survived to their scheduled necropsy and no test article related changes in clinical observations, body weight, or food consumption were noted. **Results:** This strategy only required the use of 8 dogs for the Phase I dose range finding and Phase II 28-day studies and a total of 16 dogs for phase III. **Conclusions:** Thus, for a crop protection active substance testing program, a total of 24 dogs were used for the entire canine testing program which represents a 50% - 60% reduction in animal use. A favorable toxicological profile, and the lack of sensitivity across species made this approach for the crop protection active substance testing program possible. This approach may not be viable when canines show a higher level of sensitivity; however, even in cases where the limit dose approach cannot be used for the 90-day study, the number of dogs used for a crop protection active substance testing program can be reduced to 40 animals from the normally used 48 - 58. Overall, using this strategy has established a theoretical minimum requirement to help drive our animal reduction goals.

PS 4095 Utilizing Toxicogenomics to Identify a Mechanistic Hypothesis for Species Differences in Toxicity Studies

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Background and Purpose: Hyperlipidemia (significant increases in total plasma cholesterol, high- and low-density lipoprotein cholesterol, and triglycerides) was observed in dogs but not in rodents following repeat dose administration of a novel agrochemical. No evidence of liver toxicity or steatosis was observed in any species. The species differences in hyperlipidemia and potential relevance to human safety were investigated using toxicogenomics to identify a verifiable mechanistic hypothesis. **Methods:** Transcriptomic analysis of dog liver and metabolomic analysis of dog liver and plasma clearly identified liver X receptor (LXR) activation as the mechanistic hypothesis for the hyperlipidemia observed in dogs. This hypothesis was then tested by assessing direct agonist activity of the novel agrochemical in human, dog and rat LXRA and LXRB transactivation assays. Further context was provided by comparing plasma concentrations of the novel agrochemical across species. **Results:** Toxicogenomic assessments reflected the increase in plasma cholesterol and triglycerides. Transcriptomic assessment of dog liver identified significant up-regulation of LXR target genes, including sterol regulatory element binding transcription factor 1 (SREBF1), stearyl-CoA desaturase (SCD), ATP binding cassette subfamily G member 1 (ABCG1) and inducible degrader of the LDL receptor (IDOL or MYLIP). Metabolomic assessment of liver and plasma also supported an LXR activation hypothesis. The novel agrochemical displayed dose-dependent direct agonist activity in LXR assays from human, dog, and rat, with similar potency in all species. However, plasma concentrations sufficient to reach the EC50 were only noted in dogs, which had ten-fold higher plasma concentrations than rats at similar externally administered doses of the novel agrochemical. **Conclusions:** Toxicogenomics successfully identified a verifiable mechanistic hypothesis for the hyperlipidemia observed in dogs, enabling an assessment of the relevance for human safety. The hypothesis of direct LXR activation was tested and supported using *in vitro* transactivation assays in rat, dog and human LXR. The species differences in hyperlipidemia effects were attributable to a large difference in systemic exposure demonstrated using traditional pharmacokinetic assessments and pharmacokinetic modelling. This approach confirmed that LXR activation resulted in hyperlipidemia in dogs and established lack of human safety relevance, as predicted human exposure falls well below concentrations required for LXR activation.

PS 4096 Quantifying Military and Non-Military Pesticide Exposure is US Veterans Using a Cumulative Exposure Index (CEI)

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Background and Purpose: There is a lack of literature addressing various exposures faced by those who have served in the armed forces. We created a cumulative exposure index (CEI) to quantify military and non-military exposure to pesticides in United States veterans with Parkinson's disease (PD). **Methods:** Participants were recruited nationally and mailed a questionnaire that detailed exposure, residentially, and employment history. The participants were also asked to provide a saliva sample to check for genetic polymorphisms. The CEI was generated by incorporating questions that measured exposure, and answers to all exposure questions were given a rank (low/medium/high exposure). These ranks were multiplied by exposure, frequency, and duration. All ranks were added together, resulting in the total potential exposure. An industrial hygienist with military background reviewed the construction of the CEI. Two additional CEIs were created to estimate potential military exposure

and potential non-military exposure. **Results:** The data was abnormally distributed, with one major outlier in non-military exposure and one major outlier in the military exposure category. The military CEI had a shorter duration of exposure but higher intensity, while the non-military CEI had a longer duration with a lower intensity. Of the 155 veterans surveyed for this study, N=14 (9%) reported applying pesticides as a part of their military service, N=95 (61%) reported applying skin creams to repel pests while in the military, and N=54 (34%) reported personally mixing or applying pesticides. A significant negative correlation was identified between age and military-related pesticide exposure reported (Spearman's ρ : -0.22, $p=0.02$), but no other significant correlations were found. **Conclusions:** Our next steps are to evaluate potential gene-environment interactions using the participant saliva samples and create CEIs for specific groups of pesticides. The overarching goal of this research is to utilize the CEI created to evaluate military exposure to pesticides and the relationship to PD in cases versus controls.

PS 4097 Rabbit Developmental Toxicity Studies: Are They Needed for Agrochemical Pesticides?

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Background and Purpose: Agrochemical pesticide active substances (A.S.) are heavily regulated and subject to rigorous testing guidelines to gain approval for commercial use. To identify potential hazards relevant to human health risk assessment, toxicity evaluations are conducted in rodent and non-rodent species. Meeting the stringent data requirements to develop global data packages for agrochemical pesticides necessitates a high animal burden. One such data requirement is the evaluation of developmental toxicity end points in two species: the rodent and the rabbit. Guidelines requiring the rabbit for developmental toxicity studies were developed following the post-market identification of fetal abnormalities associated with maternal use of thalidomide. More recently, the research community has taken part in an effort to reduce, refine, and replace animals in testing. Due to this initiative, recent work has focused on evaluating the utility of rabbit-based endpoints in risk assessment and exploring the use of *in silico* models to potentially reduce *in vivo* requirements in the future. The goal of this work aimed to determine the frequency of risk assessment endpoint derivation from rabbit studies, and whether developmental toxicity can be predicted by commercial software packages. **Methods:** The EU Pesticides database was filtered for registered substances which are commercialized, industrially synthesized, and currently registered in Europe. Classification for each substance was confirmed using the ECHA Classification and Labelling Inventory, considering only classification for developmental toxicity. Endpoints for risk assessment for each substance were obtained from the most recent review report or EFSA conclusion. SMILES codes were utilized for *in silico* modeling to predict developmental toxicology or teratogenicity. Risk assessment endpoints, classification status, and *in silico* predictions were then compared to evaluate how frequently the rabbit is relied upon for developmental toxicity classification, as well as to evaluate the reliability of *in silico* predictions from readily available modeling systems. **Results:** Of the 194 active substances evaluated, 63 have at least one risk assessment endpoint based on the rabbit developmental toxicity study. However, nearly all these 63 were driven by maternal, rather than fetal, endpoints. 27 of the AS are classified for developmental toxicity. 14 of the 27 classifications were based, at least in part, on rabbit developmental toxicity endpoints. Of the 63 AS with rabbit-based risk assessment endpoints, only 9 are classified by ECHA-RAC. Available *in silico* modeling systems provided inconsistent results. Derek Nexus predicted only 8 substances for potential developmental toxicity, none of which are classified for developmental toxicity based on available *in vivo* data. VEGA/CESAR predicted 127 developmentally toxic AS, while only 10 of those were classified based on the rabbit. And finally, 59 substances triggered alerts in the OECD QSAR toolbox, with only 6 classified on the basis of the rabbit study. **Conclusions:** Frequently, the use of rabbit derived endpoints to drive risk assessment reflects maternal, rather than fetal, toxicity of the test item. Further, as the rabbit is often the lowest overall NOAEL, it is commonly used as the default endpoint for risk assessment, which may cause over reliance on the species in testing. However, of the 14 AS with both developmental toxicity classification and risk assessment endpoints in the rabbit, 5 had a lower fetal NOAEL in the rabbit than in the rat and showed fetal observations in the rabbit that were absent in the rat, suggesting the use of the rabbit is not redundant in all cases. In addition, *in silico* predictive models did not prove to be sufficiently powerful to substitute rabbit developmental toxicity studies in pesticide AS development. Thus, A tiered approach to *in vivo* testing, and further refinement of available *in silico* models are necessary to reduce animal use in testing, while ensuring the safety of new pesticidal AS.

PS 4098 Application of short-term transcriptomic point of departure to inform human health risk assessments in replacement of chronic studies: A case study with a novel insecticide

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Background and Purpose: Studies required for new plant protection product registration are resource intensive and take many years to develop due to extensive safety testing requirements. For human health, this typically involves a comprehensive testing program largely focused on rodent testing, ranging from acute to chronic exposures, which require thousands of animals. Fulfilling the 3R (Refinement, Reduction, and Replacement) principles and supporting animal welfare regulation (e.g., Directive 2010/63/EU), new approach methodologies (NAMs) such as toxicogenomics are critical tools for future refinement of new product testing programs. Prior evidence suggests that short term toxicogenomic points of departure (tPODs) can closely approximate chronic traditional apical points of departure (PODs). **Methods:** A toxicology testing program for a novel insecticide active ingredient, (N-4-chloro-2-(pyridin-3-yl)thiazol-5-yl)-N-ethyl-3-(methanesulfonyl)propanamide, was leveraged to compare chronic PODs with short-term tPODs. CD-1 mice were exposed via the diet to three dose levels of insecticide and control for 28 days (liver tPOD and apical endpoints collected), 90 days (liver tPOD and apical endpoints collected), and 18 months (apical endpoints collected). Wistar rats were exposed via the diet to three dose levels of insecticide and control for 28 days, 90 days, and one year, and both liver tPODs and standard apical endpoint data were collected. To compare apical PODs with a short-term tPOD, a customized 7-day study was performed in male and female Wistar rats. Male rats were exposed to insecticide at doses of 0, 44, 221, and 525 mg/kg/day (n=5/group) and female rats were exposed at doses of 0, 49, 240, and 563 mg/kg/day (n=5/group) for seven days and whole genome sequencing was performed on liver to generate a tPOD via benchmark dose analysis. **Results:** The liver was the main target organ identified in all repeat dose studies in both mice and rats, and no treatment-related liver tumors were identified in any study. The tPODs in mice were similar across sexes and timepoints (39 and 44 mg/kg/day tPOD in males and females, respectively, in 28-day study, 56 and 25 mg/kg/day tPOD in males and females, respectively in 90-day study). The apical PODs from the mouse cancer bioassay NOAEL were 88 mg/kg/day for male mice and 315 mg/kg/day for female mice, indicating that short-term tPODs were more conservative compared to traditional chronic apical PODs. The tPODs in rats were also similar across sexes and timepoints (116 and 120 mg/kg/day tPOD in males and females, respectively in 28-day study, 160 and 220 mg/kg/day tPOD in males and females, respectively in 90-day study, and 212 and 235 mg/kg/day tPOD in males and females, respectively in the one-year chronic study). The 28-day rat no observed adverse effect levels (NOAELs) were 232 and 423 mg/kg/day in male and female rats, respectively. The 90-day rat NOAELs were 40 and 226 mg/kg/day in male and female rats, respectively, indicating close approximation to the tPODs. For the 7-day transcriptomic study in rats, BMD-derived tPODs from liver samples were determined to be 150 mg/kg/day for males and 178 mg/kg/day for females. **Conclusions:** Following exposure to the active ingredient (N-4-chloro-2-(pyridin-3-yl)thiazol-5-yl)-N-ethyl-3-(methanesulfonyl)propanamide, both rat and mouse short-term tPODs were either more conservative or closely approximated long-term PODs. This adds to the weight of evidence that short-term tPODs, potentially combined with uncertainty factors to apply for short-term to long-term exposure periods, can be a conservative method to inform risk assessments without the need for additional animal testing while still protecting human health.

PS 4099 Probabilistic Dietary Exposure Analysis of Imidacloprid Residues in Food and Drinking Water: Use of Federal and State Pesticide Residue Monitoring Data for Refining Exposure

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Background and Purpose: Imidacloprid is a neonicotinoid insecticide that acts by blocking the nicotinic acetylcholine receptors in the insect nervous system. In mammals, signs of toxicity include incoordination, tremors, decreased activity, reduced body temperature, and at sufficiently high doses, death. Imidacloprid is widely used on a variety of crops and the U.S. Environmental Protection Agency (US EPA) has established tolerances (maximum allowable residues) on more than 100 commodities or commodity groups for the combined residues of the parent imidacloprid and its metabolites containing the 6-chloropyridinyl moiety. However, established tolerances do not consider a comprehensive dietary exposure from consumption of all these commodities. In this evaluation, dietary exposure to imidacloprid residues in food and drinking water from legal uses was calculated for multiple populations, including sensitive subpopulations. Accurate exposure estimation is essential for realistic risk assessment and protection of public health; therefore, DPR conducted a highly refined probabilistic approach to calculate acute dietary exposure to imidacloprid. **Methods:** DPR's dietary exposure guidance and the U.S. EPA's DEEM (Dietary Exposure Evaluation Model) software were used to estimate exposure from residues on all commodities with established tolerances and drinking water for imidacloprid for the US population and sensitive subpopulations including infants, children aged 1-2, and women of childbearing age (13-49 years old). The food consumption data were from 2005-2010 National Health and Nutrition Examination

Survey. Residue inputs included either tolerance values or measured residues of imidacloprid and its degradates and metabolites (desnitro-imidacloprid, imidacloprid urea and olefinic imidacloprid) from federal and state monitoring programs (USDA's Pesticide Data Program, DPR, or FDA). DPR's tiered approach includes a) Tier 1: Deterministic assessment, using tolerance values for all commodities; b) Tier 2: Deterministic assessment, using the highest detected residue values from monitoring data; and c) Tier 3: Probabilistic assessment, using residue distributions from monitoring data adjusted for the percent of the crop treated, combined with food consumption distributions via Monte Carlo simulation to generate exposure distributions. **Results:** A total of 478 food forms were analyzed. Of these, 210 were assigned residue distributions from monitoring databases, while the remaining 268 were assigned point estimates: either tolerance values, maximum measured residues on the food or on an appropriate surrogate, or one-half the limit of detection, as appropriate. Residues were further refined using percent crop treated and hydration/processing factors. Monte Carlo simulations were used to generate exposure distributions, and the 99.9th percentile of exposure was calculated for multiple subpopulations. Estimated acute dietary exposure to imidacloprid ranged from 2.52 to 7.22 µg/kg/day across all subpopulations. The non-Hispanic-other population group had the highest calculated exposure. Major contributors to infant and child exposure included corn (field) syrup in baby food, drinking water, grapes, cassava in baby food, apple juice, and bananas. For women of childbearing age, Hispanic, and non-Hispanic-other subpopulations, key contributors included hops, corn (field) syrup, artichokes, Belgian endive, grape wine, and sherry. **Conclusions:** DPR conducted a highly refined Tier 3 acute probabilistic dietary exposure analysis using the extensive imidacloprid residue data from national and state food and drinking water monitoring programs. Compared to deterministic methods where the tolerance or highest measured residue is assumed, it provides more realistic exposure and health risks estimates, while remaining highly health protective. The refined exposure estimates support more accurate risk assessments for diverse subpopulations.

PS 4100 Predictive Power of the GHS Mixtures Equation for Determining the Acute Oral Toxicity (LD50) of Spray Adjuvants

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Background and Purpose: Spray adjuvants are products used in conjunction with pesticides to enhance their application or effectiveness. While exempt from federal pesticide registration by the US Environmental Protection Agency (US EPA), California law requires their registration as pesticides before sale or use. An evaluation was conducted to assess the suitability and acceptability of the United Nations Globally Harmonized System (GHS) Mixtures Equation for predicting the acute oral toxicity of spray adjuvants. The objective was to determine whether the GHS Mixtures Equation could serve as an acceptable alternative to *in vivo* animal testing, thereby supporting efforts to reduce animal use in the registration of spray adjuvants. **Methods:** The GHS Mixtures Equation estimates the acute oral toxicity (LD50) of a mixture by mathematically integrating the LD50 values of its individual components. In this study, the GHS Mixtures Equation was applied to all spray adjuvant mixtures registered in California (n=51). The LD50 values of all the individual components in these mixtures were sourced and validated in two phases using multiple publicly available toxicological databases. After calculating the estimated LD50 value for each mixture using the GHS Mixtures Equation, the resulting acute oral toxicity categories were directly compared against those obtained from *in vivo* acute toxicity studies for the same spray adjuvant formulation. **Results:** In Phase I, acute oral LD50 values were identified for only 175 of 237 relevant ingredients using the Integrated Chemical Environment (ICE) database. This limitation allowed the GHS Mixtures Equation to be applied to only 67% of the dataset (n=34 mixtures). To improve data completeness, the search was expanded in Phase II to include additional public databases (e.g., CompTox, ECHA) and peer-reviewed literature. This broader search yielded LD50 values for 35 of the 62 previously unassessed chemicals, increasing the equation's predictive power to 96% of the dataset (n=49 mixtures). When the GHS Mixtures Equation-derived toxicity categories were compared to those assigned using the US EPA classification system, 82% concordance was observed. Discrepancies included 6% of mixtures being over-classified and 12% under-classified by one toxicity category. In comparison with the US Occupational Safety and Health Administration's GHS (OSHA GHS) classification system, the equation achieved 88% concordance, with 12% of mixtures over-classified by one toxicity category and no instances of under-classification (n=49). **Conclusions:** Although the study's small sample size was a notable limitation, the findings indicate that the GHS Mixtures Equation may serve as a viable alternative to animal testing for predicting the acute oral toxicity of low-toxicity spray adjuvant formulations (i.e., those with LD50 values > 2000 mg/kg body weight), provided that LD50 values for the individual components in the mixture are available from vetted and validated sources.

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Background and Purpose: Studies from the University of Washington - Center for Child Environmental Health Risks Research (CHC) on the agricultural children's cohort, located in the lower Yakima Valley in Washington State, have analyzed levels of pesticides in biological and environmental samples of both farmworkers and non-farmworkers. An agricultural exposome was characterized by determining levels of over 47 pesticides in house dust. These pesticides were examined as potential neurodevelopmental toxicants based on EPA Registration Eligibility Decisions (REDs). Organophosphate exposures were significantly increased in farmworker versus non-farmworker households, and these increases were dependent upon season and agricultural activities. Biomarkers of response were also evaluated in these CHC studies, including miRNA urinary profiles, and we observed an upregulation of 6 miRNAs miR-223, -518d-3p, -597, -517b, -133b, and -28-5p associated with organophosphate exposure patterns. Current literature suggests miRNAs are of importance for pyrethroid impacts and may correlate with neurotoxicity potential. Pyrethroid (PT) levels in dust were stratified by occupational group, year and agricultural season. PTs are of interest in our cohort as EPA recommended their use as substitutes for OPs as their uses are phased out. **Methods:** Analysis compared total pyrethroid levels per sample in 2005 to samples taken in 2011, to determine differences between farmworkers and non-farmworkers as well as variances across seasons. This variability was analyzed using a Bayesian mixed effects model and brms package in R studio. Pyrethroids identified as developmental neurotoxicant potency were characterized using EPA REDs documents and included cyphenothrin, deltamethrin, etofenprox, imiprothrin, permethrin and s-bio allethrin. Preliminary analysis of pyrethroid levels in house dust showed higher levels in farmworker households than non-farmworker households in both years. **Results:** Total pyrethroid levels are the lowest in the non-spray season for both years as well, which is associated with agricultural activities and regulatory changes. Although the levels of pyrethroids in non-farmworker house dust are lower overall, the pyrethroid levels increased during the thinning and harvest seasons in both years, showing pesticide use correlated with exposure of non-pome fruit workers. **Conclusions:** The new PT results allow us to relate miRNAs with both OP and PT exposure. Further analysis will be done to test our hypothesis that miRNAs are of interest in this cohort by using KEGG pathway annotations to see if these relate with pesticide neurodevelopmental toxicity potential.

PS 4102 Weight of Evidence (WoE) Approach Based Assessment of Skin Irritation from Antimicrobial Cleaning Products Using the Phenion Full-Thickness Human Skin Model

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Background and Purpose: Skin irritation, i.e., reversible damage to the skin, is one of the key endpoints assessed in the acute toxicity tests required as a part of pesticide registration, informally known as the acute "six-pack." This testing battery is used globally by regulatory bodies to determine hazards of pesticide formulations. Until the recent advancement of *in vitro* alternatives to animal testing, governmental agencies had relied on *in vivo* tests using rabbits to assess potential skin irritation hazards. In the present study, DPR has independently compared a novel model called Phenion® Full-Thickness (FT) Skin model for its utility and predictability, compared to other *in vitro* methods and previously DPR-reviewed *in vivo* data, in determining skin irritation potential of antimicrobial cleaning products (ACPs). This model is comprised of a fully stratified epidermis that includes a multilayered stratum corneum and dermis and is considered more physiologically accurate. The aim of the present study is an independent evaluation to determine the skin irritation hazard of an ACP using a Weight-of-Evidence (WoE) approach which incorporates results from an *in vitro* skin irritation study using the Phenion® (FT) test system along with results from a historical *in vivo* test to determine skin irritation potential. **Methods:** As part of the WoE approach, an *in vitro* skin irritation study was conducted on an ACP formulation (ACP 1) using the Phenion™ FT human tissue model. The study protocol is based on a modified *in vitro* human keratinocyte model of the OECD Test Guideline 439. A volume of 60 µL each of the undiluted test substance was topically applied directly to the Phenion skin tissue for an exposure duration of 15 ± 1 minutes at room temperature. Following this, the tissue was incubated for 42 ± 1 hours at 32°C. Cytotoxicity (irritancy) was measured using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For this modified OECD method, a tissue viability greater than 70% (mean of three tissue replicates) was selected as the cut-off threshold for discriminating non-irritants (US EPA Toxicity Category IV). Conversely, a tissue viability of 70% or less will result in classification of the chemical as an irritant necessitating further analysis to determine if it falls under US EPA Toxicity Category I, II or III. Results from a previously conducted *in vivo* rabbit test on a substantially similar ACP (ACP 2) were included in the WoE approach. The *in vivo* test was performed according to the standard guidelines on six New Zealand White rabbits, with 0.5 mL test substance applied to each site for a single, 4-hour dermal exposure. The results from both studies were used by DPR as a WoE approach to determine skin irritation hazard. **Results:** In the Phenion assay, the relative mean

tissue viability obtained after exposure to ACP 1 compared to the negative control tissues was 72%. Since the mean relative tissue viability for the test material was above 70% after a treatment duration of 15 ± 0.5 minutes, the test material is classified as a non-irritant (US EPA Toxicity Category IV). In the rabbit *in vivo* test, grade 2 erythema was observed in all 6 animals one hour after unwrapping, while at 24 hours, grade 1 erythema was noted for one animal and grade 2 for 5 animals. At 72 hours, grade 1 erythema was observed in all 6 animals, and on days 4 and 5, it was seen in 5 out of 6 animals, which persisted in 2 out of 6 animals up to days 6 and 7. The Draize rabbit *in vivo* skin irritation test resulted in a combined PDI score of 1.4, and ACP 2 was assigned US EPA Toxicity Category IV for primary dermal irritation hazards. **Conclusions:** DPR's analysis of results from the Phenion FT *in vitro* and historical *in vivo* studies, used in the proposed WoE approach, showed concordance in detecting non-irritancy or US EPA Category IV hazard classification. The use of the Phenion model could widen the applicability domain of other *in vitro* tests in determining skin irritation hazard. Overall, this WoE approach provides greater confidence in determining non-irritancy (US EPA Toxicity Category IV). Moreover, results support that additional *in vivo* data are not required to determine skin irritation potential of ACPs identified as US EPA Toxicity Category IV, thus supporting the reduction in use of animals in toxicity testing.

PS 4103 Sex-Specific Disruption of Gut Microbial Communities Link with Altered Liver Gene Expression in Acute Exposure to Azoxystrobin

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Background and Purpose: Azoxystrobin (AZO) is a widely used fungicide linked to reproductive, neurological, and developmental defects. While this chemical disrupts gut microbial communities, it remains unclear if these microbial perturbations contribute to the host harms associated with AZO exposure. Our objective was to investigate the effects of acute exposure to environmentally relevant concentrations (5-500 mg/kg) of AZO on the host and gut microbiota in zebrafish. **Methods:** Adult zebrafish (N=60) were fed AZO-contaminated food (0, 5, 50, 500 mg/kg) for 14 days. Fecal samples (Days 0, 7, 14) were profiled using 16S rRNA and shotgun metagenomic sequencing. Livers (Day 14) were analyzed via RNA-seq and H&E staining. Microbial data were processed using DADA2 (16S) and HUMAnN3 (metagenomics). Host (DESeq2) and microbial (PERMANOVA, GLMMs) data were then statistically analyzed to identify significant alterations and associations. **Results:** AZO exposure resulted in significant alterations in microbiome composition (PERMANOVA, $R^2 = 0.333$, $P = 2 \times 10^{-4}$) and functional potential in a dose- and sex-dependent manner. Microbial communities of exposed animals showed increased pathways associated with xenobiotic metabolism (e.g., atrazine degradation, $P = 1.18 \times 10^{-2}$) and decreased pathways for bacterial motility (e.g., flagellar assembly, $P = 4.20 \times 10^{-2}$) and lipopolysaccharide biosynthesis ($P = 2.14 \times 10^{-2}$). Liver injury and transcriptional reprogramming were also noted, with 143 DEGs identified ($FDR < 0.1$). These were characterized by increased expression of genes involved in oxidative stress (e.g., xdh, mt2) and enrichment of downregulated genes in pathways like lipid metabolic process (GO, $FDR = 8.09 \times 10^{-5}$), also in a sex-specific manner. Importantly, changes in host gene expression were linked with alterations in the abundance of specific microbial taxa (2,431 unique associations), such as *Pseudomonas* abundance positively correlating with xdh and mt2 expression ($FDR < 0.15$). **Conclusions:** Our findings demonstrate that acute AZO exposure is associated with both gut microbial disruption and altered hepatic gene expression related to oxidative stress and lipid metabolism. These results identify the microbiome as a key target of AZO toxicity. The strong, sex-specific associations between microbial taxa and host gene expression suggest a potential gut-liver interplay that warrants mechanistic evaluation. Our ongoing work aims to evaluate the mechanisms that underpin these associations.

PS 4104 Assessing Impacts of Aldicarb Exposure on Gut Microbial Communities

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Background and Purpose: Aldicarb is a highly toxic restricted use pesticide with neurotoxic effects and links to cancer. While the impacts of aldicarb on the brain, liver, and other organs have been well described, the effects of exposure on host associated microbial communities is poorly understood. Gut microbial communities are both sensitive to xenobiotic exposures and highly capable of transforming these compounds and their impacts on the host. Here we aimed to define the impacts of aldicarb on gut microbial communities, isolate microbes that exhibited differential sensitivity and resistance to exposure, and to identify potential mechanisms that underpin these differences. **Methods:** To evaluate the acute impacts of aldicarb on gut microbial communities we exposed 60 adult tropical 5D zebrafish to one of three diets containing aldicarb (0.5, 5, and 50µg/kg) or a control diet with no aldicarb. Fecal samples were collected from individually housed animals at day 0, 7, and 14 of exposure. The V4 region of the 16S rRNA gene was amplified from fecal DNA using barcoded 515F and 806R universal primers and amplicon libraries sequenced on an Illumina MiSeq instrument. Shifts in microbial diversity and composition were quantified with DADA2 and custom R scripts. Potential mechanisms of resistance of differentially abundant taxa were identified by searching protein sequences of aldicarb-de-

grading genes encoded by environmental microbes against a zebrafish microbial protein catalog using DIAMOND. Resistance profiles of these taxa were further characterized by analyzing isolate growth kinetics in mineral salts media contain increasing concentrations of aldicarb (0.01-1.0mg/ml). **Results:** Acute exposure was associated with moderate disruption of the gut microbiome. Interestingly, responses to aldicarb were delayed in males and were only apparent at day 14 (PERMANOVA, $R^2 = 0.23$, $p = 0.005$), while female disruption began at day 7 (PERMANOVA, $R^2 = 0.23$, $p = 0.01$). Exposure was associated with increased abundance of several genera whose members can metabolize aldicarb, including *Arsenibacter*, *Pseudomonas*, *Rhizobium*, *Mycoplana* and *Methyloversatilis*. A homology search of aldicarb degrading genes in a zebrafish gut microbial gene catalog confirmed the presence homologs of these genes in common zebrafish gut microbes. To further evaluate if increasing taxa may utilize aldicarb degradation we isolated dozens of *Pseudomonas*, *Rhizobium* and *Mycoplana* strains from *ex vivo* culture of zebrafish fecal microbial communities in selective aldicarb-containing media. Several of these isolated taxa were capable of minimally disrupted growth even at 1mg/ml concentrations of aldicarb. Of note, *Pseudomonas* isolates varied substantially in their ability to grow in aldicarb suggesting differential sensitivity of this genus to aldicarb exposure. **Conclusions:** Our results suggest that zebrafish gut microbiota are sensitive to acute exposure to aldicarb and taxa that increase during exposure may possess machinery to metabolize aldicarb. Ongoing work seeks to identify the mechanistic underpinnings of resistance to aldicarb in specific taxa. We hope to leverage this knowledge to identify microbes that may encode similar functions in humans and to design resilient microbial communities capable of mitigating aldicarb impacts on the host.

PS 4106 Knockout of TNIP1 Protein in HaCaT Human Keratinocytes Provides a NAM Platform for Gene-Environment Interactions in Psoriasis

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Background and Purpose: Psoriasis has experienced a 73% increase in global health burden as represented by Disability-Adjusted Life Years (from 1990-2017). While there is a genetic predisposition associated with psoriasis, environmental toxicants are known to trigger the disease in susceptible individuals. Developing new approach methodologies (NAMs) mimicking psoriasis provides advanced, human-relevant methods to study the complex gene-environment interactions involved in the disease. TNF α -induced protein 3 Interacting Protein 1 (TNIP1) acts to repress inflammatory signaling. GWAS and protein deficiencies in affected patient tissues link TNIP1 protein and chronic hyper-inflammatory diseases. While psoriasis is a complex disease state with multiple genetic factors, we hypothesize that TNIP1 deficiency sensitizes keratinocytes to toxicant-induced inflammation. Herein, we developed and characterized a TNIP1-null line of keratinocytes as a NAM for studying psoriasis. **Methods:** A TNIP1-null line of HaCaT spontaneously immortalized human keratinocytes was developed using gene editing with knockout confirmed by Sanger sequencing and western blotting. Bulk RNA-seq identified differentially expressed genes (DEGs) between parental and TNIP1-KO cells. The resulting data was processed with DESeq2 and analyzed via gene correlation plots comparing the TNIP1-KO DEGs to those of clinical psoriatic samples and functional pathway enrichment analysis. For both cell lines, levels of key inflammatory and keratinocyte differentiation protein markers were detected and quantified using a Jess Automated Western Blot system. To analyze late differentiation keratinocyte markers, organotypic keratinocyte models were developed by growing HaCaT cells on a fibroblast-containing collagen raft. Confluent rafts were raised to the air-liquid interface (ALI) to induce differentiation. Tissue structure and stratified differentiation markers were analyzed via histology and immunohistochemistry (IHC). **Results:** Sanger sequencing confirmed an exon 3 frameshift mutation in the TNIP1 gene, which was corroborated by absence of protein in western blotting. RNA-seq of the parental and TNIP1-KO cell lines revealed DEGs positively correlated with tissue from psoriasis patients. Additionally, of the top 20 DEGs by fold change, the majority of genes are highly associated with a psoriatic phenotype (e.g., those encoding inflammation proteins IFIT3 and PI3, structural protein IVL, and ECM remodeling proteins MMP1 and MMP10). Pathway analysis corroborated these findings with most terms directly related to the psoriatic phenotype (e.g., decreased cornified envelope formation and cell-cell adhesion) or the underlying mechanisms (e.g., increased IFN signaling). Western blotting confirmed changes in key psoriatic biomarkers: inflammation-associated proteins (TNFAIP3, KRT6A) and the psoriasis hallmark S100A7/psoriasin were increased, while the structural protein IVL was decreased. Finally, H&E staining of organotypic models showed decreased cell layers and increased collagen protrusion. IHC analysis of basal cell proliferation (Ki67) and suprabasal differentiation (KRT10, IVL) biomarkers showed retention of biomarker compartmentalization by the TNIP1-KO cells. **Conclusions:** The TNIP1-KO HaCaT line recapitulates key features of the psoriatic phenotype at both the transcriptomic and proteomic levels. This is evidenced by the positive correlation with clinical psoriatic DEGs, the upregulation of key inflammatory and psoriasis-associated markers (e.g., S100A7, KRT6A), and the downregulation of structural proteins (e.g., IVL). Furthermore, the 3D organotypic model displayed significant structural alterations. Appropriate protein marker compartmentalization occurred across the KO strata despite a reduction in cell layers suggesting the model represents a psoriatic phenotype of impaired tissue integrity and barrier function, rather than simple

hyperproliferation. This model serves as a promising, human-relevant NAM platform for investigating how environmental toxicants trigger or exacerbate psoriatic inflammation in genetically susceptible individuals.

PS 4107 New Approach Methodologies for Photoallergy: Results of a Set of Reference Photoallergens in the Photo-DPRA, Photo-Keratinosens, and Photo h-CLAT

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Background and Purpose: The Research Institute for Fragrance Materials (RIFM) evaluates fragrance ingredients for both photoirritation and photoallergenicity. Photoallergy evaluation that avoids testing in animals is challenging due to a lack of validated *in chemico* or *in vitro* methods. RIFM has focused its photosafety research program on new approach methodologies (NAMs) for photoallergy. The test methods investigated are modifications of existing skin sensitization assays under OECD TG 442C, 442D, and 442E with inclusion of an irradiation exposure. To determine the suitability of these assays, we evaluated a set of reference photoallergens. Herein we describe the results of testing in the photo-direct peptide reactivity assay (photo-DPRA), the photo-Keratinosens assay, and the photo-human cell line activation test (photo h-CLAT). **Methods:** 3,3',4',5-Tetrachlorosalicylanilide, Musk ambrette, 6-Methylcoumarin, Fenticlor, Lomeflaxacin, Sulfanilamide, Chlorpromazine, 4-Methyl-7-ethoxycoumarin, 7-Methoxycoumarin, and Bithionol were chosen based on compelling evidence in the literature to support that classification with a focus on clinical relevance. In the photo-DPRA the percent depletion of a cysteine-containing peptide was evaluated immediately after 5 J/cm² of UVA exposure (herein referred to as irradiation), and then again after 24 hours of exposure to a test material. The peptide depletion was determined by HPLC. The photoreactivity was measured by comparing the peptide depletion in the presence and absence of irradiation. The photo-Keratinosens assay evaluated the potential of a test material to activate antioxidant response element (ARE) dependent genes in keratinocytes, as measured by the induction of luciferase in the KeratinoSens™ cells, a reporter cell line which contains an ARE and a luciferase gene. Photoallergy potential was evaluated by comparing the luciferase induction after exposure to the test material in the presence and absence of irradiation. The photo h-CLAT identifies changes in the expression of cell surface markers CD54 and CD86, which are associated with dendritic cell activation in the human leukemia cell line, THP-1, following exposure to a test substance and irradiation. The cell surface marker expression is measured by flow cytometry following cell staining with fluorescently labelled antibodies for CD54 and CD86, and photoallergy potential was evaluated by comparing the expression of CD54 and CD86 after exposure to the test material in the presence and absence of irradiation. **Results:** Nine materials demonstrated increased depletion of a cysteine containing peptide >13.89% in the presence of irradiation and were considered photoreactive in the photo-DPRA. One material resulted in co-elution and could not be evaluated. In the photo-Keratinosens assay, 3 materials produced a luciferase induction in the presence of irradiation at least 1.5 times greater than the induction in the absence of irradiation at corresponding concentrations, indicating activation of ARE-dependent genes. Six materials failed to induce a fold induction ratio >1.5-fold in the presence of irradiation compared to the absence of irradiation. One reference material, 6-Methylcoumarin, is used as the positive control in the assay; for the purposes of this analysis, the results from this material are not included herein. In the photo h-CLAT, 9 materials were found to have the potential to activate dendritic cells, as indicated by the ratio of relative fluorescence intensity for the irradiated cells compared to non-irradiated cells; 1 material did not. **Conclusions:** Currently, there are no validated NAMs for the assessment of photoallergy. Data from these preliminary studies are promising. In particular, the photo-DPRA and the photo h-CLAT showed a high level of accuracy. Results from the photo-Keratinosens assay were unexpected for many of the reference photoallergens. More studies are needed to evaluate the suitability of these assays to evaluate photoallergy, with reference chemicals that include photoirritants, skin sensitizers, and skin irritants. That work is currently underway.

PS 4108 Use of New Approach Methodology to Assess Inhalation Toxicity Risk from Three Ingredients in Pesticide Products

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Background and Purpose: N-Octyl bicycloheptene dicarboximide (MGK-264) and piperonyl butoxide (PBO) function as synergists when formulated with insecticides such as pyrethrins. Collectively referred to as the Trio actives, these three compounds are commonly used in combination in pesticide product formulations. Although these compounds exhibit low acute toxicity (EPA Category IV), EPA guideline subchronic inhalation studies in rats have reported laryngeal and nasal epithelial hyperplasia and squamous metaplasia at comparatively low exposure levels, prompting regulatory concern. Project Trio was established to develop a next-generation, human tissue-based inhalation risk assessment framework integrating new approach methodologies (NAMs) with quantitative dosimetry to assess the risk of these effects

to humans. The hypothesis is that *in vitro* human airway models combined with target-tissue dose modeling provide a more relevant estimate of human risk than direct extrapolation of rat lowest-observed-adverse-effect-concentrations (LOAECs) from subchronic inhalation studies. **Methods:** Realistic consumer exposure scenarios were evaluated for a water-based aerosol, an ultra-low-volume (ULV) fogger, and a pet care powder. Chamber studies measured time-weighted average airborne concentrations of sentinel compound PBO and particle-size distributions associated with product use. Computational Fluid-Particle Dynamics (CFPD) and Multiple-Path Particle Dosimetry (MPPD) models were used to translate these concentrations into deposited and retained doses (mg/cm²) in both rats and humans. **Results:** Chamber monitoring studies following product use showed transient exposures that declined rapidly post-application. Subsequent concentration-response studies conducted in the human tissue assays identified clear no-observed-adverse-effect-concentrations (NOAECs) and LOAECs for all three actives. For human risk characterization, *in vitro* human tissue NOAECs were used as Points of Departure (PODs) combined with modeled retained human doses to derive Margins of Exposure (MOEs). Across all product types and airway regions (nasal, bronchial and bronchiolar regions), MOEs exceeded regulatory thresholds by large margins, demonstrating negligible respiratory risk. **Conclusions:** The integration of human *in vitro* airway models, realistic exposure data, and quantitative dosimetry provides a scientifically robust NAM-based framework that enhances human relevance, reduces reliance on animal testing, and confirms that the Trio actives do not pose a respiratory hazard or risk when used in the products evaluated.

PS 4109 hTERT/SV40Tag-immortalized human hepatocytes preserves functional and transcriptomic integrity for molecular toxicology applications

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Background and Purpose: Primary human hepatocytes are indispensable for modeling xenobiotic metabolism and hepatotoxic mechanisms, yet their limited proliferative capacity and rapid loss of function in culture restrict long-term molecular investigations. Immortalized non-tumor hepatic lines such as THLE-2 and THLE-3, generated using SV40 large T antigen alone, offer practical alternatives but lack telomerase activation, leading to progressive telomere shortening, phenotypic drift, and limited culture stability. In contrast, tumor-derived lines such as HepG2 and Huh7, while metabolically active, harbor oncogenic mutations and dysregulated signaling pathways that compromise physiological relevance. To overcome these constraints, we developed a defined dual-component immortalization strategy using hTERT and SV40Tag, designed to extend proliferative lifespan while preserving hepatocyte identity, metabolic competence and transcriptomic integrity for mechanistic toxicology research. **Methods:** Primary hepatocytes were transduced with lentiviral vectors expressing hTERT and SV40Tag. Immortalization efficiency and growth kinetics were assessed by IncuCyte live-cell imaging and proliferation curves. Functional assays included albumin and urea secretion, CYP450 expression, and Seahorse-based mitochondrial and glycolytic profiling. RNA-seq was performed to characterize transcriptional reprogramming during immortalization, followed by GSEA, GO, and KEGG enrichment analysis to identify pathway-level changes. Expression of hepatocyte, telomerase, cell-cycle, and senescence-related gene signatures were examined by hierarchical clustering and ssGSEA scoring. **Results:** The immortalized lines exhibited stable, non-tumor morphology and sustained proliferation beyond 30 passages while maintaining albumin and urea synthesis, and demonstrated active CYP450 expression and energy metabolism. Principal component analysis of global transcriptomes distinguished primary, immortalized, and tumor-derived hepatocytes, confirming defined but controlled reprogramming. Immortalization induced cell-cycle and extracellular matrix pathways while preserving hepatic metabolic and xenobiotic gene networks. Signature enrichment confirmed active hTERT and SV40Tag programs with repression of senescence-associated genes. Importantly, ADME/Tox and CYP450-related pathways remained functionally intact and comparable to HepG2 and THLE2. **Conclusions:** This study establishes a scalable, dual-component, and reproducible method for hepatocyte immortalization that maintains molecular integrity and functional metabolism. The resulting hTERT/SV40Tag lines provide physiologically relevant, genomically defined platforms for molecular toxicology and xenobiotic metabolism research, offering a validated alternative to existing hepatic models.

PS 4110 An In Vitro Self-Renewing Human Intestinal Epithelium for Evaluation of Gut Toxicity and Repair

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Background and Purpose: Damage to the intestinal epithelium and disruption of crypt-villus homeostasis underlie many forms of gut toxicity, inflammation, and disease. A major challenge in evaluating intestinal safety and recovery *in vitro* is the lack of models that recapitulate the cellular organization, renewal capacity, and repair dynamics of the *in vivo* human gut. We developed a self-renewing two-dimensional intestinal epithelial model that mimics the architecture and function of the crypt compartment, enabling assessment of epithelial injury, regeneration, and long-

term responses to cytokines, chemicals, and drugs. **Methods:** Non-permeable cell culture inserts were engineered with 91 laser-drilled microholes to generate localized gradients of growth factors from the basal compartment through an overlaid hydrogel scaffold. Primary human intestinal stem cells cultured on these inserts formed organized epithelial tissues with spatially distinct proliferative (Edu⁺), secretory (MUC2⁺), and absorptive (ALP⁺) zones that were maintained for at least 14 days. The resulting polarized tissue was pulsed for 24 hours with proinflammatory cytokines to induce damage in the presence or absence of preventative agents and allowed to recover over 7 days. **Results:** Exposure to the cytokine IL-22 increased proliferative Edu⁺ cells, an effect that reversed after a 7-day washout, demonstrating the model's ability to capture epithelial repair. A 24-hour exposure to an inflammatory cytokine cocktail (TNF α , IFN γ , and IL-1 β) induced LDH release and loss of epithelial confluence, reflecting acute barrier disruption. Co-treatment with anti-inflammatory drugs adalimumab or tofacitinib mitigated these effects. Upon cytokine removal and return to normal media, epithelial confluence and lineage allocation recovered over the subsequent 7 days, confirming the model's sensitivity to inflammatory injury, therapeutic modulation, and repair dynamics. **Conclusions:** This physiologically relevant crypt-mimetic platform, henceforth called RepliGut® Crypt, enables quantification of both epithelial toxicity and regenerative capacity in response to diverse chemical and biological stressors. By capturing self-renewal and recovery processes that are absent in conventional monolayer systems, the model provides a human-relevant assay for evaluating epithelial resilience and compound safety. Applications span the chemical, food ingredient, and pharmaceutical industries, supporting preclinical toxicity assessment, mechanism-of-injury studies, and discovery of agents that promote intestinal protection and repair.

PS 4111 Microengineered 3D Vascular Injury Model for Immune-Mediated Drug Toxicity Assessment

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Background and Purpose: Drug-induced vascular injury (DIVI), which refers to blood vessel (endothelial cells and/or smooth muscle cells) damage caused by pharmaceuticals, is a challenge in drug development. DIVI can result from various classes of drugs, including both small molecules and biologics, with many compounds inducing vascular injury via activation of immune pathways, known as immune-mediated DIVI (iDIVI). This involves the release of proinflammatory cytokines and subsequent vascular disruption. Early identification of these candidates could thus help mitigate unintended toxicity, reduce delays, and lower costs in drug discovery and development. In preclinical settings, screening for iDIVI-inducing compounds traditionally involves a combination of *in vitro* and *in vivo* models to try and predict clinical outcomes. However, standard *in vitro* models have often lacked the physiological cues and diversity in cell types necessary to accurately predict iDIVI, while *in vivo* models pose challenges regarding interspecies translatability and ethical considerations. The U.S. FDA Modernization Act 3.0 supports complementing animal models in preclinical studies with new approach methodologies (NAMs). This has encouraged the development of more translatable *in vitro* models for screening drug candidates. **Methods:** In this study, a vascular coculture model was developed using OrganoPlate® 3-Lane 64 and compared against traditional Transwell® setup, combining human umbilical vein derived endothelial cells (HUVECs) and peripheral blood mononuclear cells (PBMCs) as an *in vitro* NAM to detect iDIVI. HUVECs were seeded against a collagen matrix to form endothelial tubules under gravity-driven flow. After seven days, PBMCs were introduced to the endothelial tubules along with test compounds [e.g. LPS (a toll-like receptor (TLR) 4 agonist) and Resiquimod (R848; a TLR7/8 agonist, or commercial drugs associated with vasculitis adverse events)] for 24 hours. To detect changes in endothelial function, trans-endothelial electrical resistance (TEER), morphological analysis, immunostaining, and proinflammatory cytokine (TNF- α , IFN- γ , IL-1 β , and IL-6) measurement were performed. Additionally, to confirm the involvement of inflammatory pathways for barrier damage, a one-hour pretreatment of cells with a pharmacological inhibitor for MAPKKK (aka TAK1; nodal kinase in proinflammatory pathways) was employed prior to stimulation with drugs. **Results:** Both OrganoPlate® and Transwell® coculture models demonstrated iDIVI responses, identified by disruption of the endothelial barrier when stimulated with immunomodulators only in the presence of PBMCs. However, OrganoPlate® model showed R848-induced responses at 10x lower concentrations compared to Transwell®, which were captured by real-time TEER measurement, high-content microscopy-enabled direct visualization of cell morphology, and increased expression of ICAM-1 as well as proinflammatory cytokines. When screening commercial drugs (daclizumab, docetaxel, etc.) that have been reported to cause vasculitis as a rare adverse event, several showed varying degrees of vascular damage. Notably, daclizumab (now off-market) exhibited concentration-dependent iDIVI responses starting at 1 μ g/mL [clinical C_{max} (30 μ g/mL)]. Importantly, inhibition of proinflammatory signaling using a TAK1 inhibitor effectively rescued the iDIVI phenotype induced by LPS and daclizumab. This protective effect was evidenced by blockade of TEER and junctional VE-cadherin loss, suppression of ICAM-1 upregulation, reduction in proinflammatory cytokine release (IL-1 β and IL-6), and decreased leukocyte adherence to the HUVECs. **Conclusions:** OrganoPlate® provided a robust model for characterizing the role of immune cells in DIVI by mimicking immune-endothelial interactions in a microfluidic *in vitro* model. Multiple classes of drugs including small molecules and monoclonal antibodies showed iDIVI in this model, and perturbation

with a pharmacological inhibitor allowed for proof of applicability for mechanistic studies. Thus, Organoplate® iDIVI model is expected to have broad utility for understanding the iDIVI risk for different classes of drugs, reducing the need for animal use in early screening, and ultimately helping to facilitate development of clinically safer drugs.

PS 4112 Assessment of Endothelial Network Formation and Immune-Endothelial Cell Interactions Using Different Commercially-Available Microphysiological Vascular Devices

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Background and Purpose: Perfusable, immune-competent *in vitro* models of human vasculature can serve as powerful decision-making tools to assess the effects of novel therapeutics and chemicals on the vasculature as a whole and also on the leukocyte-endothelium interactions. This study aimed at evaluating robustness and reproducibility of the methods for establishment of perfusable vascular networks and induction of inflammatory response. For this, we used two commercially available human-vasculature microphysiological systems (MPS): the IdentTX-40 (AimBiotech), based on vascular self-assembly process, and the Mimetas' Organoplate®, relying on endothelial cells forming a tubule in a hollow channel. Specifically, we evaluated how endothelial cell type, cell culture medium composition, stromal cell support, and pro-inflammatory stimuli may shape vascular architecture and immune-endothelial cell interactions. **Methods:** In IdentTX MPS, endothelial progenitor cells (EPCs) and human umbilical vein endothelial cells (HUVECs) were seeded in a fibrin-thrombin gel with either a manufacturer's recommended medium or a supplemented medium with or without primary lung fibroblasts. Vascular network morphology and pro-inflammatory signaling were assessed. In parallel, we also evaluated the 2-lane 96 Organoplate® for immune cell adhesion following LPS stimulus. This MPS does not rely on vascular self-assembly and a single, perfusable tube can be formed using endothelial cells, allowing for increased control of the vascular architecture. Fluorescently tagged THP-1 monocytes and M0/M1 macrophages were perfused through the endothelial channels, and cell adhesion and ICAM-1 expression were evaluated. Building on this, a 3-lane Organoplate® model, incorporating EPCs exposed to TNF- α and chemokine gradients (CCL2 or CXCL12), was also tested to investigate immune cell extravasation. We quantified monocyte migration toward the chemokine channel as an indicator of directional immune trafficking across an inflamed endothelium. **Results:** As expected, fibroblasts markedly improved HUVEC vessel stability and reduced ICAM-1 expression. Interestingly, EPCs formed robust, perfusable networks regardless of the presence of fibroblasts. EPCs cultured in the highly supplemented medium exhibited the highest ICAM-1 levels, suggesting that nutrient-rich formulations used to induce vessel formation may also increase undesirable baseline inflammation. Fluorescent imaging demonstrated immune cell migration toward the chemokine channel within 24-48 hours, suggesting functional modeling of directional immune trafficking across an inflamed endothelium. **Conclusions:** Together, these studies highlight differences in biology and functionality between different vasculature MPS and support a cross-platform comparison approach to leverage the strength of individual experimental tools depending on the context of use. By utilizing the benefits of IdentTX for reliable self-assembly vessel formation and Organoplate® for precise control over immune cell dynamics and signaling, we present examples for customizable MPS for studies of endothelial barrier, inflammatory activation, and immune trafficking. This integrated approach supports the development of more physiologically-relevant vascular MPS for disease modeling and studies of hazardous chemicals and drugs.

PS 4113 Bridging the Gap Between *In Vitro* Models and Human Physiology: The Advantages of HUB's Intestinal Patient-Derived Organoids in Toxicology

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Background and Purpose: Gastrointestinal toxicity (GIT) is among the most frequently observed drug-induced adverse effects, occurring both in clinical trials and after market approval. Although generally non-life-threatening, GIT often requires dose adjustments or co-medication, which can compromise therapeutic efficacy and patient quality of life. Preclinical safety assessments for predicting drug-induced toxicity typically rely on animal models, two-dimensional (2D) cell cultures, or primary tissues. However, animal models exhibit limited clinical translatability (e.g., in rodents) or are constrained by ethical and cost considerations (e.g., in non-rodents). Among *in vitro* systems, CaCo-2 cell lines are widely used but represent only a single absorptive enterocyte-like phenotype, lacking the cellular diversity, tissue architecture, and physiological relevance of the human intestinal epithelium. Consequently, these limitations hinder the accurate prediction of human gastrointestinal responses to drug exposure. In contrast, intestinal organoids provide a three-dimensional, multicellular, and physiologically relevant model that more closely mimics native human intestinal tissue, offering a promising alternative for improv-

ing the translational value of preclinical toxicology studies. **Methods:** To overcome these limitations, we established a human-relevant *in vitro* platform based on Patient-Derived Organoids (PDOs). PDOs are generated from adult intestinal stem cells and retain the genetic, phenotypic, and functional properties of native intestinal tissue. They can be derived from different regions of the gastrointestinal tract, enabling the study of region-specific responses, and can be differentiated into various intestinal cell types, including enterocytes. In addition, the 3D organoid format is compatible with high-throughput screening, enabling efficient, reproducible testing of large compound libraries for toxicity profiling. **Results:** To assess the suitability of our intestinal organoid platform for evaluating drug-induced GIT, ten compounds with known *in vivo* outcomes—five inducing GIT (e.g., *Gefitinib*) and five non-inducing (e.g., *Flecainide*)—were tested. Compound effects were evaluated in 3D organoids cultured in either expansion medium (enriched in stem and transit-amplifying cells) or enterocyte differentiation medium (enriching for enterocytes) by measuring cell viability via an ATP-based assay. Additionally, 2D organoid-derived enterocyte-enriched monolayers were treated with the same compounds, and both cell viability (ATP-based) and epithelial barrier integrity (transepithelial electrical resistance, TEER) were assessed. In the 3D assay, all ten compounds were correctly classified, achieving 100% sensitivity and specificity. In contrast, in the 2D monolayer assay, only the non-GIT-inducing compounds behaved as expected (100% specificity), whereas half of the GIT-inducing compounds did not reduce viability or TEER values, suggesting that alternative or complementary readouts may be required to detect GIT toxicity in 2D systems. **Conclusions:** These findings demonstrate that HUB's intestinal Patient-Derived Organoids offer a robust, physiologically relevant, and predictive platform for assessing drug-induced GIT. This system provides superior sensitivity and specificity compared with conventional 2D assays, making it a valuable tool for preclinical safety evaluation and drug development and helping bridge the gap between *in vitro* models and human physiology.

PS 4114 Comparative Toxicity of Alkylated and Parent Polycyclic Aromatic Hydrocarbons in Primary Human Bronchial Epithelial Cells

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Background and Purpose: Polycyclic aromatic hydrocarbons (PAHs) constitute a broad class of structurally diverse environmental contaminants primarily derived from incomplete combustion of organic materials and petroleum products. Current understanding of PAH-related health effects is largely based on studies of unsubstituted or parent PAHs. However, alkyl-substituted PAHs are among the most abundant PAHs found near contaminated sites, occurring in both vapor and particle phases in the air. Despite their abundance, little is known about the respiratory toxicity of alkyl-PAHs after inhalation exposure. Moreover, alkylated PAHs are typically excluded from routine measurements and risk assessments, raising concerns that overall health risks associated with PAH exposure may be underestimated. To address this gap, we investigated the toxicity of a range of 2-5-ring alkylated PAHs in primary human bronchial epithelial cells (HBEC) *in vitro* across multiple endpoints of toxicity compared to parent PAHs. **Methods:** Normal primary human bronchial cells were expanded and seeded in 96-well plates and maintained until they reached 95% confluency. Cells were then exposed to four increasing doses of each chemical (ranging from 0 - 400 μ M) in a 0.5-1% DMSO (assay dependent) solution for 24 hours. Nine parent PAHs, anthracene, benz(a)anthracene, benzo(a)pyrene, benzo(c)phenanthrene, chrysene, dibenz(a,h)anthracene, naphthalene, phenanthrene, and pyrene, and 52 alkylated PAHs were tested in HBEC across dose-response for endpoints of cytotoxicity, mitochondrial membrane potential, oxidative stress, and DNA damage. Based on preliminary data in other test systems, we hypothesize that alkylated PAHs will exhibit equivalent or higher toxicity compared to the parent form. **Results:** Overall, the results showed that across parent groups, many alkylated products had equal or higher toxicity than their parent compound, depending on the endpoint that was being tested. The Benz(a)anthracene family is the largest in this screening, with 15 alkyl forms tested. Of those 15, 13 caused significant cytotoxicity, and nine caused significant mitochondrial membrane damage, at a concentration equal to or less than the parent form. We see similar results in the other chemical families. In addition, we observed that alkylated PAHs may cause toxicity through unique mechanisms compared to their parent forms, potentially involving genotoxic effects. These data are being used to model the dose-response relationship and quantify the relative potency between the parent and alkylated forms using benchmark dose modeling. **Conclusions:** Overall, these data indicate that current risk assessments may underestimate PAH risk from inhalation exposure due to a lack of data for alkylated forms. Additional research is required to close the data gap on the toxicity of alkylated polycyclic aromatic hydrocarbons.

PS 4115 A New Approach Methodology (NAM) Emphasizes hypothesis-driven Experimental Timing for Air-liquid Interface Studies: A Proof-of-Concept Investigation Using Human Primary Bronchial Basal Stem Cells

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Background and Purpose: Several studies have been conducted on air-liquid interface (ALI)-cultured human bronchial basal cells (HBBCs) at different time points. Traditionally, experimental applications in ALI cultures are typically performed after day 21, assuming complete epithelial differentiation. Nonetheless, gene expression dynamics may vary across differentiation stages, and a fixed time point may not always be optimal for studying specific biological processes. **Methods:** To determine this, we conducted a targeted screening of key markers involved in DNA damage responses (*DDIT3*, *TP53*) and inflammatory pathways (*TNF-α*, *IL-10*, *IL-8*), alongside the gene expression markers of differentiation for basal cells (*ITGA6*, *KRT5*, *TRP63*), goblet cells (*MUC5AC* and *MUC5B*), ciliated cells (*FOXJ1*) and Club cells (*SCGB1A1*) at multiple time points (Day 0, 7, 14 and 21). In addition, we measured the TEER as a proxy for tight junction formation and pro-inflammatory mediators IL-8, IL-6, TNF-α, and IL-1β. **Results:** We show a distinctive pattern of gene expression and protein levels across three stages: proliferative, differentiation, and maturation. This shows that at each stage of epithelial development, different genes are at play. **Conclusions:** This novel approach may enhance the reproducibility and accuracy of airway disease modelling and drug testing, ensuring that studies are conducted at the most functionally relevant stage of differentiation. To the best of our knowledge, this is the first time such an experimental approach has been proposed. Our findings do not challenge the conventional 21-day approach for ALI studies but suggest a data-driven strategy to determine the optimal experimental window based on pathway-specific gene expression.

PS 4116 Comprehensive In Vitro Safety Assessment of Acorus calamus Rhizome Oil Using OECD Compliant New Approach Methods

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Background and Purpose: *Acorus calamus* (sweet flag) is widely used in traditional medicine, yet its dermal safety profile remains insufficiently defined under modern regulatory standards. The objective of this study was to comprehensively evaluate the skin irritation, corrosion, and sensitisation potential of *A. calamus* rhizome oil using new approach methodologies (NAMs) test battery under GLP conditions. **Methods:** Hydro-distilled *A. calamus* oil's chemical characterization was performed via HPLC and LC-MS/MS to detect and quantify α, β, and γ asarone content. The oil was evaluated for skin irritation/corrosion potential using SkinEthic™ reconstructed human epidermis skin irritation test (OECD TG No. 439) and corrosion test (TG No. 431). Furthermore, the oil was also evaluated for skin sensitisation potential using Direct Peptide Reactivity Assay (OECD TG No. 442C), KeratinoSens™ transcriptional activation assay (OECD TG No. 442D), and human Cell Line Activation Test (OECD TG No. 442E). The risk assessment was conducted using the SARA-ICE Extended Model and SCCS default exposure parameters. **Results:** The *A. calamus* rhizome oil was predicted as a Category 2 skin irritant, non-corrosive, and GHS Category 1B skin sensitizer. Chemical analysis revealed β-asarone as the major constituent (~40.75%). The reconstructed human epidermis models established reversible irritation without corrosion. Mechanistic concordance across the Direct Peptide Reactivity Assay, KeratinoSens™, and human Cell Line Activation Test showed activation of the three key events of the skin sensitisation adverse outcome pathway. Using the "2-out-of-3" Defined Approach with the KE3/1 sequential strategy allowed the hazard classification into GHS Category 1B. Quantitative risk modelling using SARA-ICE models and SCCS parameters yielded conservative safe-use concentrations ranging from 0.13-0.78% (w/w) for leave-on products and up to 7.46% (w/w) for rinse-off formulations. **Conclusions:** The combined evidence from the NAM-based assays showed that *A. calamus* rhizome oil is a moderate sensitizer and irritant but not corrosive, providing critical data for risk assessment and regulatory decision-making, which was previously unknown. The SARA-ICE PoD-derived safe-use concentrations provide guidance for cosmetic formulators to ensure consumer safety, particularly in leave-on applications such as face and hand creams, where sensitisation risk is highest. This study demonstrates the utility of NAMs for botanical safety assessment and regulatory decision-making.

PS 4117 Predicting gastrointestinal toxicity using the assay-ready, primary-derived OrganoReady® Colon Organoid model

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Background and Purpose: Gastrointestinal (GI) toxicity is among the most common adverse effects observed in clinical trials and after drug approval, often resulting in treatment delays, reduced patient quality of life, or even therapy discontinuation. Accurately predicting GI toxicity for novel drugs remains challenging, as many existing intestinal models fail to capture the full complexity of the gut epithelium. **Methods:** To address this limitation, we developed the OrganoReady® Colon Organoid model, which employs automated seeding and tubule formation of primary-derived colon organoids. Our platform integrates 64 microfluidic chips within a single plate, each supporting fluid flow and providing access to both apical and basolateral compartments, thereby enabling high-throughput screening of small- and large-molecule therapeutics. Staining analyses confirmed effective polarization of the gut tubules and the presence of key differentiated colon cell types. Functional assays demonstrated that the OrganoReady Colon Organoid tubules recapitulate essential intestinal functions, including drug transport, metabolism, and barrier integrity. **Results:** We exposed the OrganoReady Colon Organoid model with a representative panel of GI-toxicity-inducing compounds. After 72 hours, we assessed both viability and barrier integrity using Trans-Epithelial Electrical Resistance (TEER) measurements. The Colon Organoid tubules displayed greater sensitivity than conventional Caco-2 models. To evaluate reproducibility, we quantified intra- and inter-plate variability. Results showed minimal variation between chips within a single plate ($Z' \geq 0.5$) and strong reproducibility between plates. Additionally, the model remained stable during shipment under controlled temperature conditions, allowing end users to perform large-scale compound screening following a short recovery period. **Conclusions:** Together, the OrganoReady Colon Organoid represents a robust and highly predictive platform for GI toxicity testing, offering multiple functional readouts to support safety-related decision-making. Furthermore, this platform lays the groundwork for future GI toxicity studies incorporating donor diversity.

PS 4118 A Ready-to-Use 3D Human Blood Vessel model for Scalable Assessment of Endothelial Toxicity and Immune Activation

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Background and Purpose: The vascular endothelium is essential for maintaining homeostasis, controlling permeability, and regulating immune cell trafficking, processes often disrupted by pharmacological compounds. Conventional *in vitro* models lack the dynamic barrier function and immune responsiveness required for accurate prediction of vascular toxicity and inflammation, emphasizing the increasing demand for physiologically relevant and scalable models. Microphysiological systems (MPS) offer improved translational value by bridging the gap between static cultures and *in vivo* studies. Previous studies have demonstrated the utility of endothelial models in the OrganoPlate® platform for vascular and immunological applications. **Methods:** In this study, we present the OrganoReady Blood Vessel HUVEC model, a ready-to-use, membrane-free, high-throughput platform that forms a robust and modulatable endothelial barrier suitable for compound screening. The model was established in the OrganoPlate® 3-lane platform, which supports parallel culture of 40 to 64 endothelial vessels. Under gravity-driven flow, each chip enables the self-organization of HUVECs against an extracellular matrix to form a perfused, tubule-like endothelial structure. **Results:** Barrier integrity, assessed using the OrganoTEER® system, confirmed the formation of tight endothelial tubules with trans-endothelial electrical resistance (TEER). The model responded sensitively to inflammatory stimuli such as TNF-α and IFN-γ, showing reduced barrier function and morphological changes indicative of endothelial stress. High-content imaging confirmed ICAM-1 upregulation and altered morphology upon cytokine exposure, demonstrating vascular inflammation and supporting ICAM-1 as a marker for endothelial activation. Building on this inflammatory response, immune-endothelial interactions were evaluated using a PBMC adhesion assay. Following 16-hour stimulation with TNF-α and IL-1β, VE-cadherin disruption and a marked increase in immune cell adhesion were observed compared to non-inflamed controls. The model showed high reproducibility across experiments, confirming its robustness for screening applications. **Conclusions:** Together, these results validate the OrganoReady Blood Vessel HUVEC model as a reliable and scalable platform for high-throughput assessment of vascular toxicity, inflammation, and immune-endothelial interactions, addressing key needs in pharmaceutical compound evaluation.

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Background and Purpose: Modern toxicological assessments increasingly rely on *in vitro* assays for chemical prioritization, often using potency-based ranking. While nominal concentrations have traditionally been used, more biologically relevant exposure metrics useful. Mass balance modeling supports this by predicting compound bioavailability, accounting for non-specific binding, partitioning, and intracellular distribution, typically under steady-state assumptions. This study explores the impact of selecting dose metrics when applying mass balance modeling, using case examples where transcriptomic points of departure (tPoDs) were derived from *in vitro* screening assays to inform prioritisation in new active ingredient research projects. **Methods:** High-throughput transcriptomic profiling was performed using six commercially available human cell lines (HepRG, HEK293, SHSY-5Y, U2OS, iCell cardiomyocytes, H295R) via the DRUG-seq methodology. Cells were exposed to 20 research agrochemical active ingredients for 24 hours across an 8-point dose-response curve (maximum 100 μ M; half-log dilutions; 5 replicates). Benchmark dose (BMD) modeling was conducted using BMDExpress3. Gene-level BMDs were calculated from modeled concentrations using a Williams Trend Test prefilter (FDR < 0.05; fold change > 1.5), followed by BMD estimation using polynomial, exponential, and Hill models (benchmark response > 1 SD). Compound-level tPoDs were defined as the median BMD of the lowest significantly enriched GO Biological Process term (FDR < 0.05; gene sets of 5-100 genes; BMDL/BMDU ratio < 40). Compound distribution within the assay system was predicted using the mass balance model described by Armitage et al. (2014, 2021). BMDs calculations were performed by adjusting BMDs calculated on nominal concentrations and also by mass balance adjustment prior to BMD calculation. Compounds were ranked by potency in each compartment in the mass balance model. **Results:** Chemical ranking by BMD potency was different and depended on the choice of dose metric. The basis of these differences were driven by divergent physicochemical properties (log P, solubility, and pKa/pKb) within the selected compounds. High log P increased non-specific binding (reducing free concentrations) but also enhanced cellular uptake. Low solubility reduced both free and intracellular concentrations due to precipitation. pKa/pKb influences cellular localization and uptake, as ionic form and compartmental pH affect membrane interactions and organelle distribution. The consequence of this was (1) reinterpretation of low potency responses after accounting for insufficient bioavailable concentrations at cellular targets; (2) divergent ranking outcomes when correcting nominal BMDs post hoc versus adjusting full concentration-response curves prior to BMD modelling; and (3) divergent compound ranking within chemical series when using free or intracellular concentrations instead of nominal media concentrations. **Conclusions:** Dose metric selection critically influences tPoD derivation and potency-based compound ranking. While distribution modeling is a powerful tool for predicting compound behaviour *in vitro*, careful consideration of metric choice and associated uncertainties is essential for robust AI screening and toxicological decision-making. The relevant dose metric should ideally be related to the sub-cellular location of the molecular initiating event.

PS 4120 Unidirectional, pumpless, scalable: Perfusable vascular networks with long-term stability using a gravity driven microfluidic system.

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Background and Purpose: This study introduces a novel microphysiological system designed to enhance both physiological relevance and throughput in vascularized tissue models. Traditional *in vitro* models often lack the complexity and physiological relevance, whilst many microphysiological systems rely on pumps, valves, and a network of tubing to create recirculation setups for unidirectional flow, which can limit scalability and increase complexity. **Methods:** The OrganoPlate Graft 32 UniFlow (UF), our newly developed microphysiological system addresses these limitations by enabling pumpless, unidirectional perfusion of a vascular plexus cultured within a fibrin gel. The ability to create long-term stable vascular beds is the basis for more complex tissue building and can facilitate development of lung, skin, gut, liver or tumour models of greater physiological relevance. The system comprises a custom design based on the 384-well titre plate format with 32 microfluidic chips per plate, each engineered to support vascular network formation and unidirectional perfusion. The microfluidic device uses #1 coverslip thickness glass sheets, allowing for optimal compatibility with automated imaging equipment. Each chip consists of a vascular network region which is flanked by a supporting tube, and a bypass channel; a pressure drop has been designed across the vascular network region to allow for unidirectional perfusion. By leveraging a gravity-driven mechanism, the system generates flow through passive levelling, during the one-minute active phase a height difference is used to create a pressure gradient that drives unidirectional flow across the vascular network region and through the supporting tubule; changing the rocking orientation allows the system to reset through a bypass channel in a 15 second reload phase. This design utilised an open well format with access to the vascular bed via a hole above, it eliminates the need for external pumps, and reduces complex-

ity while significantly increasing throughput. Up to 16 plates (512 chips) can be accommodated on a single rocking unit, enhancing scalability for medium-throughput applications. **Results:** In experimental setups, the system demonstrated consistent formation of vascular beds within a fibrin gel and maintained perfusable vessels. Vessels within the network were seen to mature over time, and first becoming perfusable between days 6 and 9. Once perfusable, vessels are seen to widen and remain stable for 8 weeks. This was consistent for multiple users, cell lots and plates. The vascular networks exhibited stable, unidirectional perfusion, allowing for the flow of beads and immune cells across the vascular network at both an early and late stage of culture. The ability to create long term stable vasculature opens the door for modelling the interaction of vasculature with a multitude of tissues and assess both acute and chronic toxicity. **Conclusions:** In summary, the OrganoPlate Graft 32 UF, our pumpless unidirectional microphysiological system provides a versatile platform for the study of vascularized tissue models with increased longevity. Increasing the stability of vascular beds and tissues over time creates opportunities for more physiologically relevant disease triggers and repeat dosing of drugs, which can contribute to the development of new therapeutic strategies.

PS 4121 Integration of Incucyte analytics and multi-organ toxplate (kidney, Small intestine, and Liver) for NAMS-based hazard and risk assessment of compounds

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Background and Purpose: Advanced cell models are key components of NAMS for hazard and risk assessment of chemicals and pharmaceuticals. To overcome the limitations of traditional single-organ systems in predicting human responses, we developed a 3D human Multi-Organ ToxPlate (MOTP) that integrates small intestine, liver, and kidney tissues in a 96-well plate format. This platform was adjusted to be compatible with Sartorius's IncuCyte® system for high-throughput automated live-cell imaging allowing to study the effect of single or multiple drugs across three organ systems simultaneously. By combining conventional toxicological endpoints such as TEER for barrier integrity, MTT for viability, and H&E staining for histology with IncuCyte® analytics, the ToxPlate delivers comprehensive outcome measurements of pharmacokinetics and pharmacodynamics. This approach improves prediction accuracy, supports early detection of off-target effects, and contributes to reduction in late-stage clinical failures. This integrated platform enables evaluation of therapeutic toxicity, metabolism, and clearance in a physiologically relevant specific context of use. **Methods:** Primary human cells representing three organ systems (liver, kidney, and intestine) were seeded into a 96-well ToxPlate, with each organ system allocated 32 wells. Cultures were maintained under submerged conditions for 1-3 days, followed by air-liquid interface (ALI) conditions for 12 days to achieve proper differentiation and stratification. To validate the workflow, we first reconstructed a single-organ plate of the 3D human kidney model and dosed it with Cisplatin (known nephrotoxicant) and Oxaliplatin (non-toxicant) at various concentrations. Cell death was monitored using IncuCyte® Cytotox Green reagent (Sartorius, Ann Arbor, MI) dosed with the test compound. Next, we developed a 96-well multi-organ ToxPlate (MOTP) incorporating 3D tissues of intestine, liver, and kidney. Drug-exposed tissues were incubated at 37°C and imaged in an IncuCyte® S3 Live-Cell Analysis System housed within the incubator. Green fluorescence images were acquired at 10x magnification every 4 hours for 48 hours. Global mean fluorescence values were calculated using the Basic Analyzer module of the IncuCyte® software, where higher fluorescence indicates greater cell death. Since IncuCyte® imaging and analysis is non-invasive, TEER, MTT viability, and histological measurements were performed after IncuCyte® data collection. **Results:** The study demonstrated a dose-dependent decrease in TEER following Cisplatin treatment of the 3D kidney tissue model. IncuCyte® live-cell analysis revealed EC50 values for Cisplatin ranging from 11 to 25 μ M, consistent with previously reported manual measurements. No drug-induced acute kidney injury was observed in Oxaliplatin-treated tissues at the tested concentrations. We then expanded the study by testing three drugs Cisplatin, Fialuridine, and Troglitazone. Post-treatment incucyte analysis and TEER measurements indicated that Cisplatin was toxic to all three tissue models at higher doses. Fialuridine, liver toxicant, exhibited selective hepatotoxicity without affecting kidney or intestinal tissues. Troglitazone showed toxicity toward liver and kidney at higher concentrations. Finally, a 48-hour, seven-dose range finding study was performed using Cisplatin and Oxaliplatin on ToxPlate containing kidney and intestinal tissues integrated with the IncuCyte® workflow. Cisplatin exposure resulted in a clear dose-dependent reduction in TEER and MTT viability. IncuCyte® analytics also revealed a difference in drug response for Cisplatin (17 μ M) compared to Oxaliplatin (88 μ M) in 3D kidney tissue model. Post-IncuCyte® analysis showed that Oxaliplatin, however, TEER values exhibited a dose-dependent decrease, suggesting TEER may be a more sensitive endpoint for this compound. **Conclusions:** The integrated MOTP-IncuCyte® workflow provides a robust and economical platform for drug toxicity testing. This trio-tox assay system accelerates *in vitro* toxicity predictions, enhances physiological relevance, and supports early detection of organ-specific and off-target effects and reduces animal experimentation.

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are abundantly used in a plethora of products with applications in daily life. As a result, PFAS are widely distributed in the environment, raising concerns regarding accumulation in humans. Recent data show impairment of bile acid metabolism by legacy PFAS, yet the potential of short-chain and alternative PFAS as well as the mechanistic basis of these cholestatic effects remain elusive. The present study relied on an *in vitro* test battery mechanistically anchored in an adverse outcome pathway network to assess the cholestatic potential of legacy, short-chain and alternative PFAS. Focus was put on the effects of PFAS on molecular initiating events, in particular transporter changes and bile canaliculi dynamics. **Methods:** Cultures of human HepaRG liver cells were exposed to 8 types of PFAS in 3 concentrations. Fluorescently labeled probes were used to investigate effects on the functionality of bile acid transporters. Effects at the transcriptional level were studied by means of quantitative reverse transcriptase polymerase chain reaction analysis. Bile canaliculi dynamics were monitored *via in situ* immunostaining and phase-contrast imaging. An integrated weight-of-evidence approach was applied to process the results of the *in vitro* test battery. **Results:** PFAS inhibit the expression and activity of various bile acid transporters, including organic anion transporting polypeptides and multidrug resistance-associated proteins. Some PFAS inflicted morphological disruption of bile canaliculi. The highest overall cholestatic scores were observed for perfluorooctane sulfonic acid, perfluorobutanoic acid and perfluorobutanoic acid, while C6 PFAS exhibited low cholestatic potential. **Conclusions:** This study shows the power of *in vitro* testing to elucidate mechanisms underlying hepatotoxicity induced by PFAS and, simultaneously, to quantify the cholestatic potential of these ubiquitous “forever chemicals”.

PS 4123 A real-time, multiplexed assay for evaluating compound-induced disruption of barrier integrity

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Background and Purpose: Cell barriers, such as epithelial and endothelial layers, act as protective interfaces that tightly regulate the passage of ions, molecules, and pathogens between compartments. When these barriers are compromised, exposure to toxins increases, contributing to disease development. Assessing barrier integrity is therefore critical for understanding compound-induced toxicity, including cytotoxicity and changes in permeability. **Methods:** In this study, we introduce an assay that simultaneously evaluates barrier permeability through real-time impedance measurements and live-cell imaging. EA.hy926, an immortalized human endothelial cell line, was used to model endothelial barrier function. Cells were seeded at 5,000 cells per well on a biosensor plate and monitored continuously for approximately seven days using the xCELLigence RTCA eSight system. Barrier formation was indicated by a plateau in impedance, reported as the Cell Index. **Results:** Thrombin, a well-established barrier disruptor, was applied to increase permeability. Its addition caused a dose-dependent, reversible decline in Cell Index, followed by recovery. Live-cell imaging of the same monolayer revealed normal morphology with sparse open areas, confirming that the Cell Index drop reflected increased permeability rather than reduced viability. Pretreatment with Argatroban, a direct thrombin inhibitor, completely prevented the thrombin-induced decline in Cell Index, demonstrating its protective effect on barrier integrity. This impedance- and imaging-based assay was further validated by a conventional FITC-Dextran permeability assay and immunofluorescence staining of the tight junction protein Zonula occludens-1 (ZO-1). **Conclusions:** This integrated approach enables early detection of adverse effects of pharmaceuticals, environmental chemicals, and consumer products on barrier permeability.

PS 4124 Cynomolgus iPSC-Derived Neural Models as a Translational NHP In Vitro Platform for Preclinical Neurotoxicity Testing and NAM Alignment

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Background and Purpose: Nonhuman primates (NHPs) remain an essential model for assessing neurotoxicity and neuropharmacology in CNS drug development due to NHPs close genetic and physiological similarity to humans. However, *in vivo* NHP studies are costly, time-consuming, and ethically challenging. Current regulatory frameworks advance drug candidates from human *in vitro* (i.e., neurons) directly to *in vivo* NHP studies; changing both model type (*in vitro* to *in vivo*) and species (human to NHP), which weakens translational reliability. Drug candidates often fail late in the drug development process at the preclinical safety step when candidates are tested in *in vivo* NHP animal models. Exir Bio has developed cynomolgus macaque iPSC-de-

rived neural models that enable *in vitro* NHP testing alongside human systems to better anticipate *in vivo* outcomes, and refine and reduce animal dependence. These models support post-study mechanistic investigations when neurotoxicity is observed *in vivo*. **Methods:** Cynomolgus macaque (cyno) iPSCs were differentiated into NGN2 neurons, astrocytes, and oligodendrocytes via transfection with respective doxycycline-inducible vectors. Motor neurons (MN) were differentiated by generating via embryoid-body formation followed by terminal differentiation by supplementing with small molecules. **Results:** Cyno NGN2 and MN populations expressed canonical terminal differentiation markers by qPCR and immunocytochemistry: MAP2, ISL1, TUJ1 for NGN2 neurons and MAP2, ISL1, and HOX6 for MN, respectively. Multielectrode array (MEA) demonstrated that the cyno NGN2 and motor neurons had comparable firing rates to human iPSC-derived neurons. Co-culture with human astrocytes supported synaptic connectivity and neurite outgrowth. **Conclusions:** Exir Bio's cyno iPSC-derived *in vitro* models fill a key gap in the ability to examine *in vitro* to *in vivo* translatability between NHPs and animal models in four cell types from the neural progenitor cell lineages including NGN2 cells and motor neurons. These cells express the functional markers of their respective terminally differentiated neurons, confirmed via qPCR and immunocytochemistry staining, and perform equivalently in functional assays. These models provide translational NHP *in vitro* platforms for neurotoxicity and preclinical drug testing that embody the 3Rs of modern drug development.

PS 4125 Modeling repeated ENDS exposure response trajectories in adolescents using a co-culture model of the bronchial epithelium

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Background and Purpose: The use of primary *in vitro* models for inhaled chemical testing often relies on single cell models, specifically, differentiated primary human bronchial epithelial cell (dpHBEC) cultures. While dpHBECs hold physiological relevance based on their ability to form cilia, tight junctions, and mucus production, they are only one of many cell types in the bronchial epithelium. Incorporating multiple cell types increases biological complexity of *in vitro* models while also providing added context of interactions occurring *in vivo* to better predict human health outcomes. Additionally, primary *in vitro* models from human donors may capture heterogeneous responses to toxicant exposures, including response trajectories in susceptible populations. The rapid popularity of Electronic Nicotine Delivery Systems (ENDS) in adolescents had led to a knowledge gap in understanding the cumulative and long-term health impacts in humans. The effects and underlying mechanisms of inhaled test articles, such as ENDS aerosols, are often modeled using acute exposures and only one cell type commonly from human immortalized cell lines. In realistic exposures, ENDS usage typically involves repeated exposures over time and interaction of numerous cell types in the bronchial epithelium. Therefore, to increase physiological relevance of ENDS exposures in adolescents, we conducted repeated exposures to ENDS aerosols using a co-culture model containing dpHBEC air-liquid interface (ALI) cultures and donor-matched primary human lung fibroblasts (pHLFs) from adolescent donors, known as the trans-epithelial exposure model (TEEM). **Methods:** We assessed the impact of repeated ENDS exposures in the TEEM using 6 sex-balanced adolescent donors with no known health issues or history of smoking. Cultures were exposed to either vehicle control containing propylene glycol: vegetable glycerin (PG:VG) or ENDS aerosol containing benzaldehyde and nicotine for 1 (1X) or 5 (5X) days in CelTox units while maintaining ALI conditions. Exposure groups were compared against a matched ALI control. Total aerosol mass from *in vitro* exposures was compared with the *in vivo* peak modeled deposition in humans using Multiple-Path Particle Dosimetry (MPPD) model. Following repeated exposures, we evaluated impacts on common *in vivo*-relevant endpoints, such as ciliary beat frequency (CBF), trans-epithelial electrical resistance (TEER), cytotoxicity, MUC5AC secretion, and pro-inflammatory cytokine secretion: interleukin 1 α (IL-1 α), IL-6, and IL-8. We assessed transcriptional changes in epithelial and fibroblast cell types using RNA sequencing. **Results:** Aerosol mass per cm² in CelTox units was slightly decreased compared to the MPPD modeled predictive *in vivo* peak deposition in humans. ENDS aerosol exposure for 5-days led to increased cytotoxicity among all donors when compared against controls. TEER was not impacted by exposures. CBF and MUC5AC showed a robust increase in response following 5-days of exposure. Donor specific sensitivities were seen in cytokine secretion with 4 donors classified as responders to ENDS aerosol effects. RNA-seq revealed cell type dictated variation with the greatest difference in gene expression between dpHBECs and pHLFs. Additionally, pHLFs showed distinct gene expression patterns between 1 and 5-day exposures with greater induction of oxidative stress, DNA damage, fibrosis, and lung cancer pathways following 5X exposures. Gene expression patterns showed minimal upregulation in dpHBECs with a similar pattern amongst control and exposure groups. RNA-seq data indicated that model complexity can drive experimental outcomes. **Conclusions:** This study demonstrates the need to characterize primary *in vitro* systems through understanding cell type-specificity and inter-individual variability in outcomes by including susceptible population groups and increased donor numbers in toxicological studies to capture response trajectories that represent the human population. Utilization of the TEEM and repeated exposures to ENDS aerosols was able to capture complex and cumulative biological responses that single-cell or single-exposure models may miss, which better predicts adverse health outcomes in humans.

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Background and Purpose: There is a regulatory need within the U.S. EPA's Endocrine Disruptor Screening Program (EDSP) to develop new approach methodologies (NAMs) for identifying and evaluating chemicals that may interfere with the thyroid endocrine system in human. To address this need, an integrated tiered testing strategy has been established for assessing thyroid hormone (TH) disruption. Tier 1 assays typically employ cell lysates, rat or human thyroid microsomes, immortalized cell lines (e.g., FRTL-5, Nthy-ori 3-1), and engineered cells (e.g., HEK293, HeLa) over-expressing thyroid-related target genes such as thyroid peroxidase (TPO), sodium iodide symporter (NIS), and iodothyronine deiodinase-1 (DIO1). These assays focus on single molecular initiating events (MIEs) but lack thyroid-specific phenotype and the capacity for TH synthesis. Tier 2 assays incorporate organ-on-a-chip and organoid models that provide enhanced physiological relevance. This study evaluated the applicability of cryopreserved primary human thyrocytes as a 3D physiologically relevant *in vitro* model for multi-pathway screening of thyroid-disrupting chemicals (TDCs), using both T₄ and T₃ synthesis as an endpoint quantified by both ELISA and LC-MS/MS. **Methods:** Primary human thyroid epithelial cells (passage 1) obtained from healthy adult donors (≤55 years old; BMI ≤35) were cryopreserved in a serum-free bio-preservation medium. Cells from individual donors (n=3) were thawed in human thyrocyte plating medium (HTPM) and seeded on Matrigel-coated 96-well plates at 7,500 cells per well. Beginning on Day 2, cultures were maintained in an h7H-based medium supplemented with 1 mIU/mL bovine thyroid-stimulating hormone (hTSH). The effects of two reference TDCs, methimazole (MMI) and potassium hexafluorophosphate (KPF₆), known to decrease the T4 level in plasma by decreasing the TH synthesis in human were assessed using a 3D thyroid microtissue model in 96-well format. After a 6-day exposure (Days 8-14), medium samples were collected at day 14 for TH quantification via LC-MS/MS or ELISA. The limit of detection (LOD) for T₄ and T₃ by LC-MS/MS was 0.1 ng/mL and 0.05 ng/mL, respectively, while the ELISA sensitivity for T₄ was 0.33 ng/mL. **Results:** All three thyrocyte lots met quality criteria for TDC applications, forming uniform microtissues on Matrigel and synthesizing >1.0 ng/mL T₄ by Day 14. The cryopreserved thyrocytes maintained follicular morphology and T₄:T₃ ratios of >2.5. Both MMI and KPF₆ inhibited T₄ and T₃ synthesis in a dose-dependent manner. The average IC₅₀ values for T₄ were 0.16 μM and 1.49 μM, and for T₃ were 0.29 μM and 3.68 μM, respectively. IC₅₀ values for T₄ were consistent across LC-MS/MS and ELISA, demonstrating assay reliability. **Conclusions:** In summary, the primary human thyrocyte 3D microtissue assay using either ELISA or LC-MS/MS analytical approaches represents a promising strategy for evaluating and prioritizing potential TDCs in human health risk assessment. Moreover, the model enables the identification of compounds acting directly on the thyroid and supports simultaneous evaluation of multiple MIEs (e.g., TPO, NIS) for small-molecule screening in both agriscience and therapeutic research.

PS 4127 Bridging Physiological Accuracy and Reproducibility: The EpiVaginal Model Integrated with Simulated Vaginal Fluid

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Background and Purpose: The EpiVaginal model (MatTek), a 3D vaginal tissue based on a normal human ectocervical epithelial cells, has been developed as an *in vitro* alternative to the Rabbit Vaginal Irritation (RVI) assay to evaluate the safety of vaginal products. This highly differentiated 3D tissue model mimics the structural, physiological, and functional features of native vaginal mucosa and has been successfully applied in studies of vaginal irritation, inflammation, drug delivery, and microbicides. EpiVaginal aligns with the goals of New Approach Methodologies (NAMs) by providing a physiologically relevant, reliable, and reproducible assay for specific context of use for hazard and risk assessment of feminine products such as lubricants, hygiene products, microbicides, spermicides, and medical devices. While commercially available in the U.S. for years, the model was only recently introduced in Europe. In this study, we assessed reproducibility and robustness of manufacturing the tissue model by comparing ET-50 values, the exposure time required to reduce tissue viability by 50%, across two production sites (U.S. and Europe). ET-50 values serve as a standardized metric for batch-to-batch and site-to-site comparisons was used to evaluate the model's reliability and reproducibility. To further evaluate discriminatory power, we tested a panel of widely used vaginal ingredients, including exposures with Simulated Vaginal Fluid (SVF) to enhance physiological relevance. **Methods:** EpiVaginal tissues were produced simultaneously by MatTek in the U.S. and Europe (Bratislava). Tissues were produced from normal human vaginal ectocervical (VEC) cells obtained from healthy donors undergoing hysterectomy for benign conditions. Cells were cultured on 0.6 cm² inserts in serum-free medium for ~2 weeks, forming multilayered, highly differentiated 3D models that recapitulate native vaginal epithelial

structure. Each batch underwent QC testing via topical exposure to 1% Triton X-100 (positive control) for 0.5, 1, and 2 h to determine ET-50 values. Ultrapure water exposure for 1 h served as a negative control. ET-50 values between 0.70-1.70 h were used as acceptance criteria. We evaluated batch reproducibility for lots produced in 2024 in the U.S. (n=20) and 2024-2025 in Slovakia (n=31). Additionally, tissues produced at MatTek Europe were exposed to 100 μL of two concentrations of five feminine care ingredients for 24 h at both MatTek Europe and the National Institute of Public Health (Prague). Endpoints included MTT viability and TEER for barrier integrity. For selected test articles, ET-50 assays were conducted following 1, 4, and 18 h exposures, comparing cytotoxicity profiles with and without SVF. Irritation potential was evaluated based on MTT, TEER, and histology. **Results:** Across 51 lots (U.S. n=20; Slovakia n=31), all ET-50 values for 1% Triton X-100 met QC criteria (0.70 < ET-50 < 1.70 h). Following 24 h exposure to feminine care ingredients, the average difference in %MTT viability across eight test articles was 7.2%, with only two exceeding 10%. The average difference in %TEER was 6.4%, with two exceeding 10%. Toxicity profiles of Boric acid (6%), benzalkonium chloride (0.06%), and benzocaine (5%) showed ET-50 >18 h with minor SVF-related shifts (e.g., BZK: early TEER dip with partial recovery; BEN: gradual TEER decline). Miconazole (2%) exhibited a modest ET-50 right-shift (~17.0 → ~17.8 h). Nonoxonyl-9 (2%) caused early, severe loss of MTT/TEER regardless of SVF, whereas lactic acid (2%) seems protected by SVF (ET-50 >18 h vs <1 h) with higher MTT and partial TEER rescue. Overall, SVF buffered acidity-driven effects but not surfactant-type injury. **Conclusions:** ET-50-based QC criteria were reproducible across two laboratories in 51 production lots, confirming lot-to-lot and site-to-site consistency. Furthermore, SVF improved physiological relevance by mitigating acidity effects (e.g., lactic acid), highlighting its importance in irritation testing. The findings support EpiVaginal as a reliable non-animal alternative for screening feminine care products.

PS 4128 A Mechanistic Add-On to Human DNT NAMs for Detecting Endocrine Disruption via RAR and GR Signaling in Neural Progenitors

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Background and Purpose: Endocrine-disrupting chemicals (EDCs) pose a significant risk to the developing human brain, yet current regulatory frameworks lack tools to detect endocrine disruption-mediated developmental neurotoxicity (ED-DNT), particularly via non-EATS pathways such as retinoic acid receptor (RAR) and glucocorticoid receptor (GR) signaling. Both pathways reduce neural progenitor cell (NPC) proliferation, a key neurodevelopmental process, to enable terminal differentiation. While the human Neurosphere Assay, an established and scientifically validated component of the DNT *in vitro* battery (DNT-IVB), captures NPC proliferation, it cannot determine whether effects are hormone receptor-mediated or detect RAR/GR antagonism in the absence of physiological ligands. Here, we developed the NPC1_RAR_GR assay as a hormone receptor-specific add-on to the DNT-IVB, extending the NPC proliferation endpoint with mechanistic confirmation of hormone receptor involvement. **Methods:** The NPC1_RAR_GR assay uses a two-tiered design anchored in the DNT-IVB Neurosphere Assay methodology. Tier 1 quantifies NPC proliferation after single-compound exposure. Tier 2 determines whether Tier 1 effects are mediated through RAR or GR, and detects receptor antagonism that cannot be observed in Tier 1. To test agonism, compounds are co-exposed with selective antagonists for both receptors (AGN193109 for RAR, mifepristone for GR). A right-shift of the concentration-response curve indicates receptor-mediated agonism. To test antagonism, compounds are co-exposed with both receptor agonists (all-trans retinoic acid, dexamethasone). Antagonism is defined as a rescue of the anti-proliferative effects of the hormones above a predefined threshold. Seventeen environmental chemicals and a human biomonitoring-based prenatal mixture (MIX N) of seven urinary biomarkers were tested. Fetal brain concentrations for MIX N components were estimated using a human gestational PBTK model. **Results:** Model RAR and GR agonists and antagonists were reliably detected, confirming assay specificity. Among the 17 environmental chemicals, several organochlorine pesticides (DDT, aldrin, dieldrin) showed clear RAR/GR antagonism, a mode-of-action not detectable in the classical DNT IVB assay. Our results are confirmed by ToxCast RAR/GR receptor-level activity, however, for DDT at significantly higher benchmark concentrations compared to our cellular assay. Interestingly, the metabolite DDE showed only weak partial activity. Other DNT-IVB positives (e.g. cadmium chloride, tributyltin chloride, valproic acid) reduced NPC proliferation in tier 1 but were not rescued by co-exposure to antagonists, indicating non-RAR/GR mechanisms. The prenatal exposure mixture MIX N (BPA, BPF, MEP, MBzP, DPHP, TCP, and 3- PBA), tested at modeled fetal brain concentrations and up to 10 000× higher, produced no effects in tier 1 or tier 2. Consistently, all mixture components were inactive in ToxCast RAR/GR receptor-level assays except for weak activity of BPA and MBzP, neither of which translated into cellular responses. **Conclusions:** The NPC1_RAR_GR assay provides a mechanistically anchored, receptor-specific extension to the DNT *in vitro* battery, enabling differentiation between receptor-mediated and receptor-independent effects and detecting receptor antagonism, a mode-of-action not captured by standard DNT-IVB assays. Strong alignment with ToxCast RAR/GR receptor binding activity for OCP effects and absence of effects for a human biomonitoring-based prenatal mixture,

demonstrates both specificity and translational relevance. These findings support the NPC1_RAR_GR assay as a promising NAM for integration into regulatory ED-DNT assessment frameworks.

PS 4129 RosetteArray® Platform for Quantitative High-Throughput Screening of Human Developmental Neurotoxicity and Modeling of Neurodevelopmental Disorders

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Background and Purpose: Human pluripotent stem cell (hPSC)-derived neural organoids (hNOs) are prominent *in vitro* models of human central nervous system (CNS) development. Formed through spontaneous self-organization of neurally differentiating cell aggregates, hNOs recapitulate extensive aspects of CNS tissue morphogenesis. As such, they are excellent tools for toxicological screening, disease modeling, and drug discovery. Yet, emergence of their 3D structure and morphology is variable limiting their reproducible use as a scalable screening platform. Here, we present the RosetteArray® platform which enables scalable derivation of nascent forebrain (198.9 ± 11.6 per well) and spinal (793.6 ± 125.7 per well) hNOs with reproducible single-rosette structure in 96-well plate formats. Coupled with semi-automated confocal imaging and RosetteDetect™ software for artificial intelligence-based image analysis, the RosetteArray platform enables hNO-based quantitative high-throughput screening (qHTS) for assessing the risk of chemicals/drugs to cause human developmental neurotoxicity (DNT) or neurodevelopmental disorders. **Methods:** We present use of micropatterned culture substrates to standardize formation of 3D, nascent forebrain and spinal cord hNOs with consistent single-rosette structure (Knight et al. *ELife* 2018). 96-, 24-, and 6-well RosetteArray plates can be manufactured with micropatterned culture substrates as the plate bottom. Then, the plates can be used to standardize derivation of 100's-1000's of single-rosette hNOs per well from direct seeding of cryopreserved hPSCs or hPSC-derived neuromesodermal progenitors (NMPs, Lippman et al. *Stem Cell Rep.* 2015). After 8 (forebrain) or 5 (spinal) days of culture, RosetteArray assays are analyzed via standard fixation, immunostaining, and confocal imaging steps followed by image analysis using artificial intelligence-based RosetteDetect software to quantify salient metrics: # of DAPI+ cells per tissue; % of PAX6+ neural stem cells per tissue; % of tissues per well displaying a singularly polarized neural rosette structure. **Results:** To assess reproducibility and scalability, WA09 hPSCs seeded into 96-well forebrain RosetteArray plates, either manually or via a liquid handler, generated arrays with 95.46% ± 5.81 and 91.46% ± 5.81 (manual) or 91.83% ± 5.80 and 86.67% ± 6.84 (liquid handler) single rosette emergence efficiency under control conditions. Also, WA09-derived, cervical and lumbosacral spinal NMPs seeded into 96-well spinal RosetteArray plates generated 80.63 ± 8.00 or 94.38 ± 6.57 single neural rosette emergence efficiency, respectively, under control conditions. Second to assess suitability for DNT, dose-response assays were conducted with glycolic acid as a negative control and methotrexate, valproic acid, novobiocin, and benomyl, i.e., well-known Spina Bifida risk factors, as positive controls. Sensitive detection of toxicity was observed with differential profiles both across RosetteArray metrics and regions (forebrain vs. spinal). This was followed by screening a 30 compound library of pharmaceuticals and pesticides using forebrain RosetteArrays. The screen yielded 0.96 sensitivity and 1.0 specificity values with several chemicals demonstrating differential toxicity levels depending on whether they were pre-digested with S9-pooled liver fraction to simulate human metabolism. Lastly, the RosetteArray platform was used to model Spina Bifida risk caused by both chemical and genetic disruption of folate metabolism (Lundin et al. *BioRxiv* 2024, <https://doi.org/10.1101/2024.04.01.587605>) and Autism Spectrum Disorder risk caused by a SYNGAP1 genetic mutation (Birtele et al. *Nat. Neurosci.* 2023). **Conclusions:** Collectively, these results support the RosetteArray® platform's utility for qHTS of chemical DNT and modeling risk factors that cause neurodevelopmental disorders such as Spina Bifida and Autism Spectrum Disorder. Moreover, they motivate its inclusion in OECD's DNT *In Vitro* Testing Battery.

PS 4130 Innovative Assays for the Characterization of Seizure Liabilities

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Background and Purpose: A BMS program lead BMS-X caused unexpected, seizure-like adverse effects in toxicity studies in both mouse and dog. To identify alternate leads devoid of this liability, we have implemented multiple new approach methodologies (NAMs). The purpose is to leverage these assays to identify compounds with reduced risk for CNS Tox potential. **Methods:** A human-relevant *in vitro* multielectrode array (MEA) assay was established using stem cell-derived glutamatergic neurons. This assay captures key parameters of neural synchrony which are predictive of seizurogenic potential *in vivo*, and its implementation facilitates rapid program compound screen. Additionally, we have qualified and applied zebrafish larvae, a known neurotoxicity model, to assess compound toxicity in a whole organism with

an intact blood-brain barrier. Behavioral changes were recorded in parallel across the wells of a 96-well plate using Ramona's Multi-Camera Array Microscope, with videos captured at 90 fps. AI-based methods were then used to quantify behavioral endpoints such as distance traveled, speed, orientation, and tail position. **Results:** Leveraging both the MEA and zebrafish larvae assays, we have identified several internal program compounds that are non-excitatory at concentrations of up to 100 µM with *in-vivo* correlation. **Conclusions:** We have shown that these NAMs have greatly accelerated triage of program compounds prior to resource-intensive rodent studies without requiring compound scale-up, or program compounds.

PS 4131 Evaluating luminal-basal cellular plasticity using a dual transduced reporter MCF10A cell line

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Background and Purpose: The human breast epithelium is comprised of various cell types, with 2 major defining cell types being luminal and basal cells. These cells express cytokeratin intermediate filaments in a cell type-specific manner. Luminal cells are characterized by the expression of keratin 8 (KRT8) and basal cells are defined by keratin 14 (KRT14). Onset and progression of cancer is marked by loss of cellular identity and cellular plasticity, now an established hallmark of cancer. This cellular plasticity could be further accentuated by exposure to common environmental chemicals. We developed a lentivirus-based reporter cell line in non-tumorigenic MCF10A human breast epithelial cells where the KRT8 promoter drives GFP expression and the KRT14 promoter drives RFP expression. We treated these cells with common environmental toxicants to study their effect on cellular plasticity. Morphological and cell state perturbations could point towards potential mode-of-action for chemicals with undetermined mechanisms. **Methods:** MCF10A cells were plated in 6-well plate at 30% confluency. Cells were sequentially transduced with KRT8-GFP lentivirus and positive cells were selected with puromycin. KRT14-RFP lentivirus was added to puromycin selected cells, and positive cells were selected using hygromycin. Dual fluorescent cells were cultured and sorted using flow cytometry and bulk RNA-seq was done to confirm KRT8+ and KRT8+/KRT14+ expression. To study the perturbation in cellular populations, cells were dosed with TGFβ-1, a known EMT inducer, at 24, 48, 72 and 96 hours with doses ranging from 3.9pM to 2µM. To study the effect of some common environmental chemicals, cells were dosed with cadmium chloride, copper chloride, p,p-DDE, lead acetate and PFOS with doses ranging from 0.03µM to 99µM for 96 hours. Cells were fixed and stained with Hoechst33342, a nuclear stain imaged on a CX5 or CV8000 high content imaging microscopes. A cell segmentation and feature extraction pipeline was setup in Cellprofiler 4.2.1. Raw data was imported in R and was normalized using the Cytominer package. Cells were classified as KRT8+, KRT14+, KRT8-/KRT14- and KRT8+/KRT14+. Generalized linear models were used to compare KRT8 and KRT14 intensities for all chemicals across concentrations. **Results:** Flow cytometry data revealed that the reporter cell line contained ~93% cells in the KRT8+/KRT14+ state. 6% cells were KRT8+ and ~1% cells were KRT14+. After TGFβ-1 treatment, an increase in KRT14+ and KRT8+/KRT14+ populations was observed at 24 and 48h. KRT14+ only population showed an increase at 72h, with a decrease in KRT8+/KRT14+ cells. At 96h, an increase in KRT8-/KRT14- population was seen. When dosed with cadmium chloride, a decrease in KRT8-GFP intensity at doses above 160nM and an increase in KRT14-RFP intensity was observed at doses above 2.56µM. Copper chloride exposure at 2.6µM and 6.4µM showed a decrease in KRT8-GFP intensity and an increase in KRT14-RFP intensity. At 1.04µM p,p-DDE, we observed an increase in both KRT8-GFP and KRT14-RFP intensities. Interestingly, lead acetate showed a decrease in KRT8-GFP intensity at concentrations tested, and an increase in KRT14-RFP intensity at 2.56µM and doses at and above 16µM. Cells exposed to PFOS concentrations from 80 to 400nM exhibited an increase in both KRT8-GFP and KRT14-RFP intensity. **Conclusions:** A stable dual transduced luminal-basal cell line was generated to study cellular plasticity in non-tumorigenic MCF10A cells. An increase in a KRT14+ population and the loss of KRT8+ cells with TGFβ1 treatment for a shorter duration demonstrated TGFβ1's effect on modulating cytokeratin filaments and the responsiveness of this line to this signaling molecule. We found that common environmental toxicants like cadmium chloride, lead, p,p-DDE and PFOS can modulate luminal-basal cellular plasticity. An in-depth analysis of other parameters like cell morphology, fluorescence is ongoing to differentiate between the established cell population categories and understand functional impacts of toxicant-induced changes in expression of markers of cell state.

PS 4132 Assessment of a complex PAH-mixture from wildfires in a 3D respiratory model for asthma

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Background and Purpose: There exists a significant knowledge gap in understanding how non-chemical stressors, such as inflammation associated with pre-existing disease, interacts with chemical exposures to elicit distinct or exacerbated adverse

health outcomes compared to healthy populations. Prior studies employing a 3D respiratory model of type 2 allergic inflammation demonstrated unique functional changes and transcriptional alterations following exposure to a single polycyclic aromatic hydrocarbon (PAH), benzo[a]pyrene, indicative of altered susceptibility to chemical-induced toxicity under inflammatory conditions. This study will be among the first to evaluate the toxicological effects of an environmentally relevant wild-fire-creosote mixture of PAHs (ToxMix) using a 3D respiratory model for asthmatic inflammation. **Methods:** To compare mechanisms of toxicity between normal and asthmatic phenotypes, primary human bronchial epithelial cells (HBEC) were cultured in 3D at the air-liquid interface (ALI) in the presence or absence of 10 ng/mL interleukin-13 (IL-13) to induce the asthmatic phenotype. Fully differentiated cells at the ALI (25 days) were treated with 0 - 275 μ M ToxMix for 24 hours and collected for analysis of cytotoxicity, barrier integrity and global gene expression by RNA-seq. Expression of mRNAs after ToxMix exposure were normalized to their phenotypic controls and significantly differentially expressed genes (DEGs; q -value<0.05) were identified in order to begin investigating how different processes were affected by ToxMix in each phenotype. **Results:** While no increase in cytotoxicity was observed with treatment in the asthmatic phenotype, barrier integrity was significantly decreased (p -value<0.05) with ToxMix treatment across all concentrations in the IL-13 induced asthmatic phenotype. In the normal phenotype, ToxMix treatment had no significant impact on cytotoxicity or barrier integrity. With regard to global gene expression, more DEGs were identified after ToxMix exposure in the IL-13 induced asthmatic phenotype compared to ToxMix exposure in the normal phenotype. Across three concentrations (11 μ M, 55 μ M, and 275 μ M) of ToxMix exposure, 2,615 DEGs were identified in the IL-13 induced asthmatic phenotype compared to only 88 DEGs in the normal phenotype. Similar trends were observed when evaluating biological processes regulated within each phenotype. The most dysregulated pathways in the IL-13 induced asthmatic phenotype included cell adhesion processes and regulation of the epithelial-to-mesenchymal transition. **Conclusions:** Overall, these data are among the first to provide insight into how exposure to environmentally relevant PAH mixtures may elicit different responses between normal and asthma-like phenotypes.

PS 4133 Development of *in-vitro*-to-*in-vivo* extrapolation (IVIVE) approaches for PFOA and PFHpA

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Background and Purpose: Perfluoroheptanoic acid (PFHpA) was detected in approximately 20% of public water systems in California, including sites near airports and landfills. Due to limited animal toxicity studies, we used *in vitro* transcriptomic and *in-vitro*-to-*in-vivo* extrapolation (IVIVE) approaches to derive a transcriptomic point of departure (tPOD_{IVIVE}) for PFHpA and compared it to animal toxicity PODs. As a control, the same analysis was first applied to well-characterized perfluorooctanoic acid (PFOA). Mass-balance modeling was incorporated to refine the IVIVE. **Methods:** The Reardon *et al.* (2021) primary human hepatic spheroid transcriptomic dataset was analyzed for gene- and pathway-level responses, with raw counts normalized and processed using BMDExpress to derive benchmark concentrations (BMCs) and tPODs. Two *in vitro* mass balance models (IV-MBMs) - EQP v2.0 (Armitage *et al.*, 2021) and Qin *et al.* (2024) - were evaluated to translate nominal concentrations to free or cellular metrics; model performance was validated using PFAS concentration data from HepaRG cells. The best-performing IV-MBM was used to adjust tPODs for the estimated fraction unbound. Five IVIVE approaches were then tested (using PFOA as a test chemical) to convert tPODs to plasma-equivalent concentrations: assuming free *in vitro* PFOA concentration equals (1) free or (2) total plasma concentration, (3) estimated PFOA cellular concentration equals PFOA liver concentration, (4) estimated cellular protein-bound PFOA concentration equals liver protein-bound PFOA concentration, and (5) a novel extrapolation method based on estimated binding to fatty acid binding protein 1 (FABP1). Resulting tPOD_{IVIVE} values were compared to available human and animal toxicity PODs, and the best-performing approach was applied to PFHpA. **Results:** Seventy-eight and 123 genes passed BMC filters at 1 and 10 days of exposure to PFHpA, respectively, with median BMC 95% lower bound values (BMCLs) of 3.2 and 0.8 μ M. GO_BP and Reactome pathway analyses identified xenobiotic response as the most sensitive pathway at 10 days (BMCL = 0.5 μ M). The EQP v2.0 (Armitage *et al.*, 2021) model exhibited the lowest bias (-0.83 vs 3.91 nmol/mg protein) and predicted that 99% of PFHpA was free in media. Applying the five IVIVE approaches to the PFOA pathway BMCL produced tPOD_{IVIVE} values spanning three orders of magnitude, with approach 2 yielding values closest to available serum-based human and animal PODs - a human POD for alanine aminotransferase and an animal POD for relative liver weight. Approach 5 was the next most consistent. Application of approach 2 to PFHpA yielded gene- and pathway-based tPOD_{IVIVE} values of 0.8 and 0.5 μ M, respectively, which were one to three orders of magnitude below mouse serum-based PODs for relative liver weight and hepatocellular hypertrophy. **Conclusions:** Selection of IVIVE approach has a profound impact on the magnitude of tPOD_{IVIVE}. Approach 2 - commonly used in the literature - produced values most consistent with *in vivo* PODs. The next most consistent was approach 5, which incorporates binding to FABP1. Considering additional binding proteins, such as bile acid sequestering proteins, may further align tPOD_{IVIVE} values with *in vivo* PODs.

PS 4134 Automated Generation of 3D iPSC-Based Cardiac Microtissues for High-Throughput Calcium Transient Screening

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Background and Purpose: Transition towards *in vitro* compound screening for safety and efficacy in preclinical drug development needs physiologically relevant test systems reflecting complex biology while allowing for higher throughput applications. While iPSC technology integrates human biology, standard 2D high-throughput assays are often lacking complexity, whereas most 3D models are not allowing sufficient throughput. We have developed a physiologically relevant 3D iPSC-based cardiac microtissue (MT) model that is assembled and maintained by a robotic system and can be used for high-throughput measurement of calcium transients. **Methods:** MTs were assembled in 384w plates using a Tecan Fluent robot by combining 70% ventricular cardiomyocytes, 15% endothelial cells, and 15% cardiac fibroblasts differentiated from either a control iPSC line, or an iPSC line harboring a RyR2 mutation recapitulating a phenotype of catecholaminergic polymorphic ventricular tachycardia (CPVT). MT formation, media changes, and addition of reagents were performed by a Tecan Fluent robotic system. After 14 days of culture, MTs were loaded with FLIPR6 calcium dye and transferred to a FDSS μ Cell imager for subsequent compound addition and recording of calcium transients. **Results:** Automated formation of MTs resulted in inter-well variability of MT volume <5%, with an increase in diameter (305-375 μ m) from d1-d7 after formation. Calcium transient amplitude and peak width was comparable in several batches of MTs at day 14 after formation with an intra-well variability <10%. 0.1 μ M nifedipine reduced calcium transient peak amplitude by 42.2 $\pm$ 17.22 %, and peak rate by 11.9 $\pm$ 50.3%. 1 μ M BayK8644 induced an increase in peak amplitude of 43.4 $\pm$ 46.6%, and a decrease in peak rate by 4.5 $\pm$ 6.9%. 1 μ M Isoproterenol induced both a positive chronotropic (67.3 $\pm$ 11.1% increase in peak rate) and inotropic (45.7 $\pm$ 7.7% increase in peak amplitude) effect. To demonstrate the suitability of the system for pharmacological screening in a disease model, MTs generated from the CPVT line were treated with 2000 compounds from the Specs Repurposing Library. The screen revealed several hits with known mode-of-action against the disease phenotype, as well as compounds whose therapeutic role is currently under investigation. The average Z' factor across the screen was 0.6. **Conclusions:** Our 3D cardiac microtissue model can be assembled and maintained at scale in an automated process, displays robust calcium handling properties and is suitable for high-throughput screening in safety / tox studies. Microtissues generated from a CPVT iPSC line recapitulated the phenotype, which could be rescued using clinically relevant compounds, demonstrating that the platform can be leveraged to advance cardiac drug screening.

PS 4135 Evaluation of Isogenic Human iPSC-Derived 3D Cardiac Spheroid Co-culture Model for Predictive High-Throughput Cardiotoxicity Assessment

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Background and Purpose: The adult human heart is a complex organ providing highly regulated processes of pumping blood throughout the body. Heart tissue contains various types of cells, including (but not limited to) cardiomyocytes, endothelial cells, smooth muscle cells, and fibroblasts. Although cardiomyocytes may occupy about 75% of the total volume, they only constitute 40-50% of the total cell count. Recent publications show that 3D cardiac spheroids (aka, microtissues or "cardiospheres") enhance the maturation and functional activity of the cardiomyocytes compared to 2D cultures, thus more closely mimicking actual heart physiology. In this study, we developed a tri-culture spheroid model by mixing human iPSC-derived cardiomyocytes, cardiac fibroblasts, and endothelial cells from the same donor to generate 3D cardiac spheroids, aka, "cardiospheres". **Methods:** All human iPSC-derived cell types and optimized media formulations were from FUJIFILM Cellular Dynamics, Inc. Individual cell types were thawed and combined in defined ratios (65:15:20 of CM:CF:EN) and cultured in ultra-low attachment (ULA) plates. Typically, 5-10K cells per well were used in 96- or 384-well format and spheroids formed within 48 h. The structures started to contract spontaneously and regularly after 4-5 days. Spheroid formation (size and morphology) was tracked over time using live cell imaging in cultures maintained under serum-containing and serum-free conditions. Additionally, different cell types in the tri-culture system were immunostained using antibodies for Troponin T (cardiomyocytes, CM), COL1A1 (cardiac fibroblasts, CF), and VE-Cadherin (endothelial cells, EN). Spontaneous beat rate activity of 3D cardiospheres was investigated by recording the calcium oscillations on a high-throughput kinetic screening instrument (i.e., FLIPR Penta or FDSS/ μ Cell). Pharmacological modulation was performed by exposing the cells to a set of known small molecule inotropes, including isoproterenol (1-500 nM), dobutamine (0.01-10 μ M), epinephrine (1-300 nM), and Bay-K 8644 (0.01-0.3 μ M). Cell viability was also assessed following treatment with a panel of compounds with known cardiotoxicity, including doxorubicin, sunitinib, staurosporine, and others. **Results:** Importantly, we observed significant increases (approx. 2-fold) in the oscillation rate or peak count (positive chronotropic response) and increased the peak amplitude 1.2-1.5-fold (positive inotropic response) with compounds like isoproterenol. Other cardioactive drugs, including HERG blockers E-4031 (1-100 nM) and dofetilide (0.1-30 nM) and ion channel blockers nifedipine (10-300 nM) and mexiletine (0.550 μ M) demonstrated changes in the calcium oscillation patterns consistent with expected mode of action. Compounds

with previously demonstrated cardiotoxicity resulted in decreased cell viability, albeit with some differences compared to 2D format or 3D mono-culture. **Conclusions:** The work demonstrates the value and biological relevance of using human iPSC-derived cell types assembled into 3D cardiac spheroids compared to traditional 2D cardiomyocytes cultures. The 3D model represents a promising *in-vitro* model for drug discovery screens and preclinical screening, enabling high-throughput assessment of human cardiac microtissues

PS 4136 Human Liver-on-a-Chip Systems for Enhanced Mechanism-Based Toxicity Screening

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Background and Purpose: High-quality hepatocytes are essential for *in vitro* toxicology studies because they recapitulate critical liver functions, including metabolism, detoxification, and drug response. Their integration into advanced model systems improves prediction of drug-induced liver injury (DILI), reduces reliance on animal models, and enables early identification of hepatotoxic compounds. This study evaluated cryopreserved HepatoXcell™ Pro hepatocytes within a liver-on-a-chip platform under dynamic, physiologically relevant conditions, aiming to establish a robust system for predictive toxicology and pharmacokinetic assessment. **Methods:** Primary human hepatocytes (HepatoXcell™ Pro) were incorporated into a liver-on-a-chip system featuring 3-D liver tissue architecture within a dynamic fluidic environment. The platform was optimized for hepatocyte viability and function, enabling measurement of liver-specific markers, toxicity-related enzymes, and pharmacokinetic parameters. Standardized protocols were developed to assess drug-induced cytotoxicity via viability assays and enzyme activity profiling. System performance was benchmarked against qualification guidelines for preclinical toxicology models. **Results:** The liver-on-a-chip platform maintained hepatocyte morphology and function under dynamic flow conditions. HepatoXcell™ Pro exhibited stable expression of liver-specific markers and metabolic enzymes, supporting accurate assessment of hepatotoxicity. Toxicity testing with therapeutic compounds revealed dose-dependent cytotoxicity and changes in enzyme activity consistent with known DILI profiles. The platform demonstrated reproducibility across multiple runs, meeting qualification criteria for predictive toxicology applications. **Conclusions:** Integrating HepatoXcell™ Pro into a liver-on-a-chip system enhances predictive accuracy for DILI and supports mechanism-based toxicity screening in human-relevant models. This approach reduces reliance on animal testing and strengthens early-stage safety assessment, facilitating the development of safer therapeutic agents. Broad adoption of this technology could significantly advance translational toxicology and risk assessment in drug development.

PS 4137 Predicting PARP Inhibitor-Induced Bone Marrow Toxicity Using a Multi-Lineage *in vitro* Platform

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Background and Purpose: Poly(ADP-ribose) polymerase (PARP) inhibitors (PARPi) are vital therapeutics, but their use is often limited by dose-dependent toxicities, most notably neutropenia (hematotoxicity) and gastrointestinal (GI) adverse events. To better predict these risks early in drug development, we established a comprehensive, human-relevant *in vitro* platform to evaluate the toxicity profiles of various clinical agents, such as talazoparib, olaparib, and veliparib. **Methods:** Compound toxicity was assessed over relevant concentration ranges, using three human cell-based models. Granulocyte/macrophage and erythroid toxicity was quantified using human progenitor cells in both the gold-standard colony-forming unit (CFU) assay and a high-throughput HemaTox™ assay. Effects on GI liability were evaluated using a human intestinal organoid monolayer assay to measure cell viability and barrier function. **Results:** All tested PARPi demonstrated dose-dependent toxicity across the hematopoietic models. Both the CFU and the HemaTox™ assays produced a consistent hierarchy of toxicity: talazoparib > olaparib > veliparib. This *in vitro* ranking strongly validated the platform, as it accurately mirrored the reported differential clinical incidence and severity of neutropenia for these agents. The IC₅₀ values from the Erythroid and Myeloid HemaTox™ assay ranged from 0.0041 - 0.0066 µM for talazoparib, 0.61 - 1.44 µM for olaparib, and 2.1 - 9.5 µM for veliparib. Collectively, these results demonstrated the platform's suitability for comprehensive assessment of drug toxicity. **Conclusions:** The predictive capacity demonstrated in the hematopoietic models successfully correlates with the clinically observed hierarchy of PARPi-induced myelosuppression. These *in vitro* models provide a rapid, translational, and robust collection of tools for evaluating the differential multi-organ toxicity risk of novel therapeutics.

PS 4138 In-Vitro cytotoxicity assays using RTgill-W1 cells for Toxicity Identification and Reduction Evaluation (TIE/TRE) Strategies for Wastewater and Ambient Water Analysis

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Background and Purpose: Current fish toxicity testing methods are vital for the chemical registration and aquatic toxicity of pollutants dissolved in water. However, advancements with *in vitro* ecotoxicity techniques have continued to be highlighted for their ethical alternatives and more informative approach, such as providing insight into the Mode of Toxic Action (MoA). Specifically, these cell-based systems directly address the key aspects of the 3 R's (replacement, reduction, refinement), offering a screening tool that significantly decreases the reliance on live vertebrate toxicity tests. The rainbow trout (*Oncorhynchus mykiss*) gill cell line, RTgill-W1, has shown promise for incorporation into Whole Effluent Toxicity (WET) regulatory testing techniques. Moreover, while these New Approach Methods (NAMs) offer a promising animal alternative component, their primary strength lies in the utility of better understanding MoAs essential to Toxicity Identification/Reduction Evaluation (TIE/TRE) strategies, an aspect where current *in-vivo* assays are often deficient. **Methods:** The RTgill-W1 cells were maintained and cultured in Leibovitz's L-15 medium and utilized for cytotoxicity testing (Scott et al., 2023). For the assays, cells were seeded at a density of 79,000 cells per cm² in 24 multi-well plates. Cell viability was measured following a 24-hour exposure to effluent samples using the Multiple Endpoint Viability Assay, which was performed with sample adjustment (ISO 2019). This assay simultaneously measured three key endpoints: alamar Blue for Metabolic Activity, CFDA-AM for Membrane Integrity, and Neutral Red for Lysosomal Integrity. To evaluate the predictive capacity of the *in vitro* system, results were compared against traditional Fathead Minnow (FHM) larvae assays, which measured mortality at 96 hours and determined the lethal concentration 50% (LC50). The resulting cell effect concentration of 50% (EC50) values were directly compared to the LC50 values. Furthermore, various TIE treatments were systematically performed on the effluent samples to identify and remove toxicants, with cytotoxicity assays subsequently used to confirm the success of the removal process. **Results:** The RTgill-W1 cell line has been utilized to predict the mortality of acutely toxic effluents to fish larvae, covering a broad range of wastewater facility types, operations, and sample sources (i.e. pulp and paper production, agriculture, chemical production, food and beverage, and superfund sites). In this capacity, RTgill-W1 cell viability successfully predicted all toxic effluent samples, with six of these samples exhibiting greater sensitivity than the Fathead Minnow (FHM) larvae assays. Furthermore, studies have demonstrated the efficacy of TIE treatments (i.e., chemical addition, mechanical filtration and sample adjustments) in removing toxic effects to both fish and cells, and the TIE treatments were successfully applied and shown to remove toxicants from the tested effluents. This removal was subsequently confirmed through cytotoxicity assays which demonstrated a corresponding decrease in toxic effects when comparing measured subcellular endpoints before and after the TIE process. Additionally, RTgill-W1 cytotoxicity assays were able to highlight specific MoAs of wastewater samples, distinguishing between specific classes of toxicants based on sample type and subcellular endpoints. Ongoing research also indicates the promise of these cells for modeling key chronic exposure endpoints. **Conclusions:** Our results underscore the substantial utility of RTgill-W1 cells, highlighting their ability to effectively identify toxicants of concern relating to WET failures. This demonstrates that the cell line can be used for routine TRE treatments similar to established fish larvae tests, offering a fast, ethical, and mechanistically informative tool for environmental monitoring and regulatory compliance.

PS 4139 A New Approach Methodology for Hepatocyte Proliferation: EdU-Based High-Content Imaging in Human and Rodent Primary Cells

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Background and Purpose: Hepatocyte proliferation is a critical key event (KE) leading to adverse outcome (AO) to liver tumorigenesis, particularly in the context of chemical exposure in rodent. The mode of action (MOA) provides a mechanistic framework linking molecular initiating events (nuclear receptor activation) to downstream biological KEs that culminate in tumors. Determining human relevance of xenobiotic induced hepatocyte proliferation is essential for agrochemical risk assessment. Currently, there is no *in vitro* test guideline to address this KE. Building on prior applications of 5-ethynyl-2'-deoxyuridine (EdU) incorporation for assessing proliferative responses (e.g. Yamada et al., 2015), this work advances new approach methodologies (NAMs) by evaluating donor variability, time course and pregnenolone-16α-carbonitrile (PCN) as rodent-specific nuclear receptor control (to demonstrate specificity). This study aims to qualify rat and human hepatocytes and optimize *in vitro* method in hepatic proliferation following nuclear receptor activation using high-content imaging (HCI) for analysis. **Methods:** Cryopreserved primary human hepatocytes and pooled Wistar Han rat lots were plated on collagen-coated 4 chamber glass slides (3 well/slide). Dimethyl sulfoxide (DMSO) was the vehicle control (VC). Time-course comparison at 24 and 48 hours (h) used 2 rat lots and 2 human donors (1 slide/lot/donor/time point) with 200ng/mL HGF or PCN (5 or 20µM) as a pretest to assess onset of proliferation and select optimal timepoint. For main study, 4 human

donors (2 male; M and 2 female; F) and 6 rat lots (3M and 3F) were exposed to 2 doses of HGF (25 or 200ng/mL) or PCN (5 or 20μM) for 48 h (2 slides/lot/donor). Treatment began 6 h post-plating, repeated every 24 h, with 10μM EdU in final dose. After exposure slides were stained with 5μg/mL Hoechst. Images were captured using Opera Phenix Plus high content screening system with Harmony® v5.1 (Revvity, Waltham, MA). Percent EdU-positive nuclei were calculated as number EdU-positive cell divided by total Hoechst-positive/field. Data were expressed as fold-change (FC) relative to VC and evaluated with SAS Software. Data was transformed to meet the assumptions of statistical tests. The mixed-effects model used to provide estimates FC and confidence. Unpaired t-tests used to assess whether observed FC was significant than a threshold. Qualification was based on post thaw viability, morphology, and proliferation responses. **Results:** All 6 rat lots and 4 human donors had acceptable viability (>70%) and normal morphology. Average cell counts of 13382.6 per rat lot and 19824.3 per human donor were quantified, an order of magnitude higher to historical manual scoring standard 1000 cells typically used in EdU studies providing improved assay sensitivity. At 24 h (time course), treated hepatocytes did not demonstrate significant responses. In human, response was less than 2 FC for both HGF and PCN, while in rats the response was 1/2 donor achieved greater than 2 FC after HGF while both less than 1.5 FC after PCN exposure. At 48 h, human and rat showed greater than 2 FC increase in response to HGF. In contrast, PCN induced greater than 1.5 FC in rats but not in humans, as expected. Therefore 48 h (2 doses) was selected for main study. In the main study, analysis showed donor variability however, no sex-based differences were observed. Reproducible response greater than 2 FC following HGF exposure was seen in 5/6 rat lots and all 4 human donors. PCN response was greater or equal than 1.5 FC in 5/6 rat lots, whereas no human donors exhibited a response greater than 1.5 FC. Overall, these studies showed consistent donor variability without sex-based differences, with strong HGF-induced proliferation in rat and human cells and selective PCN response in rats. **Conclusions:** This work demonstrates the development of a quantitative, EdU-based primary hepatocyte proliferation assay. Confirmed donor variability and time point improving assay reliability. Incorporated PCN as alternative rodent specific nuclear receptor control supporting broader use in mechanistic liver toxicity assessments. The assay provides a foundation for screening reference chemicals to expand species comparability and support human relevance determinations. The results support development of human-relevant, non-animal methods for regulatory toxicology and risk assessment.

PS 4140 ATCC's Human-Relevant Cancer Models for Mechanism-Based Toxicity and Drug Response Profiling

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Background and Purpose: The Human Cancer Models Initiative (HCMI) is a global collaboration led by the National Cancer Institute (NCI) that aims to advance translational oncology by developing patient-derived cancer models. Traditional cell lines often fail to capture the complexity of human tumors, which limits their utility in drug discovery and precision medicine. HCMI addresses this gap by generating biologically relevant 2-D and 3-D models derived directly from patient samples, ensuring high genomic fidelity and clinical relevance. ATCC contributes to the initiative by developing, manufacturing, and distributing these models worldwide. The HCMI portfolio includes over 300 models spanning 28 tissue types, including colorectal, pancreatic, brain, and esophageal cancers, as well as rare malignancies such as Wilms tumor and Ewing's sarcoma. These models reflect diverse clinical diagnoses, stages, age ranges, and racial backgrounds, providing researchers with tools to study tumor heterogeneity and health disparities. Comparative analyses show strong concordance with The Cancer Genome Atlas (TCGA), retaining over 80% of oncogenic drivers and preserving transcriptional and epigenetic landscapes. The purpose of this work is to provide researchers with clinically relevant models that improve preclinical testing, support biomarker discovery, and enable precision oncology strategies. **Methods:** Organoids were cultured in 3-D extracellular matrix conditions using ATCC Organoid Growth Kits and passaged to form uniform structures prior to drug screening. Models were seeded into 96-well plates and treated with a panel of chemotherapeutic and targeted compounds using 12-point or 8-point dilution curves. Drug response was assessed via ATP-based luminescent viability assays and live-cell fluorescence imaging. Genomic characterization was performed using whole-exome or targeted sequencing, and data were compared to matched patient tumors and TCGA datasets to confirm model fidelity. Z' factor scores were calculated to validate assay robustness, enabling correlation of genomic alterations with drug sensitivity. **Results:** ATCC has expanded the HCMI portfolio to include 91 colorectal and over 54 pancreatic cancer organoids, each annotated with clinical, genomic, and drug-response data. Colorectal models, primarily adenocarcinomas, mirror TCGA mutation profiles and exhibit genotype-dependent drug sensitivity across 24 compounds. Pancreatic models similarly reflect key driver mutations (KRAS, TP53, SMAD4) and exhibit differential responses to targeted agents, including KRAS inhibitors. High-throughput assays demonstrated strong reproducibility (Z' >0.7), and models preserved patient tumor morphology and molecular features. Sequencing and clinical metadata are publicly available via the HCMI portal. **Conclusions:** The HCMI portfolio, distributed by ATCC, represents a next-generation resource for oncology research and toxicology. These patient-derived models enable mechanism-based toxicity screening in human-relevant systems, improving prediction of drug-induced cytotoxicity, off-target effects, and population-level variability. Their

genomic fidelity and 3-D architecture provide a robust platform for safety pharmacology and risk assessment. Ongoing expansion to include breast, glioblastoma, and ovarian cancer models will further strengthen translational toxicology and precision medicine efforts.

PS 4141 Primate NAMS : Development of Human and Cynomolgus Macaque iPSC-based Assay for *in vitro* Developmental Toxicity Evaluation of Protein Degraders

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Background and Purpose: Targeted Protein Degraders (TPDs), including Proteolysis Targeting Chimeras (PROTACs), represent a promising drug class capable of targeting otherwise "undruggable" proteins in cancer and other diseases. Despite their promise, developmental toxicity, including off-target toxicity remain key challenges, particularly degradation of critical developmental transcription factors such as SALL4, implicated in teratogenic outcomes associated with thalidomide analogs. Because of their close physiological and developmental homology to humans, nonhuman primates (NHPs) remain an essential animal model for evaluating developmental and drug safety profiles of TPDs. However, traditional *in vivo* NHP studies—particularly those involving pregnancy—pose significant ethical, temporal and financial barriers. To address this gap, we developed a New Approach Methodology (NAM) using Cynomolgus macaque (cyno) pluripotent stem cell model and a human pluripotent stem cell model to evaluate SALL4 degradation *in vitro*. These cross species, reporter based systems enable quantifiable, high content assessment of developmental toxicity prior to *in vivo* testing. **Methods:** Cyno and human pluripotent stem cells (PSCs) were first validated for SALL4 expression using immunocytochemistry and Western blot. We then generated SALL4-TdTomato reporter lines via AAV-mediated tagging of the SALL4 3' exon, enabling non-destructive, longitudinal tracking of protein degradation. Cells were exposed to known degraders (Thalidomide, Lenalidomide, and Pomalidomide) at varying concentrations. Degradation kinetics and EC₅₀ values were quantified via fluorescence imaging and proteomic validation using Data-Independent Acquisition (DIA) mass spectrometry. **Results:** SALL4 expression was robust and comparable across cyno and human PSCs. Both Western blot and reporter-based imaging confirmed dose-dependent SALL4 degradation following Thalidomide and Thalidomide analog exposure, with consistent EC₅₀ trends between species. Proteomic profiling verified SALL4 loss and revealed overlapping degradation signatures in cyno and human cells, supporting translational relevance of the model. Importantly, we observed that cell density influenced degradation efficiency, underscoring the value of dynamic, live-cell reporter assays over endpoint-based methods. **Conclusions:** This study establishes a scalable, ethically sustainable, cross-species NAM platform for evaluating developmental toxicity of TPDs. By integrating reporter-based readouts with global proteomics, our human-cynomolgus PSC system enables *in vitro* prediction of *in vivo* teratogenic risk, offering regulators and sponsors a translational bridge between human safety assessment and NHP data. The platform supports the 3Rs principles (Replacement, Reduction, Refinement) and provides a powerful preclinical tool for ranking compound safety prior to animal testing, further accelerating safer drug development of next-generation protein degraders.

PS 4142 The Utilization of *In Vitro*, Human on a Chip Cortical Networks to Assess Neurocognitive Impairment for Drug Safety and Efficacy

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Background and Purpose: Currently, the methods for assessing disease pathology to improve the prediction of compound efficacy and safety for neurodegenerative disorders are limited to animal sources. In an effort to minimize the need for animals and improve biological accuracy of drug effects, there has been an impetus for developing *in vitro*, human-based models, particularly for modeling difficult-to-source, complex neural networks. The development and validation of human-based functional platforms would not only lead to accelerated timelines for preclinical drug assessment at a fraction of the cost of developing and testing animal models, but also emphasize the importance of employing functional readouts relevant to clinical diagnoses cases that can be supplemented with biomarker assessment. To achieve this, long-term potentiation (LTP), a widely accepted *in vitro* correlate for memory and learning, was employed as the main readout of synaptic function in this study. Utilizing microelectrode array (MEA) and human, induced-pluripotent stem cell (hiPSC) technology, this study sought to validate the use of hiPSC-cortical LTP for drug efficacy and safety studies of anticholinergic cognitive burden (ACB). ACB is currently diagnosed through amalgamated categories 0-3 on the ACB scale, which possesses gaps in its reflection of the complexity of ACB-induced dementia, and does not consider attributions of co-occurring network dysfunction, such as mild-cognitive impairment (MCI). **Methods:** LTP was evaluated as synapse-dependent, persistent enhanced activity following high-frequency electrical stimulation. To assess functional neurotoxicity, anticholinergics, drugs classified into subcategories for severity of ACB induction, were tested at five concentrations to generate dose-ef-

fect curves for LTP. Anticholinergics from all classes of severity were administered directly onto a central nervous system (CNS) MEA comprised of cortical neurons and astrocytes. To determine the potential of synergistic impairment of ACB and an MCI phenotype, the systems were acutely co-exposed to exogenous amyloid-beta42 (Aβ42) oligomers and anticholinergics at nominal concentrations. **Results:** Dose-response curves for ACB-induced neurocognitive detriment were established between ACB drug concentration and acute LTP maintenance. Concentrations of ACB drugs spanning 2 log scales produced sigmoidal curves when plotted using the Hill equation. Co-exposure of nominal concentrations of anticholinergics and Aβ42 oligomers confirmed the combination induced a significant exacerbation of cognitive impairment. **Conclusions:** Drugs from various groups, classified as inducing no, low, moderate, or severe ACB on the 0-3 ACB scale, respectively, were assessed independently to provide insight to potential inconsistencies with the current clinical scale. Parameters from the Hill equation and dose-response curves provided more granularity in quantifying cognitive deficits, suggesting the need for reevaluation of the current diagnostic scale. Additionally, evaluating the synergistic contributions of ACB and MCI, co-occurring implications to cognitive preservation, underscored the need for smaller-scale platforms to not only assess the complexity of neural network health, but also that could operate at a fraction of the cost and timeframe for evaluating drug efficacy and potential functional toxicity.

PS 4143 Energy Healing Dampens PFOA-Induced Stress Responses in Lung Alveolar Epithelial Cells

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Background and Purpose: Perfluorooctanoic acid (PFOA), a persistent environmental pollutant, induces inflammatory and epigenetic alterations contributing to respiratory disorders. Reiki is widely practiced as a non-invasive complementary therapy to promote stress reduction and overall well-being, although robust experimental evidence for anti-inflammatory or epigenetic effects is limited in biomedical literature. This study investigates the effects of Reiki healing on PFOA-induced inflammatory responses. **Methods:** Human lung alveolar epithelial cells (A549) were divided into three groups: control, Sham Reiki (mock Reiki), and Reiki healing. Each group included a DMSO (control) and 100 nM PFOA (24h)-exposed sets. The Reiki healing group received four healing sessions (one in-person and three distant sessions of 10 min each) from a certified practitioner, while the Sham Reiki group underwent mock sessions mimicking the real Reiki, but without actual intent. The in-person sessions were observed and incorporated hand placements and symbolic practices. While the distant healing was performed remotely in three sessions, each separated by six hours. Inflammatory markers (e.g., NF-κB, STAT3, IL-17, NLRP3), epigenetic regulators (Polycomb Repressive Complex components), membraneless organelle markers (G3BP1, TIA1, DCP1A), cytokines and chemokine levels (IL-6, IL-8, CCL-2, CCL-5) were assessed using qPCR and ELISA. **Results:** PFOA treatment significantly elevated the studied inflammatory mediators and cytokine and chemokine production compared to the DMSO-treated group, in control and Sham Reiki groups. In contrast, PFOA exposure was unable to mount the expression of any inflammatory mediator, and cytokine/chemokine production in the Reiki healing group. **Conclusions:** Reiki healing demonstrably rescues PFOA-induced activation of inflammatory signaling, stress granule marker expression and altered epigenetic signatures in lung epithelial cells. These findings provide strong support for Reiki as a viable complementary strategy to reduce environmental pollutant-related lung inflammation/toxicity and highlight the need for further translational and clinical validation.

PS 4144 High Throughput Assessment of Barrier Function Using Human iPSC-Derived Brain Microvascular Endothelial Cells

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Background and Purpose: The blood-brain barrier (BBB) is composed of specialized brain microvascular endothelial cells (BMEC) that serve to regulate the flow of substances into and out of the brain. BMEC have physical, transport, and metabolic properties that are regulated by other vascular, immune, and neural cells to create a tightly controlled microenvironment of the central nervous system. Understanding how BMECs work alone and in concert with these other cell types is essential to understand how the brain functions during health and disease. One of the key functional features of BMEC is barrier formation, and the strength and integrity of this barrier can be evaluated via measurement of the electrical resistance across the cell layer. In this study, the barrier function properties of human iPSC-derived BMEC were measured using different platform technologies, such as impedance and trans-endothelial electrical resistance (TEER). **Methods:** Human iPSC-derived brain microvascular endothelial cells (iCell® Brain Microvascular Endothelial Cells) and all media with supplements were from FUJIFILM Cellular Dynamics. These cells were differentiated similar to previously published protocols from an apparently healthy normal (AHN) male donor 01279. Development of TEER assays to measure the barrier function of BMEC were performed using 24-well or 96-well cell culture inserts (Corning) coated with Collagen-IV and Fibronectin (Sigma). Lucifer Yellow was used to measure permeability in these cell culture inserts. To increase the throughput of TEER measurements, iCell Brain Microvascular Endothelial Cells

were also plated on CytoView-Z plates and signal was recorded using the Maestro Z (Axion Biosystems). This technology monitors cell coverage (or confluence) as a resistance at high frequency (41.5 kHz) and a very sensitive TEER at a low frequency (1 kHz). These properties were monitored continuously over the course of 10 days. **Results:** Data from culture inserts demonstrated consistent TEER >1500 Ω·cm² measured using standard 96-well STX HTS EVOM™ Electrode. They also demonstrated low permeability to Lucifer Yellow on day 5 in culture. Culturing iCell Brain Microvascular Endothelial Cells on the CytoView Z plates enables measuring real-time traces of impedance over time and demonstrated lot-to-lot consistency of iCell Brain Microvascular Endothelial Cells with uncorrected TEER resistance values of ~3500 Ω at 140 hours. This assay was used to profile compounds in 384-well format (n=4 wells; 8-point dose-response curve) that are known to disrupt the barrier and decrease TEER after exposure to the cells, including bupropion, mannitol, prazosin, VEGF, and verapamil. **Conclusions:** Characterization of and testing with the individual cellular components that make up the blood-brain barrier (BBB) provides added insight to the functional aspects of this complex system. Human iPSC-derived BMEC offer a robust and reliable source of cells to interrogate the multiple properties of this specialized cell type.

PS 4145 Acute Polyamide Micro- and Nanoplastic Aerosol Exposure Alters Ciliary Motility in Primary Human Bronchial Epithelial Cells

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Background and Purpose: Micro- and nanoplastics (MNPs, <5 μm), such as polyamides (PA), are increasingly detected in ambient air and human lung tissues, often carrying co-contaminants like lead (Pb). At the same time, an increasing incidence of chronic respiratory diseases was observed worldwide. However, MNP effects on mucociliary function remain poorly understood. We examined the acute effects of PA-12 and Pb-bound PA-12 aerosols on mucociliary integrity and function in primary human bronchial epithelial cells (HBECs) differentiated at the air-liquid interface (ALI). **Methods:** HBECs from three non-smoking female donors (mean age: 52 ± 24 years) were differentiated for 21 days at ALI, and exposed for 4 h to filtered air (FA), PA-12 aerosols (11.6 ± 0.5 mg/m³; geometric mean diameters-GMD: 0.3 μm and 3.7 μm), or Pb-bound PA-12 aerosols (11.4 ± 1.3 mg/m³; GMD: 0.3 μm and 3.7 μm). Following exposure, cells were incubated for an additional 40 h before assessment (post-aerosol exposure). Endpoints included cell viability, lactate dehydrogenase (LDH) release, mucus secretion (MUC5AC), ciliary beat frequency (CBF), ciliary length, and gene expression related to ciliogenesis (*FOXJ1*, *DNAH5*), epithelial signaling (*EGFR*), inflammation (*CXCL8*, *NFKB*), oxidative stress (*NFE2L2*), and mucus production (*MUC5AC*). **Results:** Neither PA-12 nor Pb-bound PA-12 aerosols significantly affected HBEC viability, LDH release, or MUC5AC secretion at 40 h post-aerosol exposure, indicating an absence of acute toxicity or mucus hypersecretion. In contrast, CBF exhibited a transient, statistically significant increase at 20 h post-exposure ($p < 0.05$) before returning to baseline at 40 h ($p < 0.001$). Cilia length remained stable ($7.1 \pm 0.8 \mu\text{m}$ at 40 h) and no statistically significant effect on gene expression were observed. **Conclusions:** Acute PA-12 aerosol exposure transiently enhances ciliary motility without inducing significant epithelial damage. This data suggests functional modulation of mucociliary clearance as a key early defense mechanism to acute exposure to inhaled MNPs. Further studies examining the effects of repeated exposures are necessary to elucidate long-term effects of MNP aerosol exposures on human airways.

PS 4146 Is there something simpler than EASZY for zebrafish estrogenicity detection?

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Background and Purpose: Early identification of estrogenic activity is essential in chemical development to prevent the advancement of endocrine-disrupting compounds and ensure regulatory compliance. While the OECD test guideline 250 (EASZY assay) provides a validated embryo-based screen for estrogen receptor (ER) agonists, it has limitations related to imaging complexity and tissue specificity. This project aims to develop a high-throughput transgenic zebrafish assay, using the transgenic 5XERE:GFP line to detect both ER agonists and antagonists. This approach enhances screening efficiency and provides mechanistic insights into estrogenic activity. **Methods:** Embryos from the Tg(5XERE:GFP) zebrafish line were exposed to five concentrations of each compound—17α-ethinyl estradiol, 17β-estradiol, 17α-estradiol, diethylstilbestrol, bisphenol A, flutamide, dexamethasone, dibutyl phthalate, pentachlorophenol, norethindrone, 4-tert-octylphenol, tributylchlorostannane, octamethylcyclotetrasiloxane—repeatedly at 3 and 4 dpf. At 5 dpf, the fluorescence of anesthetized larvae in 96-well plates was measured using a plate reader (Ex: 480 nm, Em: 509 nm), and data was used to determine the lowest concentration inducing GFP expression (lowest effect level; LEL). In addition, an estrogenic index was calculated as the ratio of the effective concentration causing 25% embryo mortality or malformation (EC25) to the LEL. **Results:** Estrogenic activity of the tested compounds was quantified using an estrogenic index, defined as the ratio

of the effective concentration (EC25) to the lowest concentration inducing estrogen receptor (ER) activation. Preliminary results showed strong concordance with established ToxCast ER model scores. Specifically, compounds such as TBTC, dexamethasone, dibutyl phthalate, flutamide, D4, and pentachlorophenol exhibited no ER activation. Bisphenol A and 4-tert-octylphenol showed intermediate activation, while potent ER activation was observed for 17 β -estradiol, 17 α -ethinyl estradiol, 17 α -estradiol, and diethylstilbestrol. **Conclusions:** Based on preliminary findings, the 5XERE:GFP zebrafish assay represents a compelling alternative to established estrogen receptor activity screening methods. It offers reduced hands-on time and accelerated data acquisition while maintaining high levels of accuracy and sensitivity. These attributes position the assay as a valuable tool for efficient and reliable endocrine activity assessment in early-stage safety evaluations.

PS 4147 High-Precision Neurotoxicity and Neuropharmacology Assessment in CNS Models Using Field Potential Imaging

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Background and Purpose: A detailed understanding of neural circuit dynamics is essential for advancing drug discovery, neuropharmacology, and toxicity assessment. Recent advances in microelectrode array (MEA) technology, particularly the emergence of ultra-high-density CMOS-MEAs, now enable simultaneous recording of large-scale neuronal activity with single-cell resolution. This study aimed to establish a multiscale field potential imaging platform for central nervous system (CNS) models using a CMOS-MEA containing 236,880 electrodes. **Methods:** Human iPSC-derived cortical neurons, primary cultured rat hippocampal neurons, and acute rat brain slices (hippocampus and neocortex) were prepared on the CMOS-MEA. Electrophysiological activity was recorded before and after compound administration. Network-level and single-neuron parameters were quantified in iPSC-derived neurons. In rat primary neuronal cultures, network organization was further assessed using a graph theory-based connectivity analysis. In hippocampal-cortical slices, large-area recordings enabled mapping of region-specific activity propagation. **Results:** In human iPSC-derived cortical neurons, integrated analyses revealed previously inaccessible features of compound responsiveness, including modulation of synaptic strength and alterations in network bursting characteristics. In primary cultured rat hippocampal neurons, connectivity analysis demonstrated that pharmacological perturbation with picrotoxin and muscimol induced distinct reconfigurations of functional connectivity, accompanied by clear changes in firing patterns and propagation dynamics. These connectivity-based signatures identified shifts in excitatory-inhibitory balance that were not detectable through firing-rate-based metrics alone. In acute hippocampal-cortical slices, carbachol administration enhanced beta-band oscillatory power in hippocampal circuits and induced coordinated activity transitions in neocortical regions, demonstrating the system's ability to resolve drug-dependent changes in oscillatory activity and propagation dynamics *ex vivo*. **Conclusions:** Ultra-high-density CMOS-MEA-based field potential imaging enables multiscale characterization of CNS neural circuits across human iPSC-derived neurons, primary rodent neurons, and brain slices. Integrating high-resolution recordings with propagation-based and connectivity-based analyses provides a powerful framework for detecting subtle compound effects, dissecting neural circuit mechanisms, and supporting high-precision neurotoxicity evaluation.

PS 4148 Repeated daily exposure to acetic acid attenuates of the induction of *in vivo*-relevant *in vitro* markers of respiratory irritation in a donor-dependent manner.

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Background and Purpose: In addition to a range of industrial, commercial, and household applications, acetic acid is commonly found in personal care products where it is typically used to support fragrance stability, solubility, and pH control. It is also an essential component in the manufacturing of many floral and fruit scents used in fragranced products. Fragrance-related exposures to acetic acid via the inhalation route of exposure can occur during both the manufacturing of fragrances as well as the use of the finished product. In either case, exposures can occur daily. Here, we sought to define the effects of repeated daily exposures to a range of acetic acid concentrations across a battery of *in vivo*-relevant *in vitro* endpoints using a biologically complex air-liquid interface (ALI) model of the human nasal epithelium. **Methods:** Donor-matched differentiated primary human nasal epithelial cell (dphNEC)/primary human nasal fibroblast (pHNF) co-cultures (Epithelix MucilAir-HF Nasal) from five individual donors were exposed to a range of acetic acid concentrations daily for two hours for three or five days. The effects of exposures were evaluated for cytotoxicity, epithelial barrier integrity by trans-epithelial electrical integrity (TEER), ciliary beat frequency (CBF), percent active ciliated surface area (PACSA), apical release of mucin 5AC (MUC5AC), and basolateral secretion of the pro-inflammatory cytokines interleukin (IL)-8, IL-6, and tumor necrosis factor alpha (TNF α) over five daily exposures. **Results:** Acetic acid exposures resulted in mild cytotoxicity at 120 ug/cm² that peaked after the first exposure and was progressively attenuated

following repeated exposures. Epithelial barrier integrity was significantly increased following both Day 3 and Day 5 of exposures at 120 ug/cm² with greater TEER following Day 5 than Day 3. While CBF was not affected, PACSA was significantly reduced following treatment with 120 ug/cm² following both Day 3 and Day 5 exposures. Apical secretion of MUC5AC decreased following Day 3 and Day 5 exposures to 120 ug/cm² acetic acid. Secretion of IL-8, IL-6, and TNF α increased following the Day 3 exposure to 120 ug/cm². IL-8 and TNF α were increased to a lesser extent following Day 5 exposure and IL-6 was not significantly altered. While acetic acid exposures typically resulted in statistically significant contributions to observed variation in endpoint measurements, inter-individual differences also significantly impacted variation across all endpoints. TEER, CBF, MUC5AC secretion, and IL-6 release were affected more by inter-donor differences than acetic acid concentration. **Conclusions:** Considered together, the observations reported here indicate that repeated exposures cause an adaptive effect that results in the attenuation of induction of *in vitro* indicators of respiratory irritation such that the magnitude of exposure-induced changes following five exposures is lower than following three exposures. Thus, an increase in the number of exposures progressively reduced the ability of the tissue to respond to irritant insult, resulting in outcomes similar to those observed following repeated exposures to the respiratory irritant ozone in humans and animal models. These observations may also indicate that repeated acetic acid exposures result in tissue-level adaptation that involves loss of ciliated and goblet cells and remodeling of the epithelium in a manner that reduces its susceptibility to the cytotoxic and pro-inflammatory effects of repeated acetic acid exposures. This can be confirmed with histopathological analyses of the tissues. Further, the observation that contribution of inter-individual differences to the observed variation in endpoint data often exceeds that of a known irritant highlights the importance of improving our understanding of the role of donor variation in *in vitro* exposure outcomes. Accomplishing this will support the rigor of study designs, define key variables in the test system, and facilitate a more relevant understanding of how to extrapolate the outcomes of *in vitro* studies to the variable human population. Ultimately, gaining this understanding will support their safe and effective integration into product safety evaluation and regulatory applications.

PS 4149 Impacts of Exposure Method and Cell Type on Transcriptomic Concentration-Response Curves during Toxicological Screening of Respiratory Irritants

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Background and Purpose: There is a critical need to develop new approach methodologies (NAMs) to assess the health effects of inhalable substances. Proposed NAMs include several 2D or 3D lung cell culture systems at the air-liquid interface (ALI) exposed to chemical via direct liquid application (DLA) or via aerosol delivery with ALI exposure systems. *In vitro* inhalation NAMs have the potential to provide relevant, rapid toxicity data and points of departure (PODs) to be used in hazard assessments, but it is important to understand how the selected cell types, time points, or exposure methods may influence study outcomes. Assessment of gene expression changes using transcriptomics is a well-characterized tool for comparing activation of biological pathways across species and can be used to determine whether differential exposure methods of ALI airway cultures impact transcriptional concentration-response curves. Here, the effects of exposure on gene expression and biological pathways, transcriptomic biological pathway-altering concentration (BPACs), and transcriptomic PODs were evaluated following exposure to a compound known to cause respiratory irritation at the ALI and via DLA in organotypic and immortalized HBEC cell culture systems at multiple time points to characterize best practices. **Methods:** Organotypic (MatTek EpiAirway; differentiated primary human bronchial epithelial cells (dphBEC)) and immortalized human bronchial epithelial (16HBE) cells were exposed to multiple concentrations of an irritant case study chemical using both ALI and DLA approaches. ALI exposures were conducted with the VITROCELL Cloud α 12, and multiple apical volumes (10, 30, 50 μ L) were assessed. Cell lysates were collected at 6 and 24 h following exposures. The BioSpyder Tempo-Seq human whole transcriptome version 2 (hWTV2) assay was utilized and only gene probes with at least one read in at least 10% of samples were included. Differential gene expression was assessed using DESeq2 (absolute fold change $\geq 20\%$, false discovery rate < 0.05). Transcriptional benchmark dose (BMD) values at the probe level were calculated and aggregated to gene set and pathway level using BMDExpress3 (v3.20) software. The BPAC was calculated as median BMD for associated genes/pathways passing all filters in the most sensitive gene sets, with only gene sets containing at least 3 valid genes and 5 % gene set coverage retained in final analyses. **Results:** Exposure resulted in a concentration-responsive increase in number of differentially expressed genes (DEG) for all exposures. The absolute number of DEG varied across exposure system, cell type, and timepoint. Samples exposed at ALI generally had higher numbers of DEG than those exposed at any volume under DLA exposure conditions, but the specific genes and pathways were generally concordant. This study observed similar transcriptional reprogramming of DLA vehicle controls that has been reported previously for dphBEC-fibroblast co-cultures. This reprogramming appears to be volume-dependent and more pronounced in organotypic cultures. The BPACs and PODs identified in each condition varied across exposure systems, cell types, and timepoints, but were quite similar (< 5.5 -fold different). **Conclusions:** NAMs to screen inhalation hazards are a rapidly developing area, and

different exposure methods, cell types, and assessment timepoints are commonly utilized. The presented results characterize how these study variables impact transcriptomic concentration-response curves and demonstrate the need to characterize variables and harmonize study design to facilitate regulatory acceptance. [Abstract does not reflect views or policies of the U.S. EPA. Mention of specific technologies or companies does not represent endorsement by U.S. EPA].

PS 4150 Quantitative High-Throughput Screening Identifies Small-Molecule Inhibitors of Wnt/ β -catenin Signaling

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Background and Purpose: The Wnt/ β -catenin signaling pathway plays a pivotal role in development, cell fate determination, and tissue homeostasis. Aberrant activation of this pathway contributes to a wide range of human diseases, including cancer, fibrosis, and neurodegeneration. However, identifying small-molecule modulators has remained challenging due to pathway complexity and feedback regulation. In this study, we developed and optimized a T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) β -lactamase reporter assay in a 1536-well format suitable for quantitative high-throughput screening (qHTS). **Methods:** A TCF/LEF- β -lactamase reporter HEK293 cell line was optimized to identify inhibitors of canonical Wnt/ β -catenin signaling. The assay, activated by Wnt-3a and R-spondin 2, was validated with known inhibitor imatinib mesylate. The compounds from the Library of Pharmacologically Active Compounds (LOPAC) and the Toxicology in the 21st Century (Tox21) collections were screened across multiple concentrations. Screening data were analyzed for potency and efficacy, and active compounds were confirmed through repeat testing and Western blot analysis of non-phosphorylated (active form) and total β -catenin levels. **Results:** The optimized assay exhibited strong performance with Z' factor of 0.58 ± 0.07 and signal-to-background ratio of 2.9 ± 0.17 . Primary screening against the LOPAC library identified 12 potential inhibitors, including 5 known and 7 previously unreported compounds, all of which were confirmed on retesting. Western blot analysis revealed that 6 of these compounds, including 5 known inhibitors (emetine dihydrochloride hydrate, tyrphostin A9, niclosamide, ouabain, and podophyllotoxin) and 1 unreported compound, reduced levels of non-phosphorylated β -catenin and total β -catenin, confirming inhibition of canonical Wnt signaling. **Conclusions:** This study established a robust qHTS-compatible reporter gene assay for discovering small-molecule inhibitors of Wnt/ β -catenin signaling. The identification of an unreported Wnt antagonist expands the repertoire of chemical tools available for studying pathway regulation and demonstrates the assay's utility for mechanism-based toxicology and drug discovery applications.

PS 4151 Impact of Chemical Quality on High-Throughput *In Vitro* Assays - A Tox21 Study

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Background and Purpose: The Tox21 10K library has undergone analytical quality control (QC) testing after exposure to room temperature storage for 0 and 4 months (T0 and T4). Each chemical was previously assigned a QC grade based on purity, identity, and concentration. In parallel, the Tox21 10K library has been tested in ~80 *in vitro* assays in qHTS format, generating >120M datapoints to date. These data were used to analyze the correlation between chemical quality and bioassay activity, as well as chemical structure. The chemical characteristics of poor quality and unstable compounds were explored to identify structural features that should be avoided. A high-throughput assay measuring the induction of p53 activity by small molecules was used to test the Tox21 10K library at T0 and T4 to monitor compound stability. This study is the first attempt to systematically evaluate the impact of compound quality and stability on biological activity outcomes based on results from a large and diverse library. **Methods:** The Tox21 10K chemical library is an *in vitro* toxicology toolbox consisting of more than 8,900 unique chemical entities including environmental chemicals and drugs. Chemical structures were processed using the publicly available ToxPrint chemotypes (729-bit) generated within the ChemoType application and the Chemical Developer Kit (CDK) package (29 physicochemical properties) as implemented in KNIME. *In vitro* assay data of ~80 assays with 241 readouts were obtained from the Tox21 database (<https://tripod.nih.gov/pubdata/>). The p53 assay activities at T0 and T4 were compared using log AC₅₀ values. Statistical significance was determined by the Fisher's exact test for chemical features, the Student's t-test for physicochemical properties, and the Wilcoxon rank-sum test for log AC₅₀ value differences between T0 and T4. Machine learning models were built using the Random Forest method and model performance was evaluated by calculating the AUC-ROC. **Results:** The chemical characteristics of poor quality and unstable compounds were explored to identify structural features that should be avoided. In addition, one of the high-throughput assays measuring the induction of p53 activity by small molecules was used to test the Tox21 10K compound library at T0 and T4 due to its robust performance and reproducibility. Approximately 2% of compounds in the library showed a significant change in activity in the p53 assay between T0 and

T4 (active to inactive or vice versa), which also correlated with chemical stability. Here, machine learning models were constructed using bioassay data or chemical structure to predict poor quality (low QC grades at T0) and unstable (grade drop from T0 to T4) chemicals. Chemical structure was found to be highly predictive (0.75) of chemical quality and stability, whereas bioassay data was less predictive (0.66) but still showed better than random performance. **Conclusions:** A cell-based assay was utilized alongside analytical QC efforts to assess the stability of the Tox21 10K library, revealing time-dependent changes in assay activity that aligned with QC grade shifts. Comparative analyses showed that chemical quality has a significant impact on assay activity, and poor quality/degraded chemicals tend to be less active than the chemicals that passed QC. Machine learning models were used to analyze the impact of chemical quality and stability on bioassay activity, demonstrating that chemical structure was a good predictor of chemical quality. Chemical features of poor quality/unstable chemicals were identified, which can be used to guide future chemical selection and handling.

PS 4152 Evaluating Effects of Varying Serum Levels on *In Vitro* Disposition (IVD) and Partitioning of Per- and Polyfluoroalkyl Substances (PFAS) in Human Cell Models

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Background and Purpose: US EPA is developing *in vitro* new approach methods (NAMs) that efficiently evaluate bioactivity across a range of biological pathways and targets. Characterizing *in vitro* disposition (IVD), the distribution and binding of a chemical in microplate systems, is important for interpreting exposure and effects data generated by NAMs and informing *in-vitro-in-vivo* extrapolation (IVIVE). Despite increased acceptance of NAMs for risk prioritization, it is recognized that the nominal concentration is not always the concentration available to elicit an effect. This study explores the IVD and partitioning of 21 per- and polyfluoroalkyl substances (PFAS), selected to capture a range of carbon-fluorine (CF₂) chain lengths (C4-C14) and functional groups, in U-2 OS cells, a human osteosarcoma cell line model used for *in vitro* chemical testing, amenable to culture in varied concentrations of fetal bovine serum (FBS) to evaluate protein binding effects. We analyzed differences in PFAS cell partitioning in media containing 10%, 5%, or 0% FBS to determine its influence on chemical distribution *in vitro*. **Methods:** Three 96-well culture plates were used to assess the compartmental distribution of PFAS compounds after 24 hr of exposure. The media was transferred from two plates and tested for their PFAS concentration separate from the plastic. Therefore, the third plate containing cells was analyzed completely and served as a 100% representation of the PFAS concentration *in vitro*. Each well in the culture plates was spiked to contain a final concentration of 10 μ M of PFAS. Acetonitrile was used to crash the biological material and extract the PFAS from the plastic. Quadruplicate PFAS concentrations were analyzed from each plate using a Waters Xevo TQ-S micro-LC-MS/MS. Cell confluency 24 hours before and after dosing was quantified using an Opera Phenix Plus. Ionomycin served as a reference for cytotoxicity and DMSO as a vehicle control to confirm cell growth was not impacted by the PFAS. **Results:** Our study demonstrated the partitioning behavior of PFAS in *in vitro* microplate test systems across three serum levels depends primarily on CF₂ chain length. As the number of fluorinated carbons increased, the partitioning coefficient increased, attributing to lower concentrations of PFAS in the media compartment *in vitro*. There were no discernable trends nor evidence that functional group influenced PFAS plastic-media partitioning or concentrations in the media. Rather, the main driver seemed to be the CF₂ chain length. Additionally, linear regressions created using physicochemical properties dependent on molecular size all followed the same trends and were consistent throughout each serum level, suggesting that FBS did not have a significant impact on *in vitro* partitioning. However, cell-free media preparations had statistically higher concentrations of longer chain PFAS than media from the cell-based preparations, implying heightened chemical-cell partitioning. This trend was consistent with each serum level analyzed. Insolubility was only observed for the longest chain PFAS (>12 carbons) in serum free media, suggesting FBS allows chemicals to stay in solution. **Conclusions:** Our study provides important information to reduce knowledge gaps on PFAS IVD. Our findings advance understanding of how PFAS structural differences influence their behavior *in vitro*. Longer CF₂ chain lengths impact bioavailable concentrations *in vitro*, affecting NAM and IVIVE analyses. The lack of significance PFAS functional groups have on IVD can provide better insight on the administered equivalent dose (AEDs) for NAMs, since previous studies indicated sulfonated groups to have higher partitioning to membranes or polymers. Overall, there is a scientific knowledge gap on PFAS binding to various components of a cell-based *in vitro* test system in microplate format. Current use of NAMs estimating AEDs for PFAS is a conservative method using the nominal concentration to determine potency. Our study indicates that most PFAS have low partitioning out of the media, possibly reducing the free/bioavailable concentration of the chemical. *This abstract does not represent US EPA policy.*

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Background and Purpose: Advances in new approach methods (NAMs) have led to more efficient, information rich, and cost-effective strategies for next generation risk assessment (NGRA). Two NAMs-based assays, high-throughput phenotypic profiling (HTPP) with Cell Painting and high-throughput transcriptomics (HTTr) with TempO-Seq, measure changes in cell morphology and gene expression, respectively, to inform hazard assessments. Notably, points of departure (PODs) can be derived from chemical bioactivity data from these assays for use in NGRA. In this study, both assays were conducted in a biologically diverse battery of ten human cell lines and results were evaluated in the context of an example NGRA application: bioactivity:exposure ratio (BER) analysis for chemical prioritization. Here, we assessed how the individual or combined use of both assays and the cell line battery affected the ability to flag chemicals with overlapping bioactivity and predicted human exposure potential. **Methods:** A total of 41 test chemicals found in drugs or consumer products were screened in ten biologically diverse human cell lines (ASC52Telo, CHON-001, CCD-18Co, HBEC3-KT, HepG2, hNP1, Ker-CT, RPTEC/TERT1, TeloHAEC, U-2 OS) using HTPP and HTTr. Each chemical was tested at 8 concentrations and were a priori assigned to a dose range based on available bioactivity data. The dose ranges were designated "HIGH" (0.1 - 300 μ M), "MEDIUM" (0.01 - 30 μ M), "LOW" (0.01 - 3 μ M), and "VERY LOW" (3x10⁻⁶ - 30 μ M). For each cell line x chemical combination, concentration-response modeling was performed to calculate phenotypic altering concentrations (PACs) in HTPP and biological pathway altering concentrations (BPACs) in HTTr. These *in vitro* PODs correspond to the lowest test chemical concentrations where biological perturbations are observed. PACs and BPACs were used as input for *in vitro* to *in vivo* extrapolation (IVIVE) using the htk R package to calculate human administered equivalent doses (AEDs). For BER analysis, AEDs were compared to estimated human exposure levels derived from the systematic empirical evaluation of models (v3) (SEEM3) model. A BER was calculated by taking the difference between an AED and SEEM3 exposure prediction, and chemicals were flagged for prioritization if a BER was less than three or if a negative BER was detected. **Results:** Chemical PODs in both assays varied widely across cell lines in response to drug-like chemicals in the VERY LOW dose band. In some cases, PODs ranged by multiple orders of magnitude across cell lines supporting that biological context (i.e. cell type) influences response to chemicals. While PODs between assays were largely within one order of magnitude of each other for each cell line x chemical combination, there were also cases where active hits were only observed in one assay. Due to the range of PODs and differences between assay observed, analysis was conducted to assess how BER results may be influenced by assay and cell line. Results supported that combined use of HTPP and HTTr provided the greatest level of biological coverage in comparison to only using information from either assay alone. Additionally, BER results using the full cell line battery flagged a greater number of chemicals than using a leave-one-out approach or when considering only one cell line in isolation. Using one cell line alone resulted in large variations in the number of active chemicals and flagged chemicals across the battery. In addition, the identity of the cell line driving the BER varied from chemical to chemical with each cell line being represented at least once. Therefore, BER results were not driven by a single cell line. **Conclusions:** Our results showed that using a cell line battery in addition to screening in both assays was a more comprehensive approach to BER analysis for chemical prioritization as compared to using a single cell type or either technology alone. Furthermore, using a cell line battery in screening can capture how biological responses to chemicals can be influenced by cell context, as observed with our POD comparisons, which may be missed by only screening in one cell line. *This abstract does not reflect the views or policies of the US EPA or Unilever.*

PS 4154 Use of Data Derived Extrapolation Factors to Reduce Uncertainty in Organophosphate Human Health Risk Assessments

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Background and Purpose: When conducting human health risk assessments for pesticides, it is important to recognize that pharmacokinetic (PK) and pharmacodynamic (PD) differences exist between animal models and humans and from person to person. To account for these interspecies and intraspecies differences, uncertainty factors (UFs) are applied to the point of departure; the point of departure is a dose or concentration not expected to lead to the most sensitive effect for a given route of exposure. A default value of 10X is most often given to each UF, which incorporates 3X each for PD and PK considerations, for a total of 100X. Refining these UFs based on robust data results in a more accurate evaluation of real-world risk estimates. The purpose of this study is to apply data derived extrapolation factors (DDEFs) to refine the UFs of organophosphates (OPs), a large group of insecticidal pesticides registered for a wide range of uses, including agriculture and public health pest control. OPs share a well-established mode of action (MOA) involving inhibition of the enzyme acetylcholinesterase (AChE), preventing the breakdown of acetylcholine and leading to neurotoxicity. This universally accepted MOA, along with the extensive

amount of data available for OPs, provides an opportunity to utilize *in vitro* data to generate DDEFs and utilize human relevant data to inform human health risk assessments for OPs. **Methods:** This study incorporates a new approach method (NAM) using animal and human enzyme *in vitro* kinetics data for 15 registered OPs and a positive control (parathion). Red blood cell AChE was collected from Sprague Dawley rats (3 pooled samples of 5 animals/sex) and from 18 human individuals of various age, sex, and ethnicity. Measurements were conducted every minute for 5 minutes at varying concentrations of each OP to determine the bimolecular rate constant. Absorbance for duplicate samples was measured via a spectrophotometric assay, and velocity of each reaction was calculated by determining the slope of plotted absorbance as a function of time. In addition, the longest time point of 5 minutes was used to estimate the concentrations leading to 50% of maximum enzyme activity (i.e., IC₅₀) across the dose-inhibition curve for each OP. Five model options were evaluated using a 3-parameter log-logistic dose-response curve consisting of the IC₅₀, upper limit, and slope of the curve. The most appropriate model was used to determine the species-specific IC₅₀ and the ratio of the rat-to-human IC₅₀ was used to estimate the DDEF and assess the PD differences between species. From the same dose-response curves, interspecies ratios were also calculated for other inhibitory concentrations (IC₁₀, IC₂₅, and IC₇₅) in a similar manner. **Results:** Interspecies PD DDEFs ranged from 0.42 to 1.60. Values could not be calculated for 2 of the tested compounds. While outlier or convergence issues impacted a minority of the analyzed OPs, the central tendency of the interspecies DDEFs was approximately 1. This finding was broadly conserved regardless of inhibitory concentration. For some tested chemicals, the width of the 95% confidence intervals indicates some uncertainty around interspecies ratios at each of the inhibitory concentrations, which could reflect inherent variability in the *in vitro* assays and study design, such as sample size or incubation duration, or interindividual differences. Generally, confidence intervals were narrow around point estimates as inhibitory concentration increased, reflecting lower variability between individuals at high treatment response levels. **Conclusions:** *In vitro* data supports reduction of an interspecies PD UF from 3X to 1X for all but 2 of the 16 tested OPs. This health protective approach considers minor variability observed across the data, the inhibitory concentrations evaluated, and the high degree of species homology in the enzymatic structure of rat and human AChE. Additionally, other data demonstrate that PK differences, such as metabolic clearance, primarily contribute to the differences in AChE activity between rats and humans, rather than PD. Refinement of the interspecies UF through application of NAMs provides opportunity to reflect more realistic human health risk estimates for OPs.

PS 4155 Screening Level Assessment of 1470 ToxCast Chemicals Across Nine Biologically Diverse Human Cell Lines Using the Cell Painting Assay

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Background and Purpose: New Approach Methods (NAMs) have the capability to support chemical risk assessment by characterizing bioactivity and potential hazards using human cell-based *in vitro* models. There are thousands of chemicals in the environment with insufficient or absent human health hazard data where evaluation with NAMs will be less expensive and more efficient than animal studies. In this study, a set of human cell lines from diverse tissues were screened using High-Throughput Phenotypic Profiling (HTPP) with the Cell Painting assay. High-throughput profiling assays, like HTPP, can characterize chemical bioactivity across a broad range of biological processes and molecular targets. Bioactivity points-of-departure (PODs), the chemical concentration where perturbations in cell morphology are first detected, derived from these data can be compared to human exposure estimates and help inform chemical risk assessment. The ability to run HTPP in combination with other bioactivity assays and computational methods has the potential to accelerate the pace of chemical risk assessments. **Methods:** HTPP is a high-content imaging assay that uses fluorescent labeling of organelles and automated image analysis to quantify changes in cell morphology. The cell line panel screened in this study was selected using a data-driven approach based on differences in baseline gene expression and consists of hTERT-immortalized cell lines (ASC52Telo mesenchymal stem cells, HBEC3-KT bronchial cells, Ker-CT skin keratinocytes, RPTEC/TERT-1 kidney cells, and TeloHAEC vascular cells), normal cell lines (CCD-18Co colon fibroblasts), and cancer cell lines (Huh-1 hepatocellular carcinoma, MCF-7 adenocarcinoma, U-2 OS osteosarcoma). In each cell type, a total of 1,470 unique test chemicals were screened at 8 different concentrations using either a 1/2-log₁₀ dose spacing or full log₁₀ dose space, assigned based on previously collected ToxCast high-throughput screening data. After a 24h exposure period, assay plates were stained, imaged and concentration response modeling was performed using the R package *tcplfit2* to determine phenotypic altering concentrations (PACs) for each chemical x cell line combination. Cell viability was evaluated using normalized cell counts (nCC) and treatments resulting in cytotoxic or cytostatic effects were excluded from downstream analysis. HTPP PAC values were further utilized for *in vitro* to *in vivo* extrapolation (IVIVE) using the R package *httk* to calculate human administered equivalent doses (AEDs). These AEDs were compared to human exposure estimates to determine a bioactivity-exposure ratio (BER) and flag chemicals for prioritization. **Results:** Ker-CT was found to be the most sensitive cell type with 873 total active hits. CCD-18Co were found to be the least sensitive with 486 active hits. In total, 412 chemicals were found to be active across all nine cell types tested

and 300 chemicals were found to be inactive. There were 1,120 chemicals that were found to be active in at least one cell type. A subset of chemicals found to be active in a single cell type exists for all cell types tested. Chemical PAC values were found to be either consistent across all cell types tested (<1 order of magnitude) or highly variable (>5 orders of magnitude), depending on the chemical x cell type combination. The cell types ASC52Telo, CCD-18Co, Huh-1, and U-2 OS were found to have increased sensitivity to glucocorticoid steroids. Evidence of metabolism was found with the Huh-1 liver cell line where both bioactivation of 3'-azido-3'-deoxythymidine (a left shifted PAC >2 orders of magnitude) and inactivation of multiple parabens and phthalates that were active in all cell lines except Huh-1. **Conclusions:** These results indicate that the bioactivity of some chemicals is dependent on the cell type being screened and should be considered during study planning. Molecular targets of cell lines are being examined as potential sources of PAC variability for some chemicals. Subsequent analyses will use HTPP PACs, IVIVE and human exposure estimates to flag chemicals for prioritization and demonstrate the capability of these NAMs for accelerating hazard identification and informing chemical assessments. *This abstract does not reflect the views or policies of the US EPA.*

PS 4156 Evaluating Respiratory Irritants using New Approach Methods: Harmonizing Key Study Design Variables for Air-Liquid Interface and Direct Liquid Application Exposures

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Background and Purpose: New Approach Methods (NAMs) are increasingly used to assess the toxicity of inhalable substances, highlighting the need to optimize and harmonize exposure methods for airway cell cultures. While air-liquid interface (ALI) exposure systems preserve the physicochemical properties of inhalable chemicals and allow direct cell-toxicant interaction, they are often costly, low-throughput, and require specialized expertise. Direct liquid application (DLA) presents an attractive alternative to screen non-volatile chemicals because many challenges associated with aerosol delivery can be bypassed by directly applying a known amount of chemical in a liquid solution to the apical surface of ALI cell cultures. This study was designed to evaluate *in vitro* exposure parameters in support of building best practices to screen respiratory irritants using organotypic and immortalized human bronchial epithelial cell (HBEC) cultures. **Methods:** In the first phase of this study, MatTek EpiAirway tissues were exposed to a model respiratory irritant at the ALI using the VITROCELL Cloud or across a range of commonly utilized DLA volumes (10, 30, 50 μ L in 6.5 mm ALI inserts) to determine the effect of apical volumes on study outcomes. Exposures were then repeated with the lowest apical volume that maintained cell surface coverage (30 μ L) in differentiated primary human bronchial epithelial cells (dpHBEC) and immortalized 16HBE cell cultures to assess repeatability across multiple human donors and within cost-effective cell culture systems. Trans-epithelial electrical resistance (TEER), viability (ATP production), cytotoxicity (LDH release), and pro-inflammatory cytokine release were assessed at two timepoints (6, 24 h). **Results:** Both ALI and DLA exposures resulted in similar points of departure (PODs) when the most sensitive endpoints were compared. Cytotoxicity measured through LDH release was most impacted by exposure study design variables, especially at the 6 h timepoint. In general, observed differences between ALI and DLA exposures diminished at the 24 h timepoint. Notably, DLA exposures led to vehicle toxicities in a volume-dependent manner which supports the use of the lowest volume that achieves full cell coverage. Mass per surface area (μ g/cm²) is the most appropriate dose metric to report regardless of exposure method to enable *in vitro* to *in vivo* extrapolation. **Conclusions:** This study demonstrated the utility of ALI and DLA exposure methods to evaluate respiratory irritants and identified key study design variables that can be harmonized to increase repeatability of study outcomes. DLA exposures compare most favorably to ALI exposures at the 24 h endpoints compared to the 6 h measurements. Applied apical volume remains an important variable for DLA study designs and should be optimized across 6-, 12-, and 24-well formats. Application of 30 μ L in a 6.5 mm ALI insert was identified as the minimum volume that provided uniform cell coverage, and future studies should explore whether a shortened DLA submersion period reduces vehicle toxicities observed in organotypic ALI cultures (e.g., a 4 h liquid application, removal of applied material, and restoration of ALI conditions until the desired timepoint is achieved). Future studies would also benefit from direct measurements of cellular uptake to further refine dosimetry characterization across *in vitro* exposure methods. *[Abstract does not reflect views or policies of the U.S. EPA. Mention of specific technologies or companies does not represent endorsement by the U.S. EPA].*

PS 4157 Comparative Predictivity of Estrogen-Dependent Cell Proliferation Assays to In Vitro/In Vivo Reference Data

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Background and Purpose: To modernize and improve the efficiency of the Endocrine Disruption Screening Program (EDSP), computational models using data from high-throughput assays were developed. A key focus has been the estrogen receptor

(ER) agonist model—originally constructed from 16 ToxCast assays— which may be used in lieu of three EDSP Tier 1 assays, including the uterotrophic assay, an *in vivo* assay that assesses estrogen pathway agonism. Subsequent analyses revealed that a predictive ER model could be effectively streamlined to use as few as four assays, focusing on critical events in ER pathway activation: ligand binding, dimerization, transcriptional activation (ERTA), and proliferation. Some of the original ToxCast assays are no longer available or face practical implementation barriers; therefore, alternatives to these assays are being developed with the aim of building a new ER computational model comprised entirely of accessible methods. This study utilized 32 ER reference chemicals to compare five alternative cell proliferation methods to the original ToxCast proliferation assay (ACEA Biosciences), evaluating their ability to predict responses *in vitro* versus *in vivo*. **Methods:** Alamar Blue, CellTiter-Glo®, CyQuant™, and Hoechst quantify the increase in number of T47D cells by measuring cellular reduction, cellular ATP, DNA content, and nuclei counts respectively. BrdU incorporation identifies actively proliferating cells undergoing DNA synthesis. The 32 reference chemicals were tested from 0.1 pM to 100 μ M (final) in five replicate experiments. Responses were scaled 0% to 100% using the vehicle and estradiol control responses, respectively. Data were fit using the tcplfit2 R package constrained to one of three models- Hill, gain-loss, or constant with activity calls and half-maximal concentrations (AC₅₀) derived from the best fitting model. Activity calls and AC₅₀ data for the ACEA_ER_80hr ToxCast assay were retrieved from invitrodb (v4.3). The responses listed in OECD Test Guideline 455 (ERTA) were used for *in vitro* reference activity. *In vivo* reference activity was determined from uterotrophic data using only guideline-like studies with chemicals active in > 70% of studies classified as active, those active in < 30% classified as inactive and all others classified as equivocal. **Results:** For the 32 *in vitro* reference activities, each of the five alternative proliferation methods has lower balanced accuracies (67-81%) than ACEA (89%) due to lower specificities (43-71%) stemming from the misclassification of two chemicals - linuron and spironolactone. Of the 21 chemicals classified as active or inactive based on uterotrophic responses, Hoechst was more predictive than any other method (including ACEA) with a balanced accuracy of 68%. Kepone and methoxychlor were misclassified as inactive by most methods, while daidzein, ethylparaben and kaempferol were misclassified as actives by all methods including ACEA. AC₅₀ values for potent ER agonists like 17 β -estradiol were much lower (20 pM) in the alternative methods than ACEA (2 nM), but similar AC₅₀ values were observed for less potent agonists like bisphenol A. **Conclusions:** ER activation is sufficient to stimulate cell growth in some tissues; however, proliferation is a complex response to many competing factors. Reference chemicals selected based on ERTA activity may not be well predicted by cell proliferation assays if the chemical elicits other anti-growth responses and stimulates cell growth in an ER-independent manner. Reference chemicals selected based on uterotrophic response may not be well predicted in a cell proliferation assay due to the toxicokinetic differences between intact animals and cultured cells. Chemicals like kaempferol and butyl benzyl phthalate have opposing *in vitro* and *in vivo* activities. However, cell proliferation assays serve as a gateway between test methods that focus on ER mechanics (e.g., ERTA) and a complex *in vivo* response and thus are indispensable for any new ER computational model. The disparity in balanced accuracy between the ACEA and the alternative methods largely stems from a small number of false positives compounded by an imbalance in reference chemical composition (actives >> inactives). Of the alternative methods evaluated, Hoechst was the most predictive overall.

PS 4158 Predictivity Assessment of the Human Thyroid Microtissue Assay

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Background and Purpose: The U.S. Environmental Protection Agency (EPA) aims to evaluate and adopt new approach methods (NAMs) for screening endocrine disruptor chemicals, when appropriate. One NAM-based assay, the human thyroid microtissue (hTMT) assay, utilizes primary human thyrocytes in a 3D culture model to evaluate chemical effects on thyroid hormone synthesis. Previous work characterized the hTMT model, including qualification criteria for human thyrocyte selection, acceptance criteria for assay performance, and reference chemical benchmark doses (BMDs) for proficiency testing. While an interlaboratory validation study assessed the relevance, reliability, and transferability of the assay for a small reference chemical set, expanding the tested chemical space will aid in evaluating the predictive performance of the assay. The objective of this study was to assess the predictive accuracy of the hTMT assay by conducting a within-laboratory assessment with 49 test chemicals. **Methods:** A set of 49 chemicals were tested across six concentrations (0.001 - 100 μ M) in the standardized hTMT assay to evaluate disruptive effects on thyroxine (T4) synthesis. The chemical set comprised four previously established reference chemicals, and 45 chemicals curated by subject matter experts. These included known inhibitors of molecular initiating events (MIEs) within the thyroid adverse outcome pathway network, and inactive chemicals. A priori chemical classifications were established using previously published data on molecular and functional inhibition of thyroid MIEs, gland activity, and hormone composition, classifying 36 as positive (disruptive to T4 synthesis) and 13 as negative (non-disruptive). **Results:** Initial results from two of four experimental replicates demonstrated chemical-induced T4 disruption with BMDs ranging from 0.8 to 75.4 μ M. T4 disruptive activity and potencies of the reference chemical set (methimazole [0.31 μ M], 6-propyl-2-thiouracil [0.24 μ M], sodium perchlorate [0.25 μ M], and methomyl [non-disruptive]) were slightly outside of the range of established proficiency testing thresholds.

Because differences were within an order of magnitude and consistent with previous studies (e.g., interlaboratory validation), reference chemical results are considered to meet acceptance criteria. Assay performance measures for T4, for dynamic range (rS/B, 21.5 ± 11.9), precision (rCV, 4.4 ± 2.6), and screening quality (rZ' factor, 0.9 ± 00.4) also satisfy acceptance criteria. Collectively, these results demonstrate that technical proficiency was achieved and further support the robustness and reliability of the hTMT model. Among test chemicals, 24 of 36 were identified as true positives, where T4-disruptive activity was correctly matched to a priori positive classifications, and 11 of 13 were identified as true negatives, where non-disruptive activity was correctly matched to negative classifications. These outcomes were used to calculate predictive performance metrics: 67% sensitivity (ratio of true positives to all a priori positives), 85% specificity (ratio of true negatives to all a priori negatives), and 76% balanced accuracy (average of sensitivity and specificity, providing an overall measure of predictive performance). These results demonstrate acceptable predictive accuracy of the hTMT assay. **Conclusions:** Given the acceptable predictive accuracy and technical performance, this within-laboratory study supports the continued evaluation and advancement of the hTMT assay towards regulatory adoption and the implementation of NAMs in an improved screening paradigm for thyroid-disruptive chemicals. The views expressed in this presentation do not necessarily reflect the views or policies of the U.S. EPA.

PS 4159 In vitro Cardiac New Approach Methodologies Compared to Nonclinical Animal Studies and Clinical Repolarization Prolongation

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Sponsor: B. Anson

Background and Purpose: The study evaluated human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) as a non-animal new approach methodology (NAM) for assessing cardiovascular repolarization risk in drug development. The research compared hiPSC-CM data with traditional methods (hERG assays and animal studies), to determine if hiPSC-CMs could potentially support regulatory decision-making, aligning with FDA Modernization Act 2.0 goals. **Methods:** The study retrospectively analyzed Investigational New Drug (IND) applications submitted to FDA/CDER between 2020 and 2023, comparing hiPSC-CM studies using multielectrode array (MEA) and voltage sensing optical (VSO) platforms with hERG assays and *in vivo* non-rodent QT studies. The analysis focused on concordance between these methods and their ability to predict clinical QT prolongation risk. **Results:** Both hERG and hiPSC-CM assays showed similar concordance with clinical QT outcomes. hiPSC-CMs had accuracy (0.83) and likelihood ratios comparable to *in vivo* non-rodent QT studies (0.74-0.78). Both hERG and hiPSC-CM assays tested effects at higher exposure multiples than animal studies. **Conclusions:** hiPSC-CMs offer a promising alternative to extensive animal testing for cardiovascular risk assessment. However, limitations include small sample size, low prevalence of clinical QT True Positives, early-phase INDs, variability in hiPSC-CM study rigor, and challenges in establishing exposure-response relationships. Despite these limitations, this research provides evidence supporting the application of hiPSC-CMs for cardiovascular regulatory assessments. Further research is needed to establish robust protocols for integrating hiPSC-CM data into regulatory workflows, balancing the promise of reduced animal testing with the need for comprehensive safety assessment. **Disclaimer:** This abstract reflects the views of the author and should not be construed to represent FDA's views or policies.

PS 4160 Electrophysiological Evaluation of Fentanyl-Naloxone Combination using Human Neural New Approach Methodologies (NAMs)

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Sponsor: B. Anson

Background and Purpose: The FDA Modernization Act 2.0 has promoted the adoption of human-relevant models such as those using human induced pluripotent stem cells (hiPSCs) as new approach methodologies (NAMs) to reduce animal testing for pharmacological safety assessment. This study combines multielectrode array (MEA) technology with a neural co-culture derived from hiPSCs to characterize electrophysiological signatures resulting from opioid activation and antagonism as part of efforts to address the ongoing opioid epidemic through nonclinical central nervous system (CNS) drug interaction research. **Methods:** The neural NAM model we present is composed of hiPSC-neurons and hiPSC-astrocytes coupled with MEA which was used to assess the neural electrophysiology. Neural cells were treated with varying concentrations of fentanyl and naloxone following their plating onto the MEA platform. Spontaneous neural electrical activity was then continuously recorded for up to 1 hour using an Axion Maestro Pro to evaluate the functional effects. **Results:** Throughout the experimental window there was a display of robust networked electrical activity within the co-cultured neural population. When exposed to fentanyl and the combination (i.e., fentanyl and naloxone), the assay displayed a concentration-dependent modulation of neural electrophysiological activity relative to the vehicle control. Moreover, the combination mitigated the electrophysiological effects of fentanyl alone. The effects resulting from these drug interactions were

observed across spiking, bursting, and network bursting parameters which were used to characterize the degree and quality of hierarchical communication with the neural population. **Conclusions:** We demonstrate a capacity for this NAM to assess opioid and opioid antagonist interactions on a hiPSC neural population through the characterization of human networked electrical activity at baseline and following exposure to fentanyl, naloxone, and both in combination. Future studies will investigate additional CNS active compounds, polypharmacy, and reversal agents. As a whole, these data support the application of a neural MEA platform as a human *in vitro* model to assess the safety and efficacy of CNS active compounds. **Disclaimer:** This abstract reflects the views of the author and should not be construed to represent FDA's views or policies.

PS 4161 Neurotoxicity and Hepatotoxicity Testing in Organotypic Models as Part of a Tiered Screening Assessment for Force Health Protection

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Background and Purpose: Military personnel may be exposed to a variety of substances, many of which have limited data regarding health risks. A tiered screening strategy using new approach methods (NAMs) is being developed to expedite the assessment of health risks from poorly characterized substances. Initial tiers employ *in silico* and high throughput *in vitro* screening to identify potential modes of action. Higher tiers use biologically relevant organotypic models to generate points of departure (PODs) for quantitative risk assessment, with the goal of reducing the need for *in vivo* tests. To assess whether human equivalent doses (HEDs) derived from *in vitro* PODs from organotypic neurotoxicity and hepatotoxicity models are at least as conservative as *in vivo* PODs, nine organophosphates (OPs; chlorpyrifos [CPF], chlorpyrifos oxon [CPF oxon], diazinon, diazoxon, diisopropylfluorophosphate [DFP], malathion, malaoxon, tris (2-chloroethyl) phosphate [TCEP], tris(1,3-dichloro-2-propyl) phosphate [TDCPP]) and three carbamates (aldicarb, carbaryl, methomyl) were tested in a central nervous system organoid model (CNS-3D, 28bio). Six hepatotoxic chemicals (4-chlorobiphenyl [4-CBP], chlorobenzene, diazinon, diazoxon, TCEP, TDCPP) were assessed in liver microtissues (3DInSight, InSphero). **Methods:** In the CNS-3D model, acute and three-week repeat dose-response curves for OP and carbamate administration were generated for acetylcholinesterase (AChE) inhibition, viability, and intracellular calcium (Ca²⁺) flux. In the 3DInSight model, dose-response curves were generated for tissue viability, total glutathione, and triglycerides on day seven after dosing on days 0, 2, and 5. *In vitro* PODs were calculated as the benchmark dose lower confidence limits (BMDLs) for each assay. A high-throughput toxicokinetic (HTTK) model, adapted for the United States Air Force population, was used to predict lower 95th percentile oral HEDs (HED₉₅) from the *in vitro* PODs. In select cases, HEDs were also calculated using bespoke physiologically-based pharmacokinetic models. For each chemical, the lowest *in vitro*-derived HED₉₅ was compared to literature-derived *in vivo* PODs (POD_{Lit}). **Results:** AChE inhibition had the lowest HTTK HED₉₅ for most OPs and carbamates in the CNS-3D model acute testing, with values ranging from 0.0011 mg/kg for methomyl to 307 mg/kg for malathion. Sensitive acute dose-dependent responses were obtained for Ca²⁺ flux assays for carbaryl, DFP, CPF, and CPF oxon, with the lowest HED₉₅s (ranging from 0.001 mg/kg for CPF oxon to 0.038 mg/kg for carbaryl) derived from excitatory or inhibitory effects on the Ca²⁺ flux activity. More pronounced effects on Ca²⁺ flux burst strength and viability were measured after three-week exposures. Neurotoxicity HTTK HED₉₅s were generally similar to, or more conservative than, the POD_{Lit} values, with the exception of aldicarb (4-fold higher) and malathion (16-fold higher). Some PBPK HED₉₅s were comparable to HTTK HED₉₅s (aldicarb, chlorobenzene; within 2-fold), but others were much higher (diazinon/diazoxon, 50 to 200-fold) or lower (TCEP, 20-fold) than HTTK HED₉₅s. Liver microtissue testing also produced HTTK HED₉₅s that were comparable to *in vivo* values, with reduction in cell viability being the most sensitive effect for 4-CBP and TCEP, and alteration of glutathione levels being the most sensitive effect for diazinon, diazoxon, and TDCPP. **Conclusions:** The testing demonstrates the utility of using organotypic models in the later stages of a tiered approach that will support reasonable predictions of *in vivo* PODs in rapid assessments of novel or data-poor chemicals. HTTK HED₉₅s derived from organoids were at least as conservative as POD_{Lit} values when based on the most sensitive *in vitro* endpoint for each chemical, with exceptions of aldicarb and malathion, although they had HED₉₅s that were comparable to POD_{Lit} values in prior early tier testing. While the findings support that these NAM-based methods show promise for rapid risk assessment, work is continuing on their broader applicability and limitations, and for the diverse exposure scenarios encountered in military risk assessments. **Disclaimer:** No DoD endorsement implied.

PS 4162 Dose-response evaluation of lung injury in human reconstructed lung epithelium *in vitro* models following acute propylene oxide exposure

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Background and Purpose: Assessment of health risks posed by chemical exposures is a fundamental component of force health protection. Novel approaches are needed to support timely, defensible operational decisions, particularly with regard to use of new chemicals/materials or their byproducts in emerging technologies. While *in vitro* and *in silico* methods for chemical testing have advanced tremendously over the last decade, *in vitro* methods for respiratory toxicity are not nearly as well-studied as other portal of entry effects. Here, we tested multiple key events for lung injury in an established reconstructed human airway *in vitro* model and compared the dose-response results to *in vivo* data to evaluate the utility of the *in vitro* model for testing reactive gases in support of health risk assessments for novel chemicals. **Methods:** EpiAirway™ cells were exposed at the air-liquid interface to propylene oxide (PrO) for four hours at concentrations of 0, 354 ± 3.3, 756 ± 4.1, or 1250 ± 7.0 ppm (mean ± standard deviation) in a VITROCELL® 24/48 exposure system. Initial range-finding studies were used to identify the three PrO concentrations used in the definitive study. The vapor generation system used compressed PrO gas metered via a mass flow controller, diluted with temperature/humidity-controlled filtered air (36.2 ± 0.2°C, 86 ± 0% relative humidity) and transported to the VITROCELL® chamber via heated Teflon lines. Exposures were monitored with an on-line Total Hydrocarbon Analyzer with a Flame Ionization Detector that was calibrated to known PrO concentrations. Trans-epithelial electrical resistance (TEER) was measured pre-exposure, immediately following, and 22 ± 2 hours post-exposure. Tissue histology, cytotoxicity (measured by lactate dehydrogenase, LDH, and adenylate kinase, AK), inflammatory cytokines, and the ratio of reduced to oxidized glutathione (oxidative stress marker) were also evaluated post-exposure. **Results:** Two range-finding studies demonstrated dose-dependent increases in markers of cell death (LDH, AK), and morphological changes at 900 ppm. In the definitive exposure studies, 1250 ppm PrO caused a significant decrease in TEER immediately after exposure, which continued to decline 22 hours post-exposure. Increased PrO concentrations were associated with increased cytotoxic protein marker expression; the AK assay demonstrated greater sensitivity than LDH. At 1250 ppm PrO exposure, microscopic signs of barrier stress (mucus production, cell rounding, cilia loss, cell loss) were affected immediately after exposure and worsened after 22 hours. Cytokine analysis revealed increased production of the pro-inflammatory and immune cell recruitment cytokines IL-6, IL-1α, and IL-1β. The reduced to oxidized glutathione ratio also decreased at 1250 ppm, indicating increased oxidative stress at 22 hours post-exposure. In summary, no effects were seen at 350 ppm and only the most sensitive effects were perturbed (e.g., AK-indicated cytotoxicity, cilia loss) at 750 ppm. All endpoints were altered at 1250 ppm PrO. **Conclusions:** This work successfully developed and characterized an *in vitro* air-liquid interface respiratory toxicity testing strategy with PrO. EpiAirway™ tissues exposed to PrO demonstrated a concentration-dependent increase in cytotoxicity, barrier stress, cytokine production and oxidative stress in human bronchial reconstructed airway models. The EpiAirway™ model demonstrated responses that were qualitatively and quantitatively consistent with acute inhalation toxicity studies in animals, which report lethality with marked respiratory effects starting at similar concentrations (1000-4000 ppm). The data collected in this study demonstrates the utility of the EpiAirway™ model to identify acute lung injury from a reactive gas at *in vivo*-relevant doses. This work is part of a larger suite of studies aimed at developing a rapid risk assessment process for novel and poorly tested chemicals, to improve force health protection and warfighter readiness. **Disclaimer:** No DoD endorsement implied.

PS 4163 Accelerating Organoid Adoption as a NAM with New Non-Toxic Dyes that Deliver Comprehensive Insights

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Background and Purpose: The U.S. FDA and NIH are actively promoting New Approach Methodologies (NAMs) like organoids to reduce reliance on animal models, which often fail to predict human-relevant efficacy and toxicity. However, the successful adoption of organoids requires technologies capable of probing these sensitive models in a standardized and reproducible way, but also in their native, unperturbed state for optimal data capture. To address these challenges, we present a live-cell imaging approach with NucleoLIVE™ and MortaLIVE™. This new approach not only allows for real-time assessment of organoid health and response to treatments, but also for reproducible and accurate biological data capture through uniform dye penetration of 3D cultures, and the non-toxic nature of these dyes. **Methods:** NucleoLIVE™ and MortaLIVE™ were used for viability and cytotoxicity assessment in human organoid models. These dyes are two non-toxic dyes, one for nuclear staining and the other for live-dead evaluation, which were applied to heart, retinal, and brain organoids under hypoxic stress, and to endometrial organoids for preclinical toxicology screening of known therapeutics (e.g., Relugolix, Stauroporine, Tamoxifen).

Results: Our imaging studies demonstrate that these non-toxic dyes provide a robust and data-rich solution for viability assessment in 3D models. In hypoxia studies, the dyes successfully detected elevated cell death and stress. In endometrial organoids, the assay effectively distinguished between compounds with high (Stauroporine), moderate (Relugolix), and minimal (Tamoxifen) cytotoxicity. Notably, this live-cell platform was sensitive enough to identify a potential dose-dependent, off-target toxicity from Relugolix, highlighting the assay's sensitivity for detecting specific toxic thresholds. The non-toxic nature of the dyes further allowed deep staining of 3D structures, enabling optimal data capture from these valuable organoid models. **Conclusions:** By providing a reproducible and data-rich assay that's ideally suited for organoid cytotoxicity evaluation, NucleoLIVE™ and MortaLIVE™ non-toxic live-cell dyes are a critical enabling technology platform that promises to accelerate the regulatory adoption of organoids as a NAM.

PS 4164 Quantitative Assessment of Mitochondrial Toxicity in Liver Organoids Using the Seahorse XF Mito Tox Assay and XF Flex Organoid Microplate

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Background and Purpose: Drug-induced liver injury (DILI) is a major challenge in drug development and safety assessment, and mitochondrial dysfunction is a key mechanism underlying it. Liver organoids—3D structures derived from pluripotent stem cells or primary liver tissue—offer a physiologically relevant model that better recapitulates liver architecture, cellular heterogeneity, and metabolic function compared to traditional 2D hepatocyte cultures or animal models. This study demonstrates the application of the Seahorse XF Mito Tox assay for quantitative evaluation of mitochondrial toxicity in liver organoids using the newly developed XF Flex organoid microplate. **Methods:** The XF Mito Tox assay quantifies mitochondrial toxicity through the Mito Tox Index (MTI), which distinguishes between inhibition and uncoupling of the electron transport chain. MTI is a normalized, unitless metric that enables reproducible comparisons across different culture formats. While previous applications of the XF Mito Tox assay focused on 2D models (e.g., HepG2, primary hepatocytes, HUREL co-cultures), its use in 3D matrix-embedded organoids was limited due to incompatibility with plate formats. The XF Flex organoid microplate overcomes this barrier, supporting matrix-embedded organoid culture and real-time mitochondrial respiration measurements. **Results:** Using mouse hepatic organoids, we evaluated four model hepatotoxic drugs known to impair mitochondrial function. The assay successfully quantified mitochondrial toxicity and demonstrated full compatibility with the organoid format, confirming the utility of the XF Flex organoid microplate for drug safety screening. **Conclusions:** This study introduces the XF Mito Tox assay as a robust and reproducible platform for assessing mitochondrial toxicity in liver organoids. The integration of the XF Flex organoid microplate expands the assay's applicability to 3D culture systems, enhancing its relevance for predictive DILI screening in preclinical drug development.

PS 4165 Evaluation of RNA preparation for Induction Studies in Primary Human Hepatocytes in 2D and 3D Formats

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Background and Purpose: Spin-column RNA purification is widely used for hepatocyte CYP450 induction assays but adds handling steps that could affect recovery. We assessed whether an extraction-free direct-lysis approach (TaqMan™ Cells-to-CT™ HepatoExpress™; Thermo Fisher Scientific) produces induction results comparable to spin-column-purified RNA across common hepatocyte culture formats. **Methods:** Primary human hepatocytes were cultured in 24-well and 96-well plates and as 3D spheroids. Induction experiments were performed under standard conditions. For each condition, we collected triplicate biological samples and ran duplicate technical RT-qPCR replicates. RNA was prepared either by direct lysis (Cells-to-CT HepatoExpress) or by spin-column purification. Expression of CYP1A2, CYP2B6, and CYP3A4 was quantified by qRT-PCR; fold-change was calculated relative to matched vehicle controls and normalized to internal reference gene, GAPDH. Method- and format-related differences were compared within each dataset using the 2^{-ΔΔCT} method. **Results:** Across both 96-well and 24-well plating formats, induction measurements obtained from direct-lysis preparations were comparable to those from spin-column RNA for all three genes. A plate-format effect was observed: 24-well cultures displayed slightly greater induction than 96-well cultures under otherwise matched conditions for some of the CYP genes. In primary hepatocyte spheroids, direct-lysis and spin-column preparations yielded comparable expression results. **Conclusions:** Within the conditions tested, a direct-lysis, extraction-free RNA preparation produced RT-qPCR induction readouts comparable to spin-column RNA in primary human hepatocytes, including 3D spheroids. The modest increase in induction observed in 24-well versus 96-well plates indicates that culture format can influence induction magnitude and should be considered when comparing studies.

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Background and Purpose: Regulatory agencies are interested in assessing the potential toxicity of surfactants due to their use in consumer products, including household cleaners, personal care items, and pharmaceuticals. Inhalation is one of the routes by which human exposure to these chemicals can happen. To evaluate the respiratory toxicity of these chemicals, a normal human bronchial epithelial cell line (BEAS-2B), and two reconstructed human respiratory epithelium (RHRE) models—a bronchial (MucilAir, Epithelix), and an alveolar model (^hAECS; AlveoliX)—were exposed to two surfactants: oleoyl sarcosine and Triton X-100. **Methods:** The biological systems were treated with the two surfactants by pipetting (30 µL, 100 µL/cm²), either as a single (in BEAS-2B, MucilAir, and ^hAECS) or as repeated exposures (MucilAir only). For single exposures, five concentrations of the two surfactants were tested for 24 hours. Cellular effects were evaluated approximately 24 hours after, and after recovery periods of 3 days (^hAECS) and 7 days (MucilAir). For repeated exposures, MucilAir tissues were exposed to three concentrations of the two surfactants for 4 hours daily (with 20 hours of recovery between exposures) consecutively for 4 days. Cellular effects were assessed each day after exposure (~4 hours) and 7 days after the last exposure. Cellular effects assessed included cytotoxicity (lactate dehydrogenase release) and cell viability (PrestoBlue assay) for all three systems. Secretion of interleukins (IL-6 and -8 (CXCL-8; MSD V-Plex Assay) was assessed for BEAS-2B and MucilAir. Barrier integrity, using transepithelial electrical resistance (TEER), was measured for MucilAir and ^hAECS. Cilia beat frequency (CBF), average active area (AAA), and histology were assessed for MucilAir. **Results:** In BEAS-2B cells, a concentration-dependent decrease in cell viability was observed after single exposure to oleoyl sarcosine and Triton X-100, with higher concentrations of Triton X-100 needed to cause toxicity. A concentration-dependent increase in IL-6 and CXCL-8 was observed for both surfactants. In MucilAir tissues, single exposure to the two surfactants elicited concentration-dependent effects. At the two highest concentrations, both surfactants caused a marked reduction in cell viability and barrier integrity, and increased cytotoxicity, confirmed by histology showing partial to complete cell layer destruction. At the middle concentration, viability remained unaffected, but cytotoxicity increased and TEER dropped markedly for both chemicals. TEER recovered after 7 days, though oleoyl sarcosine still showed slightly reduced viability. In ^hAECS, viability decreased after exposure to either surfactant in a dose-dependent manner, but more prominently for oleoyl sarcosine. Barrier integrity was completely abolished in cells treated with oleoyl sarcosine and showed long-lasting effects. For Triton X-100, there was a concentration-dependent decrease in barrier integrity that recovered over time. In the repeat-dose assessment, both surfactants showed a concentration-dependent decline in MucilAir barrier function after the first exposure. For oleoyl sarcosine, this trend persisted with repeated dosing. For Triton X-100, after a drop on day 1, differences between treated and control groups were less pronounced over time. **Conclusions:** BEAS-2Bs and ^hAECS responded similarly and were generally more sensitive to surfactant exposure than MucilAir tissues, particularly in terms of decreased cell viability and barrier integrity. Except for single exposure in MucilAir, oleoyl sarcosine was typically more toxic than Triton X-100 and caused longer-lasting impairment of barrier integrity in MucilAir and ^hAECS. The results from this work were used to inform a multi-laboratory study, the goal of which is to establish a standardized test method that can be used to help meet regulatory needs related to inhalation toxicity.

PS 4167 **Aerosol Application for Prediction of Respiratory Toxicity using a Human Airway Model**

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Background and Purpose: Traditional *in vivo* inhalation toxicology studies are costly, require large numbers of animals, and often fail to translate effectively to human outcomes due to anatomical and physiological disparities between animal and human respiratory systems. Consequently, there is growing interest in human-relevant *in vitro* models for assessing inhalation toxicity. Human lung organotypic cultures are currently being used to assess direct toxicity of inhaled chemicals in a case-by-case basis. However, the absence of an OECD guideline limits their broader regulatory adoption. Establishing such a guideline would provide a standardized framework and promote regulatory acceptance of these models. The objective of this project is to develop an *in vitro* inhalation toxicity screening protocol for risk-based decision making. We show that an EpiAirway™ protocol has the potential to allow prediction of human hazard classifications for acute inhalation risk by applying chemicals directly to the epithelial surface at the air liquid interface in solution or suspension. We also re-evaluate a subset of chemicals using aerosol exposure to determine whether the mode of application influences the resulting toxicity profiles. **Methods:** A panel of ten chemicals with known inhalation point-of-contact toxicity, spanning a range of toxicities and mechanisms of action, were tested using the 3D

human EpiAirway™ tissue model. Acute exposures were performed by applying the test materials directly to the epithelial surface for 4 h, followed by a 20 h recovery period, according to a harmonized protocol conducted in parallel at Charles River Laboratories (CRL) and MatTek. A subset of 4 chemicals were subsequently tested using aerosol conditions with a Vitrocell 12/12 Continuous Flow Exposure System (CRL only). Flow rates, temperatures and humidity of aerosols were closely regulated and continuously monitored. Barrier integrity (TEER), cell viability (AlamarBlue) and morphology changes (H&E staining) using AI analysis algorithms (Visiopharm) were used as endpoint measurements. The drc package R was used to curve fit data, calculate EC₁₀ values (the dose required to reduce a response by 10%), and compare results from the 2 laboratories. **Results:** Comparable results were obtained between laboratories with p values of >0.05 and <2.5-fold difference denoting no biological relevance of differences. EC₁₀ generated from direct application to EpiAirway™ successfully ranked the chemicals in order of UN GHS inhalation hazard. Compounds producing EC₁₀ < 5 mg/mL for TEER or viability endpoints (e.g., potassium dichromate) corresponded to the highest hazard categories, whereas chemicals with EC₁₀ values exceeding the maximum testable concentration (e.g., lactose) were classified as non-hazardous. Samples exhibiting TEER < 50 Ωcm² generally lacked sufficient structural integrity for histological analysis. For analyzable tissues, concentration-dependent morphological alterations were evident for both labs (quantitative image analysis by Visiopharm). Aerosol application of vehicle (humidified air) did not significantly alter TEER or viability compared to incubator controls (P>0.05: one way ANOVA). Furthermore, when normalized to surface area dose (µg/cm²), the dose-response relationships obtained from aerosol exposures closely aligned with those from direct applications. **Conclusions:** These findings demonstrate that this tiered *in vitro* testing strategy shows strong potential for predicting human acute inhalation hazard classifications. The approach represents a key step toward developing a predictive, risk-based testing framework for aerosolized chemicals. The broader research program aims to establish an integrated *in vitro* protocol capable of supporting regulatory decision-making for inhalation risk assessment. This initiative directly supports the global regulatory objective of replacing animal testing with scientifically robust, translatable, and cost-efficient alternative methods. This work is funded by the American Chemistry Council's Long Range Research Initiative.

PS 4168 **prophECG: An AI-Enabled, Single-Cell, Multiplexed Kinetic Image Cytometry-based Cardiotoxicity Screening NAM for Extracting Actionable Clinical Insights from Human iPSCs in Early Drug Development**

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Background and Purpose: Cardiotoxicity (CTox) remains a leading cause of clinical trial failures, post-FDA approval drug withdrawals, and post-market adverse drug reactions. Traditional preclinical CTox studies rely on animals and are costly and laborious, yet still poorly predictive of clinical outcomes. Here, we present the latest iteration of "prophECG": a new approach method (NAM) that leverages multiplexed Kinetic Image Cytometry (KIC) of human iPSC-cardiomyocytes (hiPSC-CMs) to obtain functional readouts (calcium transients [Ca], action potentials/membrane voltage [V], and motion/contraction [Mot]) which are used to calculate margins of safety (MoS=EC₂₀/effective therapeutic "free" concentration [ETPC₁]) for rank-ordering the relative CTox of test compounds. We further use a fully-explainable, white-box AI approach to amplify the utility, scale, and holistic clinical predictivity of our *in vitro* hiPSC-CM KIC model. **Methods:** We loaded hiPSC-CMs with Cal-520 (for Ca), BeRST-1 (for V), TMRM, and Hoechst. After acute drug exposure, we used Vala's IC200-KIC automated microscope to acquire videos of interleaved Ca and V images at 30 Hz/channel (60 Hz overall), along with static images of TMRM and Hoechst. Our proprietary image analysis software, CyteSeer, extracted single-cell waveforms and transient data (Ca & V, separately; and Mot derived from both Ca & V), from which >200 measurements each for Ca (255), V (255), and Mot (233) were obtained. The prophECG platform characterized compound-induced effects in 25 manually-curated measurements empirically correlated with 6 clinical E/ECG classes. Torque clustering (TC) was then employed to classify all >200 measurements from each readout to further augment and expand on our 6 pre-defined classes. **Results:** In a previous validation study, our assay predicted the clinical outcomes of 30 out of the 31 drugs we tested (96.8% predictivity). We also previously compared our *in vitro* results with E/ECG readouts to group 25 kinetic measurements into 6 empirical classifications, corresponding to: 1) heart rate (HR), 2) the QT interval, 3) the QRS complex, 4) TU (early/delayed afterpolarizations [EADs/DADs] and triangulation), 5) rhythm (Rhy, including severe arrhythmias, e.g., alternans and cell-cell asynchrony), and 6) arrest (Arr). In the present study, we added 34 compounds to the validation set (for a total of 65, spanning varying degrees and mechanisms of CTox). We generated a (preliminary) rank-ordering of these 65 compounds according to overall CTox and by subclasses of CTox, along with CTox "fingerprints" illustrating at-a-glance relative CTox risk across the 6 E/ECG categories as a function of the calculated MoS in each class. Unsupervised clustering via TC of all >200 measurements/readout yielded 19-23 classes of measurements. Comparing our 6 empirical classes from 25 curated measurements to the TC-generated classes revealed some expected

overlap, but also implied the existence of “hidden” subclasses and/or unexpected interplay between measurements. We posit that fully comprehending the underlying relationships amongst the measurements and classes will help elucidate causal mechanisms of action/toxicity. **Conclusions:** Our single-cell approach captures cellular heterogeneity that may be obfuscated by other whole-well/-field *in vitro* platforms, leading to higher sensitivity and thus predictivity. By using explainable AI (via TC), we are working towards causal models that exploit the data-richness of prophECG to move far beyond existing single-target cardiosafety paradigms (e.g., CiPA, for *hERG*) to give a more comprehensive view of CTox through a single NAM. We envision further scaling to “Clinical Trials-in-a-Dish” to de-risk drug portfolios early in the development process to subvert the pitfalls of traditional animal testing.

PS 4169 Replacing animal testing in local tolerance assessment of an injection device: Human *ex vivo* HypoSkin model as a New Approach Methodology (NAM)

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Background and Purpose: Local tolerance testing of medical devices and injectable formulations is critical to ensuring patient safety and regulatory compliance. In line with current regulatory trends promoting New Approach Methodologies (NAMs) for non-animal safety assessment, human-relevant *in vitro* and *ex vivo* platforms are being increasingly adopted to improve translational predictivity. However, conventional preclinical systems often fail to replicate the complex immune and structural features of real human skin, particularly in the context of intradermal delivery. HypoSkin®, an *ex vivo* human skin platform that preserves native immune cell populations and tissue architecture, provides a NAM-aligned solution for assessing local tolerance and immunoreactivity. In this study, HypoSkin® was used to evaluate the impact on tissue and immune response following intradermal injection using an innovative fixed-depth and fixed-angle delivery device, in comparison to a standard 30G syringe and a reference device. **Methods:** A two-phase workflow was established to accommodate the large injection footprint of the Pharma Latch Hollow intradermal delivery device. The device and a reference device were used to either pierce the skin (sham) or inject phosphate-buffered saline (PBS; 100-500 µL) into fresh *ex vivo* human skin obtained from a single donor. In parallel, the Comirnaty® COVID-19 vaccine was administered using the test device. Following treatment, 23-mm skin biopsies were collected from injection sites to generate HypoSkin® models, which were subsequently cultured for three days. For additional comparisons, standard HypoSkin® models were produced and then injected using a conventional 30G syringe (sham, PBS, or vaccine). Local tolerance was assessed by macroscopic imaging, histopathology (H&E staining), and multiplex cytokine profiling (29-plex panel analyzed with Audacy™ bioinformatics). **Results:** Injections performed with the test device, reference device, and 30G syringe were well tolerated across all models. No macroscopic or histopathological alterations were detected compared to untreated controls, regardless of the injected compound or volume. H&E analysis confirmed preservation of epidermal, dermal, and hypodermal structures, supporting the absence of local irritation or tissue injury. PBS injections using the device, the reference device, or the 30G syringe induced only slight and device-dependent cytokine secretion, with no notable overall effects compared to the no injection control. Administration of the Comirnaty® vaccine elicited cytokine responses comparable between the test device and the 30G syringe, while levels remained within physiological ranges. The pro-inflammatory control (LPS + TNF-α) confirmed model responsiveness, exhibiting a marked induction of cytokine release. **Conclusions:** This study demonstrates the robustness and human relevance of HypoSkin® for local tolerance assessment of intradermal injection devices. The customized workflow enabled controlled, intra donor comparison of multiple delivery systems, generating reproducible data aligned with regulatory expectations. The tested device was well tolerated, showing no structural damage or excessive cytokine release, and elicited immune activation comparable to that observed with a standard 30G syringe following vaccine injection. As an *ex vivo* New Approach Methodology (NAM), HypoSkin® provides a valuable non-animal, human-relevant platform that supports regulatory-compliant evaluation of both local tolerance and immunoreactivity of injectable delivery systems, facilitating the translation from preclinical safety testing to humans.

PS 4170 Harnessing Bioconvergence to Advance Organ-on-a-Chip Technology and Help Transform Drug Discovery and Development

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Background and Purpose: Bioconvergence integrates materials science and microelectronics with organoid biology transforming organ-on-a-chip (OOC) technology for more translational drug discovery. Our bioconvergence program seeks to produce a new generation of *in vitro* and *in silico* platforms by merging human 3D cell cultures with a semiconductor-based sensor-integrated microfluidic silicon chip that simulates interconnected multi-organ physiology at a microscale. We aim to achieve greater clinical translatability compared to existing cell and animal models, while also reducing our reliance on animals. Together with imec we aim to translate our validated organoid models into a scalable platform. The system roadmap includes enzymatic metabolic sensors, Transepithelial electrical resistance (TEER), top-and-bottom live imaging, and photonic sensing for low-volume, longitudinal, CMOS-compatible measurements. **Methods:** Using efficient and reproducible differentiation protocols, we generated human gut- and liver organoids from induced pluripotent stem cells (iPSC) and patient-derived (PDO) sources. Organoids were validated for lineage identity and function by immunofluorescence for key markers, RT-qPCR for gene panels, and functional assays including TEER and enzyme activity. Integration of the validated biology with the semiconductor platform was performed by coupling them to fluidic and electrical interfaces under sterile conditions. Sensor readouts were benchmarked against established commercial reference methods to assess accuracy, sensitivity, dynamic range and temporal resolution. **Results:** iPSC-derived gut organoids demonstrated epithelial architecture and barrier integrity as well as the expression of key transporters and metabolic enzymes (for example, BCRP, MRP3; CYP3A4, UGT1A1), alongside representative lineage markers (enterocyte/brush border [Villin], goblet [MUC2], Paneth [LYZ], enteroendocrine [CHGA], epithelial [E-cadherin]). iPSC-derived mature liver organoids express hepatocyte and cholangiocyte markers (for example, albumin; Sox9, CK7, CK19) and show Phase I/II metabolic competence and relevant drug transporters (for example, CYP3A4, CYP2C9, CYP1A2; GST; MDR1/P-gp, MRP2). These organoid systems, provide a robust foundation for investigating the viability and functionality of the platform. Prototype silicon chips sustain viability and function of Caco-2 cells and colon organoids under perfusion, establishing single-organ feasibility, sensor module characterization and calibration. Planned expansions include small intestine and duodenum PDOs and iPSC-derived liver organoids, followed by modular multi-organ use cases and compound response profiling. **Conclusions:** Converging validated human organoid biology with scalable semiconductor microfluidics yields a microphysiological platform with improved translational relevance for toxicology, DMPK, and disease modeling. By enabling longitudinal, low-volume measurements and modular multi-organ coupling, the platform bridges preclinical and clinical applications and supports the development of safer, more effective therapies while reducing reliance on animal studies.

PS 4171 Evaluation of Cell Health in Microphysiological Systems (MPS) under Different Perfusion Conditions Using Metabolic Monitoring

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Background and Purpose: Microphysiological systems (MPS) are microfluidic devices designed to reproduce key physiological functions of human organs and have attracted attention as alternative approaches to animal testing in drug discovery and toxicological studies. To enhance the reliability of MPS, it is essential to maintain and continuously monitor the physiological health of cultured cells throughout experiments. Our research group focuses on real-time metabolic monitoring as an indicator of cellular health, aiming to establish a quantitative assessment framework for MPS functionality. In this study, we investigated how medium perfusion rates affect cellular viability and metabolism in a hepatocyte-based MPS model and evaluated the usefulness of glucose and lactate as metabolic indicators for cell health monitoring. **Methods:** HepG2 cells were seeded on collagen-coated microfluidic chips (ChipShop GmbH) and perfused with culture medium running at flow rates of 0.5, 2.0, and 4.0 µL/min for 72 hours. Cell viability was evaluated by Hoechst33342 and EthD-1 staining, followed by quantitative image analysis using ImageJ. Effluent medium samples were analyzed for glucose and lactate concentrations using a high-sensitivity electrochemical measurement system developed by PHC Corporation, which enables measurements with less than 1 µL of sample within 15 seconds. To further characterize the effects of lactate accumulation, static cultures were performed with exogenous lactate concentrations ranging from 2 to 15 mM. In addition, cultures were exposed to the anticancer drug doxorubicin (DOX) under different lactate conditions to assess whether metabolic changes could serve

as early indicators of drug-induced cellular effects. These combined experiments were designed to clarify the significance of controlled perfusion and to evaluate the potential of metabolic monitoring for early detection of cellular responses in MPS. **Results:** Under perfusion culture, cell proliferation was suppressed at 0.5 $\mu\text{L}/\text{min}$ but maintained at 2.0 $\mu\text{L}/\text{min}$ and above. Metabolic analysis revealed that perfused cultures maintained glucose concentrations around 5 mM and lactate below 3 mM, while static cultures showed marked glucose consumption and lactate accumulation, exceeding 7 mM. These findings suggest that appropriate medium supply is critical for sustaining metabolic balance and cellular viability in MPS. In static cultures, increasing external lactate concentrations led to reduced glucose consumption and lactate production, accompanied by decreased cell proliferation in high-lactate environments. When DOX was introduced, both glucose consumption and lactate production declined earlier than in control cultures (around 46 hours), indicating that metabolic shifts reflected the onset of growth inhibition. Moreover, in high-lactate conditions, the magnitude of DOX-induced metabolic change was smaller, suggesting that uncontrolled lactate accumulation may lead to over- or underestimation of drug effects. **Conclusions:** This study demonstrates that optimization of perfusion flow rate is essential for maintaining metabolic homeostasis and cell health within MPS. Monitoring glucose and lactate concentrations provides a valuable means to assess culture conditions and detect early cellular responses to drug exposure. These findings highlight the importance of integrating metabolic monitoring into MPS to ensure reproducibility and reliability in toxicity and efficacy testing. Building upon these results, we aim to develop an in-line metabolic monitoring system that enables real-time assessment of cell status, thereby contributing to the advancement of more reliable MPS-based toxicological and pharmacological evaluations.

PS 4172 Placentation Disruption by Perfluorooctanoic Acid and Perfluorooctanesulfonate in Human Trophoblast Organoids

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Background and Purpose: Prenatal exposure to perfluorooctanoic acid (PFOA) and perfluorooctanesulfonate (PFOS) is associated with low birth weight, a condition often resulting from placental dysfunction. However, whether and how PFOA and PFOS affect human placentation and placental-specific functions remains unclear. **Methods:** We reconstructed a human trophoblast organoid model, incorporating a near-physiological proportion of extravillous trophoblast (EVT). The organoids were exposed to PFOA or PFOS for 7 days. **Results:** Exposure to PFOA at 10 nM significantly increased the proportion of villous cytotrophoblast (CTB) cells, while reducing the proportion of EVT and syncytiotrophoblast (STB) cells at 10 and 100 nM, respectively. A similar pattern was observed with PFOS, albeit at concentrations 10 times higher than those of PFOA. Mechanistically, both PFOA and PFOS inhibited trophoblast differentiation by antagonizing the transcriptional activity of cAMP response element-binding protein (CREB). This disruption in placentation impaired placental function, as evidenced by significantly decreasing hormone secretion and invasion potential. **Conclusions:** Our investigation provides mechanistic insight into the association of PFOA and PFOS with low birth weight observed in epidemiological studies, with PFOA demonstrating a stronger effect than PFOS. These findings may aid in evaluating the toxicity of emerging PFAS and support the development or selection of safer chemical alternatives.

PS 4173 A NOVEL APPROACH TO EXTRAPOLATE EPIAIRWAY IN VITRO INHALATION TOXICITY DATA TO IN VIVO INHALATION TOXICITY ASSESSMENT

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Background and Purpose: *In vitro* inhalation toxicity was assessed to evaluate the potential respiratory toxicity of two test articles of the same organotin chemical class, a known industrial chemical Dimethyltin dichloride (DMTC) and a new organotin chemical of the same class (MBT-1). **Methods:** Two *in vitro* experiments were conducted using reconstructed 3D human airway epithelial tissues (EpiAirway AIR-100, MatTek EpiAirway™). The first experiment tested both chemicals dissolved in vehicle at concentrations recommended by the MatTek protocol (20-200 mg/mL) to determine each chemical's GSH (Globally Harmonized System Classification hazard category, 2003). Then extrapolation of the expected *in vivo* air concentration was made based on the GSH classification levels of *in vivo* toxicity. To assess the relevance of established inhalation toxicity to a real-world exposure scenarios, a second experiment was performed using significantly lower *in vitro* concentrations—0.002–0.02 mg/mL at serial dilutions—representing a 1,000- to 10,000-fold reduction from the toxic *in vitro* doses used in the first Experiment. These concentrations were chosen based on the worst-case predicted exposure levels in the human breathing zone after the possible spillage of these chemicals. **Results:** Both chemicals showed significant cytotoxicity in the first experiment at the tested *in vitro* concentrations, with the cell viability falling well below the 75% threshold, classifying them within GSH Hazard Category 1 or 2. Based on the known acute *in vivo* toxicity of DMTC, this experiment correctly placed this chemical in the GSH classification. No cytotoxic effects were observed at any of *in vitro* lower concentrations range in the second experiment, as all cell viability measurements remained above 75%

relative to vehicle controls. **Conclusions:** These findings indicate that, despite high toxicity of the tested chemicals, the levels of DMTC and MBT-1 expected in occupational settings (similar to what people might be exposed to at work) do not pose a significant risk to human respiratory health. A novel approach to correlate *in vitro* concentration range (in concentrations preprepared in liquid vehicle) to a real *in vivo* air concentration in the working breathing zone was used in this study. Additional studies with the air pollutants of other GSH classes are needed to confirm a validity of this approach.

PS 4174 Novel tox readouts: high-throughput protein profiling captures compound cytotoxicity, immunotoxicity, and distinguishes on- from off-target bioactivity

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Background and Purpose: The toxicity landscape is changing, with increasing pressure to develop New Approach Modalities (NAMs), emphasizing *in vitro* models yielding insights that translate to patients. Protein profiling is particularly well-suited to these efforts, capturing ground-truth signals of toxicity and immune responses, but adoption has been limited by the high cost of proteomic platforms. **Methods:** Here, we leverage Nomic's Omni 1000, capable of absolute quantification of 1000 proteins in high-throughput and low cost, to analyze the secretome of 20,000 samples from cell-based models, simultaneously capturing compound MOA and signs of toxicity. To achieve this, supernatants from hepatocytes, cardiomyocytes, and microglia treated for 48 hours with 510 bioactive compounds at three concentrations were analyzed. **Results:** Across all samples, >700 proteins were affected by at least 1 compound. Each cell type presented unique secretory features, though certain phenotypes were shared reflecting toxicity modes of action. For example, cytotoxic compounds such as staurosporine resulted in intracellular proteins leaking out of cells and being detected in the supernatant; TLR agonists such as resiquimod induced the expression of a variety of cytokines, which were inhibited by anti-inflammatory compounds such as corticosteroids. Critically, paradoxical pro-inflammatory effects of corticosteroids such as methylprednisolone were also captured, characterized by the induction of SAA and IL-6, reflecting the observed immunotoxicity of this compound when delivered systemically at high dose in the clinic. We used cosine similarity to identify compounds sharing the cytotoxic phenotype of staurosporine, or the immunotoxic phenotype of methylprednisolone; of 20 compounds with liver exposure in the clinic, 16 (80%) display liver toxicity in patients, highlighting the translatability of secretome analysis as a readout for tox models. Finally, secretome analysis was inherently interpretable, and thus enabled distinctions between on-target bioactivity and off-target toxicities. In the case of cycloheximide, we captured 3 distinct, dose dependent activities: low dose inhibition of protein synthesis, immunotoxicity caused by superinduction of IL-6 at intermediate doses, and high dose cytotoxicity. Furthermore, when comparing a set of 11 mTOR inhibitors, we identified shared biomarkers reflecting on-target immune modulation and a distinct set of biomarkers for compounds with distinct off-target effects. Comparing the doses at which these effects arose enabled the characterization of compound potency and specificity. Finally, we compared the sensitivity of secretome analysis to the ToxCast reference dataset, as well as published reports of image-based toxicity profiling. For 152 compounds shared with the ToxCast database, two thirds showed signs of toxicity at lower doses using Omni 1000 than traditional readouts, whereas 58 of 59 yielded signs of toxicity at lower doses in our assay than by imaging. **Conclusions:** These results highlight that secretome analysis captures toxicity with sensitivities on par with state-of-the-art readouts, while providing critical mechanistic information that translate to clinical outcomes.

PS 4175 Protocol Transfer and Reproducibility of an Electrospun Scaffold-based In Vitro Human Full-thickness Skin Model

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Background and Purpose: *In vitro* full-thickness human skin models are important tools for testing cosmetics and chemicals, screening new pharmaceuticals, and human disease research. However, these models commonly use animal-derived collagen as a main structural element of the stromal matrix, resulting in stability and contraction issues, short lifespan and poor reproducibility. Additionally, culture media used to produce these models commonly contain undesirable animal-derived components including fetal bovine serum (FBS) and bovine pituitary extract (BPE). To address these shortcomings, we recently developed a full-thickness human skin model (Bio-Spun™ FT-Skin model) using electrospun scaffolds as the stromal structural component, together with open-source FBS/BPE-free culture media. In the current work, we evaluated protocol transfer and reproducibility of the Bio-Spun™ FT-skin model in a naive laboratory. **Methods:** To test the reproducibility and robustness of the Bio-Spun™ FT-Skin production protocols and Bio-Spun™ scaffold inserts, Bio-Spun™ FT-Skin models were produced by an independent contract research

laboratory (the Institute for *In Vitro* Sciences, IIVS). Tissue morphology and baseline viability were determined by histological analysis and WST-8 metabolism assays, respectively. A time-to-toxicity (ET50) barrier assessment assay that is commonly used as a quality control test for several commonly accepted regulatory assays was also performed. Parallel tissue lots were produced at BioSurfaces (Ashland, MA) and IIVS (Gaithersburg, MD) using identical lots of Bio-Spun™ scaffold inserts, cryopreserved NHDF and HEKn, and FBS/BPE-free culture media. All materials were prepared at BioSurfaces and shipped to IIVS by overnight courier. IIVS staff received ½ day of in-person training at IIVS and further 1 ½ hour training by zoom conference prior to performing the production runs. **Results:** Well-to-well baseline viability was assessed on n = 6 random tissues from 3 independent production runs from both laboratories using the WST-8 assay. Results show a low coefficient of variation (cv) ranging between 5.87-11.64 %, and good concordance between laboratories. ANOVA showed that run #2 was significantly different from runs #s 1 and 3 (p < 0.01). No statistically significant difference was observed between run #s 1 and 3 (p = 0.55). No overall statistically significant difference was observed between laboratories (2-tailed t-test, p = 0.67). The WST-8 assay is non-destructive, allowing histological assessment of the same tissues following the WST-8 viability assay. The morphology of the Bio-Spun™ FT-Skin tissues produced by BioSurfaces and IIVS was virtually indistinguishable. A robust viable epithelium with similar thickness, showing well-developed basal, spinous and granulosum layers was produced by both laboratories. Stratum corneum development between the 2 sets of FT-Skin tissues was also similar. The Triton X-100 (TX-100) ET50 assay was conducted to assess the functional barrier of the Bio-Spun™ FT-Skin tissues produced by the 2 laboratories in 3 independent production runs. The barrier of the tissues was found to be highly reproducible within and between laboratories. The observed average ET50 of 12.51 +/- 0.37 hours (cv = 2.97 %) demonstrated a very robust stratum corneum barrier of the models. No statistically significant differences were found between production runs (1-way ANOVA p = 0.30) or laboratories (2-tailed t-test p = 0.052). **Conclusions:** Bio-Spun™ FT human skin models were produced using animal collagen-free 3D electrospun scaffolds and open-source FBS/BPE-free culture media formulations. The models provide long-term stability without contraction and stromal degradation issues. Excellent reproducibility and robustness of the production protocols, Bio-Spun™ scaffold inserts and Bio-Spun™ FT-Skin models were demonstrated following technology transfer to a naïve laboratory. These next-generation, full-thickness human skin models offer the opportunity for reduced reliance on proprietary commercial models, and show promise for completely animal-product-free testing of cosmetics/chemicals, screening of new pharmaceuticals and more human-relevant disease modeling.

PS 4176 Integrating Inter-Individual Variability and Cellular Interactions: Transcriptomic Responses to Acrolein in a Donor-Matched Lung Co-Culture Model

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Background and Purpose: There is a growing movement toward New Approach Methodologies (NAMs) to improve human relevance in chemical safety assessment. Among these, human-derived *in vitro* models coupled with high-throughput molecular profiling are increasingly important in inhalation toxicology for generating mechanistic data. Currently, many respiratory toxicity studies rely on isogenic cell lines or primary cell-based epithelial monocultures, which lack intercellular communication that occurs *in vivo* and intrinsic inter-individual variability within human populations. Thus, relying solely on these models may not accurately capture human exposure responses. Since airway responses arise from coordinated activity among multiple interacting cell types, we used a donor-matched co-culture model of differentiated primary human bronchial epithelial cells (dHBECs) and primary human lung fibroblasts (pHLFs) from 14 donors to better represent cellular heterogeneity and inter-individual variability. Cells were exposed to acrolein, a reactive aldehyde and major toxicant in cigarette smoke and air pollution, to characterize transcriptomic responses using RNA-seq as a sensitive, unbiased readout of molecular perturbations. We hypothesized that acrolein would induce both shared and cell-type-specific transcriptomic responses, and that incorporating multiple cell types from a range of human donors would reveal mechanisms not captured by conventional monocultures or cell lines. **Methods:** dHBECs at air-liquid interface were co-cultured with donor-matched pHLFs and exposed to 0.6, 1.0, 2.0, or 4.0 ppm acrolein for one hour, with filtered air as the control. RNA was isolated separately from each cell type and sequenced on an Illumina NovaSeq (~150 million paired-end reads/sample). Quality control confirmed high sequencing depth and mapping efficiency. Unsupervised principal component analysis (PCA) was used to examine major sources of variation between and within cell types. Differentially expressed genes (DEGs) were identified using edgeR for each dose versus control (FDR < 0.05, |log₂FC| > 1.5). Enriched pathways and biological themes were identified by over-representation analysis. **Results:** Both cell type and donor identity contributed to variation in transcriptomic response to acrolein. At baseline, fibroblasts exhibited greater inter-donor variability than epithelial cells. Transcriptional responses in epithelial cells were strongly dose-dependent, increasing from 35 DEGs at 0.6 ppm to 870 at 4.0 ppm. Fewer DEGs were detected in fibroblasts (8-490 across doses), with donor effects exceeding dose effects in PCA. Only a small subset of DEGs overlapped between cell types, indicating distinct transcriptional responses, yet both converged on stress- and inflamma-

tion-related pathways. Epithelial cells were enriched for oxidative, proteotoxic, and stress-adaptive responses (p53, reactive oxygen species, unfolded protein response, mTORC1, hypoxia) and pro-inflammatory signaling (TNFα/NFκB, IL6-JAK-STAT3). Fibroblasts were enriched for inflammatory and stress-adaptive pathways (p53, TNFα/NFκB, IL6-JAK-STAT3, hypoxia), reflecting activation of common defense mechanisms despite divergent gene-level responses. **Conclusions:** Acrolein induced distinct cell-type-specific transcriptomic profiles while activating shared stress and inflammatory pathways in epithelial cells and fibroblasts. Inclusion of 14 donors revealed substantial inter-individual variability, particularly among fibroblasts, highlighting the importance of incorporating biological complexity and inter-individual human variability in predictive *in vitro* inhalation models. These findings demonstrate the utility of donor-derived multicellular systems as NAMs for mechanistic toxicology and for integration into risk assessment. These models improve translational relevance of *in vitro* data and provide greater context for understanding how biological complexity and inter-individual variability shape study outcomes, which is critical to ensure these models are predictive of human responses as the field increases reliance on *in vitro* approaches for human health decision making.

PS 4178 Primate *in vitro* NAMs : A Platform to Complement Human *in vitro* NAMs Model for Efficient Drug Safety Evaluation

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Background and Purpose: With the FDA Modernization Acts 2.0 and 3.0 and the April 2025 FDA roadmap to reduce nonhuman primate (NHP) use in drug safety studies, there is growing momentum to adopt human *in vitro* New Approach Methods (NAMs) for early safety testing. However, a persistent challenge in human NAMs is limited *in vitro*-to-*in vivo* translation, as unapproved compounds cannot be tested in human subjects before preclinical safety evaluation in animal models. We propose a primate *in vitro* NAMs platform using induced pluripotent stem cells (iPSCs), autologous immune cells, and primary tissues from cynomolgus macaques (Macaca fascicularis), each linked to personalized genomic data. This platform serves as a comprehensive translational toolbox to bridge the *in vitro*-*in vivo* gap in drug safety evaluation. Three core *in vitro* systems have been developed: (1) NHP iPSC-derived neurons, (2) NHP iPSC-based developmental toxicology assays, and (3) NHP primary tissue-derived gut organoids. Together with matching genomic information, these enable human-NHP side-by-side testing before costly NHP *in vivo* studies and can also support post-*in vivo* investigative toxicology, especially when observed toxicities in NHPs are not reproduced in human *in vitro* models. **Methods:** (1) NHP iPSCs were generated from cynomolgus macaques and differentiated into NGN2 and motor neurons using induction vectors and chemical cues. (2) AAV-based SALL4 reporter cell lines were created from NHP iPSCs to evaluate potential toxicity of Targeted Protein Degraders (TPDs) such as Proteolysis Targeting Chimeras (PROTACs) in parallel with human stem cell lines to determine EC₅₀ and dose-dependent effects. (3) Cynomolgus macaque gut organoids were developed for *in vitro* evaluation of gastrointestinal toxicity observed in NHP *in vivo* studies. **Results:** NHP iPSC-derived neurons showed appropriate lineage markers and electrophysiological activity confirmed by multielectrode array (MEA) recordings. The iPSC-based developmental toxicity assay demonstrated high accuracy, identifying EC₅₀ and dose responses for known degraders (thalidomide, lenalidomide, pomalidomide) and new candidate compounds. NHP gut organoids exhibited molecular and structural features comparable to human gut organoids, providing a robust system for cross-species comparative studies. Collectively, these tools enable Weight of Evidence (WoE)-based preclinical evaluations aligned with the FDA 2025 NAMs roadmap, supporting more informed decisions on when NHP models are appropriate before initiating *in vivo* studies. **Conclusions:** Our findings demonstrate that NHP *in vitro* NAMs complement human *in vitro* systems, bridging the translational gap between *in vitro* and *in vivo* studies. In line with the FDA's April 2025 NAMs roadmap, these NHP models can serve as Weight of Evidence (WoE) in IND submissions, helping determine when NHPs are the appropriate translational models. This approach enhances predictive safety, reduces reliance on live NHP studies, and advances the 3Rs principles in drug development.

PS 4179 Exposure to welding fumes induces systemic inflammation in not only welders but also office workers working in factories

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Background and Purpose: Welding fumes are complex mixtures of airborne particles generated during the welding processes, and primarily consist of metal oxides, including iron, manganese, chromium, and other trace metals. Welding fumes can induce various lung disorders and neurodegenerative diseases. In addition, heavy

metals in welding fumes can induce oxidative stress by increasing the levels of reactive oxygen species (ROS) and altering the expression of antioxidant enzymes. Oxidative damage is recognized by the immune system as a danger signal, leading to activation of inflammatory pathways. Therefore, welding fume exposure is involved in systemic inflammation, which may contribute to the onset and progression of several disorders. Welders are required by law to wear masks in Japan. Welding fumes are airborne, and in factories with offices on the same premises, employees other than welders may also be exposed to welding fumes. However, to our knowledge, no previous studies have investigated the inflammatory status of office workers in welding factories in Japan. In this study, we aimed to estimate the fume exposure risk of welders, office workers in welding factories, and general subjects living in cities far from factories by measuring blood cytokine and chemokine concentrations. **Methods:** The concentrations of cytokines (IL-1 β , IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, IL-33, and tumor necrosis factor α (TNF α)), interferon γ (IFN γ), and chemokines (C-C motif chemokine ligand 2 (CCL2), CCL3, CCL4, CCL11, CCL17, CCL20, chemokine C-X-C motif ligand 1 (CXCL1), CXCL5, CXCL9, CXCL10, and CXCL11) in the serum were determined. **Results:** We recruited 105 welders and 129 office workers in the factories from ten welding factories. Thirty people living in Kyusyu far from welding factories (general subjects) participated in the study. The serum concentration of IL-1 β was significantly higher in office workers and welders than that in general subjects. Importantly, the serum concentrations of IL-12p70, IL-17A, IL-33, TNF α , and CCL3 in office workers were significantly higher than those in general subjects, and there was no significant difference in the levels of these inflammatory molecules between welders and general subjects. **Conclusions:** What's particularly novel about this study is that it found that office workers who work in welding factories and are not legally required to wear respiratory protection are at high risk of developing systemic inflammation due to exposure to welding fumes, just like welders. In the future, it is important to closely examine the exposure status and risk management of office workers in factories and welders.

PS 4180 Triangulation of Evidence in Reconstruction of Historical Exposure and Health Risks for Nuclear Workers

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Background and Purpose: Worker exposure reconstruction at U.S. nuclear production facilities is particularly challenging because of missing personnel records, long operational histories, multitude of hazardous chemicals, and little recognition of non-radiological hazards until after the mid-1990's. The Hanford Nuclear Reservation produced plutonium from 1942 to about 1989, followed by extensive decommissioning, demolition, waste treatment, and cleanup. Due to the lack of an OSHA-equivalent safety and health program until recently, the large work force has few records on non-radiological exposure and poor documentation of chemical use and disposal practices. Estimating worker exposures, however, is critical for conducting epidemiological studies as well as for government programs on diseases related to workplace exposures. The purpose of this study was to use triangulation to produce surrogate exposure measures or ratings for potential substance exposure by specific job tasks and employment dates for Hanford workers. Exposures were related to health effects based on substance toxicology. Triangulation methods integrate available lines of evidence with differing sources of potential bias and uncertainty. **Methods:** Information on workers and from publicly available documents were triangulated to develop exposure and health risk ratings for diseases: 1) employment dates, job titles, and work location; 2) job task analyses in later years; 3) dosimetry data; 4) historical chemical use and release from site production processes, supporting activities, construction, and demolition/decommissioning; 5) worksite characteristics; 6) time periods for different chemical processes; 7) records of upsets, contamination events by location; 8) substance exposure matrices by job title and location; 9) development of the industrial hygiene program over time; 10) limited epidemiological studies of specific job tasks for a few health endpoints or on radiation exposure and cancer; 11) substance-specific toxicology. Job exposure matrices identify substances associated with over 100 job titles and locations at the site, involving production, construction, maintenance, waste handling/treatment, demolition, and research. The matrices vary in completeness, particularly for exposure timing. Potential exposures depending on job title, work location, and time period involved 1000s of chemicals, including hydrocarbon and chlorinated solvents, aromatic hydrocarbons, aromatic amines, nitrosamines, ammonia, acids, nitrogen and sulfur-containing compounds, metals (beryllium, lead, welding fume, mercury, chromium), diesel exhaust, asbestos and mineral particles/fibers, and radiological materials. Limited worker health studies (e.g., x-ray abnormalities v. lung function decrements) provide insight on substances and relative exposure specific to jobs titles (e.g., asbestos/silica v. other respiratory toxicants). **Results:** Higher exposure and health risk identified by triangulation for rating worker exposures include: 1) aromatic amines (bladder cancer) for nuclear process operator at the Plutonium Finishing Plant, 1955 to 1989; 2) asbestos, silica (lung disease/cancer) for plumber/steam fitter in reactor, process, and tank waste areas, prior to 2000; 3) lead, solvents (neurological and obstructive lung disease) for painters. Lead paint was widely used including to cover radioactive waste spills, prior to 2000. Ratings are upgraded based on longer employment particularly pre-1994, higher radiological dosimetry and internal intake levels, exposure incidents, and frequent overtime. **Conclusions:** Substance exposures and health risks can be reconstructed qualitatively for past nuclear workers. This methodology has broader application for estimates of exposures and health risks for other operations with historically large work forces and complex work histories.

PS 4181 Odor perception versus real-world fragrance exposure via inhalation

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Background and Purpose: Odor thresholds and irritation thresholds from chemicals have broad applications across scientific, regulatory, and industrial domains, including safety assessment, product development and quality control, and environmental air quality monitoring. In the cosmetics, personal care, and household product industries, odor perception is central to fragrance formulation, inhalation safety evaluation, and overall product acceptability. Fragrances are unique organic, semi-volatile substances distinguished by their odorous properties. Upon inhalation, fragrance molecules interact with specialized olfactory receptor neurons in the nasal cavity, generating sensory stimuli perceived as smell. The lowest concentration at which an odor can be detected is referred to as the *odor threshold*. Although odor detection occurs at extremely low concentrations, the perceived intensity can often create the impression of much higher exposure levels. Chemesthesis is the chemical sensation elicited by certain compounds, which occurs when odorant molecules activate the free nerve endings of the trigeminal nerve (cranial nerve V) located in the nose, eyes, and mouth, producing sensations of warming, cooling, tingling, stinging, or burning, depending on concentration. The lowest concentration at which these sensations are perceived is known as the *irritation threshold*, which is typically an order of magnitude higher than the odor threshold. In this study, odor thresholds and irritation thresholds were determined in human volunteers for eight individual fragrance ingredients. **Methods:** 30 healthy, non-smoking males and females, aged 20-38, were recruited to participate. The fragrance materials were phenylethyl alcohol, acetic acid, linalool, eucalyptol, menthol, styrene, terpineol, and cinnamaldehyde. We used a custom-designed air-dilution olfactometer (Olfasense GmbH, Kiel, Germany) to collect both odor and irritation thresholds for eight fragrance materials. The computer-controlled olfactometer presents precise dilutions of the chemical in an air stream at 20 L/minute for odor and 8 L/minutes for irritation. For odor detection, participants were presented with a series of three stimuli, only one of which contained the fragrance, and the others were clean air. The task was to identify the 'odor' presentation. Concentrations were presented in ascending order to avoid sensory adaptation. For irritation detection, Teflon nose plugs were used to present the chemical to one nostril while clean air was simultaneously presented to the other nostril. The concentration that activated the nasal branch of the trigeminal nerve enabled individuals to say which nostril was stimulated and represented the irritation threshold. **Results:** As hypothesized, irritation thresholds were significantly higher than corresponding odor thresholds, in some cases an order of magnitude higher. For phenylethyl alcohol (PEA), no irritation threshold could be obtained, as it was not able to be localized at the highest vapor concentration tested. No sex differences were observed for either threshold. The slope of the psychophysical function for odor and irritation differed across the tested materials, indicating that intensity increases faster for some materials as a function of concentration. **Conclusions:** Nearly all fragrance chemicals have the potential to elicit chemesthesis and result in eye and nose irritation; however, the concentration at which that occurs was significantly higher than what is detectable by odor and what is necessary to be used in fragrance formulations to ensure a positive fragrance experience. Establishing the distance between the odor and irritation thresholds for fragrance materials can guide formulations and avoid unpleasant sensory effects from fragranced product use.

PS 4182 Assessing PFAS Exposure in Donor Human Milk Across Lactation Stages

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Background and Purpose: Breast milk is widely regarded as the optimal source of nutrition for infants because it delivers essential nutrients, immune support, and bioactive components. When mothers are unable to provide their own milk, donor human milk is often recommended as an alternative due to its nutritional and therapeutic benefits in comparison to infant formula. Although donor milk is screened for pathogenic microorganisms and certain drugs/medications, current milk bank guidelines do not typically address maternal exposure to environmental contaminants. This gap is particularly relevant for per- and polyfluoroalkyl substances (PFAS), a class of persistent chemicals that have been consistently detected in maternal serum and breast milk in studies worldwide. Despite growing concern, U.S.-based biomonitoring efforts remain limited, with relatively few studies evaluating PFAS levels in human breast milk and assessing how these concentrations change over time or vary across geographically diverse populations. Given the widespread detection of PFAS and ongoing gaps in national surveillance, there is a need to characterize PFAS occurrence in donor milk and to better understand the factors that influence exposure during lactation. **Methods:** In collaboration with Mothers' Milk Bank Austin (MMBA), breast milk samples were collected from ten donors, each providing three samples representing different stages of lactation (n=30). Samples were stored at -80°C until analysis and extracted using a QuEChERS-based method with isotope-labeled internal standards. 21 PFAS (8 short-chain, 13-long chain) were quantified via ultraperformance liquid chromatograph tandem mass spectrometry (UPLC-MS/MS) using external calibration and matrix-matched quality controls. Donor socio-demographic information (maternal age, occupation, education level, and race/ethnicity)

was provided by MMBA and multivariate regression models were used to assess associations between PFAS levels and donor characteristics. **Results:** Overall, 11 of 21 PFAS were detected in donors. Short-chain compounds perfluoro-n-heptanoic acid (PFHpA) and perfluoro-n-hexanoic acid (PFHxA) were detected in 93% and 70% of samples with median concentrations of 0.31 and 0.12 ng/mL. Long-chain compounds including perfluoro-n-octanoic acid (PFOA), perfluoro-n-undecanoic acid (PFUnDA), and perfluoro-n-tetradecanoic acid (PFTrDA) were detected in 93%, 80%, and 73% of samples at median concentrations of 0.28, 0.52, and 0.19 ng/mL. Regarding temporal trends, PFAS concentrations were generally stable across the lactation period for most donors, although a few donors exhibited declines in certain PFAS over time. Donor sociodemographic characteristics were not significantly associated with milk PFAS concentrations. **Conclusions:** Several PFAS were detected in donor milk samples, with short-chain PFAS appearing more frequently, consistent with the phase-out of many legacy long-chain compounds. Although concentrations generally remained stable throughout lactation, modest declines in select PFAS for a subset of donors highlight the importance of evaluating individual variability. Incorporating additional covariates such as parity, dietary patterns, and personal care product use may help determine whether these factors influence PFAS transfer into milk. More comprehensive biomonitoring studies with a focus on current-use PFAS and how these chemicals change over the course of lactation are essential for assessing potential risks and estimating infant daily intakes. Such data will help inform evidence-based breastfeeding recommendations and support the development of strategies to minimize PFAS exposure in early life.

PS 4183 Methodological Limits in Assessing Long-term Blood Lead Trends

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Background and Purpose: More than 20 years of data are available from a blood lead monitoring program in a historical mining district in Butte-Silver Bow County, Montana, USA. Since the late 1980s there has been substantial remediation of mine wastes in Butte. Residential soil and dust lead abatement programs are ongoing. Pursuant to a USEPA order, studies are conducted every five years to assess the effectiveness of these activities in reducing lead exposures in Butte children. The third study will be completed in early 2026. The Butte-Silver Bow Health Department administers the blood lead monitoring program for families participating in the USDA Special Supplemental Nutrition Program for Women, Infants, and Children. A large number of 12 to <60 month old children have been tested each year since 2003 (257 to 464 children per year through 2019). The first Butte study evaluated laboratory-analyzed capillary blood lead data collected from 2003 to 2010 with a detection limit of 1 µg/dL. Since 2012 capillary blood lead testing has been conducted with the LeadCarell device which has a much higher detection limit (3.3 µg/dL). The purpose of this study is to examine the ability to reliably identify trends in blood lead levels (BLLs) using LeadCarell data, as lead exposures have declined over time, considering the high detection limit and false positive rates. **Methods:** After deidentifying individual records, the studies have used regression models to examine differences in BLLs over time and among categories of variables including season, child age, gender, neighborhood, and house age. Neighborhoods include Uptown, characterized by a high percentage of pre-1940 housing located atop historical mine workings, and the Flats with newer housing and less mining influence. Analyses have focused on children aged 12 to <60 months. **Results:** The first study demonstrated substantial declines in geometric mean BLLs from 2003 to 2010 (3.6 to 1.7 µg/dL in Uptown, and 3.4 to 1.4 µg/dL in the Flats). In the second and third studies, very high rates of nondetect results (>75%) have limited analyses to rates of elevated BLLs. From 2003 to 2010, the percentages of 12 to <60-month-old children with BLLs >5 µg/dL declined from 41% to 15% in Uptown and from 31% to 5% in the Flats. Since about 2009, the percentage of BLLs ≥5 µg/dL among 12 to <60-month-old children living in either neighborhood appears to have leveled off, varying within a consistent range (5% - 12%) without apparent trend through the end of 2019. Similar percentages have been observed through 2024, but data limitations (including numerous disruptions in services related to the COVID-19 pandemic) complicate interpretation of more recent data. Significant differences in the likelihood of elevated BLLs were observed between categories of the house age (year built before 1940 or 1940 and later), neighborhood (Uptown/Flats), and age range (12 to <36 and 36 to <60 months) variables, none of which are solely related to historical mining influences. The first two studies found significant differences in blood lead level patterns between seasons, with a greater percentage of elevated BLLs occurring in warmer months when children spend more time playing outside. Consistent seasonal patterns were no longer apparent in the third study. A venous confirmation program begun in June 2022 has identified a high rate of false positives among LeadCarell results, with 33% false positives for results ≥5 µg/dL, and 85% among results in the 3.5 to <5 µg/dL range. The second study had a similar finding (~75% for results ≥5 µg/dL), but with more uncertainty. **Conclusions:** The high false positive rates observed with consistent venous confirmation testing suggest that LeadCarell results may not be reliable to assess trends in elevated BLLs. As the rates of elevated BLLs decline, isolated (rather than community) sources of exposure have greater influence on the observed rates. The lack of seasonal differences observed in the most recent study may indicate a reduction in the influence of soil lead as cleanup has progressed. Follow up with families of Butte children with elevated BLLs seldom identified one clear exposure source for BLLs in the range of 5-10 µg/dL. Higher BLLs were more likely

to have identifiable sources. With small numbers of affected children, such sources may alter community rates of elevated BLLs from year to year. Alternate approaches to screening with LeadCarell may be needed to discern the influence of community lead sources on childhood exposures.

PS 4184 Metal Exposure from Retained Shrapnel Fragments in War-Injured Veterans: Findings from Surveillance Efforts

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Background and Purpose: Evidence suggests that embedded metal fragments can oxidize *in situ*, thereby releasing metal ions into the systemic circulation permitting adverse target organ effects remote from the injury site. As a result, an on-going medical surveillance program was established to help inform the clinical management of war-injured veterans with embedded fragments. **Methods:** Surveillance-eligible veterans, identified through screening questions incorporated in the Department of Veterans Affairs' (VA) electronic health record system, are asked to complete an exposure questionnaire and provide a urine sample to assess systemic metal exposure. Using inductively coupled-mass spectrometry (ICP-MS), concentrations of 15 different fragment-relevant metals are measured in the urine, creatinine-adjusted, and compared to established reference values and known toxicity thresholds. In addition to considering the fragment as a likely source of a metal elevation, other sources of metal exposure, such as the presence of a metal implant or veteran reporting a metal-related hobby or occupation, are also considered. **Results:** Of almost 2,200 enrolled veterans, most are male (96.7%) and served in the Army (65.5%). The majority (72.4%) reported suffering only a blast injury, 18.5% reported both blast and bullet injuries, and 9.1% reported being injured only by a bullet. The mean self-reported age of participants at time of injury was 28.5 years and urine samples were submitted an average of 10.6 years post-injury. Most veterans (~76%) had no urine metal elevations, suggesting that if metal ions are being released from the fragment, they are not being released in excess, into the systemic circulation. When a metal elevation was observed, in most cases it was below a level of concern for a toxicity threshold. In some cases, metal elevations were significantly associated with non-fragment related sources of exposure. For example, veterans who reported having a joint implant were more likely to have elevated urine cobalt (9.8% versus 0.7%, p<0.001) and chromium levels (11.8% versus 3.9%, p=0.005) compared to those without a joint implant. However, veterans with a bullet injury were more likely to have an elevated lead level compared to those with a blast only injury (3.8% versus 1.1%, p<0.001), suggesting the fragment as the source of the metal elevation. **Conclusions:** Interpretation of urine biomonitoring results is complex; however, in the absence of other known metal exposure sources, elevated levels may indicate the release of metal ions into systemic circulation from embedded fragments. Because surgical morbidity often precludes fragment removal and injuries typically occur at a relatively young age, retained fragments can result in decades of sustained metal exposure. Thus, continued surveillance is crucial to better understand the kinetics of fragment-related metal exposure and the concentrations that may reach thresholds of injury over the long term. *This material is the result of work supported with resources and the use of facilities at the VA Maryland Health Care System, Baltimore, Maryland. The contents do not represent the views of the U.S. Department of Veterans Affairs or the United States Government.*

PS 4185 Characterizing Chemical Exposure in Human Serum Using Ion Mobility Spectrometry-Mass Spectrometry (IMS-MS): Comparison with Traditional Targeted Analytical Techniques

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Background and Purpose: Accurately characterizing human exposure to environmental contaminants is essential for evaluating potential health risks. Biomonitoring studies traditionally rely on targeted analytical techniques such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) and gas chromatography-tandem mass spectrometry (GC-MS/MS), which can quantify hundreds of known compounds with high sensitivity. However, in the context of chemical exposomics, these methods often focus on quantification of specific pre-selected analytes and depend on the availability of chemical standards, limiting their utility for the detection of emerging or unknown contaminants. High-resolution mass spectrometry (HRMS) has expanded exposomic analyses through non-targeted workflows, yet gaps remain in the comprehensive chemical characterization of complex biological samples. This study aimed to evaluate the utility of ion mobility spectrometry coupled with quadrupole time-of-flight mass spectrometry (IMS-QTOF-MS) as a hybrid, semi-targeted platform for simultaneous quantitative and non-targeted assessment of persistent organic pollutants (POPs) in human serum. **Methods:** A biphasic protein precipitation extraction was used to preserve a wide range of analyte polarities and reduce matrix effects. Serum samples from 32 individuals were analyzed using IMS-QTOF-MS (with and without liquid chromatography) and compared with LC-MS/MS and GC-MS/MS.

A suite of per- and polyfluoroalkyl substances (PFAS) was used to evaluate quantitative performance. The calibration curves of PFAS ranged from 0.025 to 100 ng/mL and were constructed using a single surrogate internal standard. Method performance was assessed using detection frequency, calibration curve linearity, and accuracy of quality control (QC) samples. **Results:** Both LC-IMS-QTOF-MS and LC-MS/MS consistently detected PFOS, PFHxS, and PFDoA in all serum samples, with comparable concentration trends between methods. LC-MS/MS achieved lower absolute detection limits overall. Calibration curves exhibited strong linearity for both techniques (coefficients of determination > 0.9 for all PFAS), with more than 50% of PFAS analyzed by LC-IMS-QTOF-MS and over 80% by LC-MS/MS achieving coefficients of determination > 0.99. LC-IMS-QTOF-MS demonstrated higher average accuracy and lower variance in QC quantification, suggesting strong reproducibility. Distinct drift time profiles were observed for individual PFAS in LC-IMS-QTOF-MS analyses, indicating enhanced selectivity in complex mixtures. **Conclusions:** LC-IMS-QTOF-MS generated quantitative data comparable to LC-MS/MS for major PFAS while offering the added benefit of ion mobility separation to resolve co-eluting compounds. The strong calibration linearity and improved QC reproducibility demonstrate its potential as a complementary platform for quantitative and semi-targeted biomonitoring. Expanding this workflow to additional chemical classes, such as pesticides and polycyclic aromatic hydrocarbons (PAHs), and including nontargeted analyses may enhance detection coverage and advance the comprehensive characterization of human exposures. This hybrid analytical approach represents a promising step towards improving the accuracy and scope of exposomic assessment in toxicology.

PS 4186 Metabolic Reprogramming in Gut Microbiota Exposed to Lithium

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Background and Purpose: Lithium (Li), a key component in rechargeable batteries and industrial products, is increasingly released into the environment due to the rapid expansion of the electric vehicle and energy storage industries. The widespread presence of Li in soil and water raises concerns about its potential toxicological impacts on human health, including homeostasis of the gut microbiome. In this pilot study, we hypothesize that Li exposure will negatively affect gut microbiota composition and function, in which metabolic reprogramming plays an important role. **Methods:** We conducted both *in vitro* and *in vivo* experiments to evaluate the biological effects of Li exposure. Three representative gut bacterial strains—*Escherichia coli* MG1655, Nissle 1917, and *Lactobacillus rhamnosus*—were individually cultured in anaerobic conditions and exposed to various concentrations of lithium chloride (0, 10, 20, 50, 100, and 500 µg/mL). Bacterial growth and viability were assessed, followed by untargeted and targeted metabolomics using liquid chromatography-mass spectrometry (LC-MS). In parallel, C57BL/6 mice were orally exposed to Li via drinking water for 4 weeks. Fecal samples were collected for 16S rRNA sequencing and metabolomic profiling to investigate Li-induced alterations in gut microbial composition, function, and metabolism. **Results:** Li exposure reduced bacterial viability in a dose-dependent manner and induced broad metabolic reprogramming across several key pathways, such as sulfur metabolism, amino acid metabolism, and nucleotide metabolism. In mice, we observed significant shifts in microbial taxa, including a decrease in beneficial *Lactobacillales* and disruption in energy-producing pathways such as glycolysis/gluconeogenesis and the pentose phosphate pathway. **Conclusions:** This study showed that lithium exposure may cause comprehensive alterations in gut microbial metabolism and composition. The results will contribute to understanding the mechanistic basis of Li-induced metabolic disturbances and potential adverse health, offering insight into environmental lithium toxicity at the gut-microbiome interface.

PS 4187 Use of *in vitro* reconstructed intestinal epithelium to study effects of repeated aluminum exposure

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Background and Purpose: Aluminum (Al³⁺) is one of the most prevalent elements in the environment and is commonly found in dietary products. The free cation, Al³⁺, is physiologically reactive and thus might affect metabolism when ingested. The gastrointestinal tract plays a pivotal role in its absorption, and it is estimated that approximately 40% of ingested aluminum is retained in the intestinal tissues. The present study aims to evaluate morphological alterations, barrier effects, and gene expression pattern changes after repeated exposure of *in vitro* intestinal tissue models to aluminum. **Methods:** Al³⁺ was applied twice a day for two hours at 5, 20, and 50 ppm for 12 days onto the *in vitro* reconstructed intestinal epithelium EpiIntestinal (MatTek). The tissue integrity was monitored over the course of the experiment using trans-epithelial electric resistance measurement (TEER). At the end of the dosing period the cumulated Al³⁺ absorption was assessed using Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) analysis and the morpho-

logical effects were analyzed by standard histological assessment and transmission electron microscopy (TEM). RT-qPCR was used to assess expression of genes associated with oxidative stress. **Results:** Barrier integrity monitoring via TEER did not reveal any significant differences between tissues exposed to Al³⁺ and controls except for the highest concentration. These findings were consistent with observed expression patterns of genes associated with tight junctions and with level of metallothionein genes associated with different amounts of Al³⁺. Histological and TEM analyses revealed that the highest Al³⁺ concentration was associated with vacuolization and microvilli shortening, while no pathological changes were observed in other samples. **Conclusions:** Overall, the results of this study suggest that the repeated exposure to Al³⁺ did not lead to significant detrimental effects of the 3D intestinal tissue model except for the highest concentration used. These findings contribute to our understanding of the safety of Al³⁺ exposure within the context of the gastrointestinal system since physiological concentrations of aluminum in blood stream are very low, typically < 10 µg/L (0.01 ppm). Nonetheless, further research may be needed to explore any potential long-term effects and to understand the possible implications for human health especially for chronic exposure at high concentrations. The observed morphological changes may deserve further attention.

PS 4188 Quantitative Assessment of Microbial Activity in a Veterinary Clinic Environment: Integrating ATP Bioluminescence Surface Assays with Airborne Culture-Based Sampling

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Background and Purpose: Veterinary clinics are complex microenvironments where microorganisms coexist with animal dander, disinfectants, and organic debris. This study quantified microbial activity within a mixed-species veterinary facility to evaluate spatial and temporal variations in bacterial and fungal presence, focusing on potential occupational exposure pathways relevant to toxicology and human health. **Methods:** Sampling was performed across nine functional zones (wash, grooming, wards, treatment, and control areas). Airborne microorganisms were collected using SKC BioStage impactors operating at 28.3 L/min for 3 minutes and plated on Tryptic Soy Agar (bacteria) and Malt Extract Agar (fungi). Plates were incubated for 72 hours at 36 °C for bacteria and 27 °C for mold, and results expressed as CFU/m³. Surface cleanliness was evaluated using ATP bioluminescence (Hygiena SystemsSURE Plus), with swabs taken above and below breathing level (floors, rails, counters, scales, and kennel surfaces). Temperature and relative humidity were concurrently recorded. Morning and afternoon samples were collected to capture operational variability during clinic activity. **Results:** Mean bacterial counts were highest in the grooming (523.9 CFU/m³ in the morning; 348.1 CFU/m³ in the afternoon), wash (2,504.1 CFU/m³ in the morning; 384.9 CFU/m³ in the afternoon), and ward areas (5,223.5 CFU/m³ in the morning; 279.4 CFU/m³ in the afternoon), suggesting persistent microbial loading where animals were bathed, handled, and housed. Fungal (mold) counts were also elevated in these zones, with morning means of 357.2 CFU/m³ (ward) and 279.7 CFU/m³ (grooming), reflecting favorable growth under moist, humid conditions (mean RH 68-74%). Office and control areas exhibited substantially lower microbial loads (bacteria ≈220-379; fungi ≈170-250 CFU/m³). ATP surface activity paralleled these patterns, averaging 523 RLU below breathing level in animal-contact areas versus 119 RLU above. Floors, scales, and kennel surfaces showed the highest readings (up to 1,276 RLU) in morning sessions, suggesting organic residue buildup and potential biofilm initiation. Morning values generally exceeded afternoon levels across CFU assays, indicating cumulative microbial loading associated with daily animal movement, grooming, and cleaning cycles. Bacterial counts consistently surpassed fungal counts, suggesting greater aerosol mobility and persistence of bacteria, whereas mold activity clustered in moist, poorly ventilated zones. Elevated mold levels in the wash, grooming, and ward areas may indicate sustained moisture retention and suboptimal air exchange despite disinfectant use, conditions favorable for fungal sporulation and mycotoxin production. **Conclusions:** Our preliminary data suggest that veterinary clinic environments represent dual biological exposure sources: bacterial aerosols with potential inflammatory and endotoxin activity, and fungal bioaerosols capable of allergenic or mycotoxic effects contributing to respiratory irritation. Compared with other occupational environments, microbial loads in the wash, grooming, and ward zones appear elevated and are more consistent with animal-handling or agricultural settings, implying comparable exposure potential. These trends may indicate that veterinary staff experience measurable bioaerosol exposures requiring targeted ventilation and humidity management. The observed concordance between ATP and viable counts suggests ATP bioluminescence can serve as a practical, real-time indicator of microbial contamination and exposure potential. Our preliminary findings point toward the utility of integrated ATP-culture surveillance for assessing occupational bioaerosol exposure and identifying biomarkers of inflammation or sensitization. Ongoing work aims to extend this dataset through antibiotic-resistance gene profiling and evaluation of sustainable ("green") disinfectants to inform One Health-based risk mitigation strategies.

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Background and Purpose: Drinking water (DW) is a potential pathway for human exposure to per- and polyfluoroalkyl substances (PFAS), prompting increased monitoring and regulatory attention. To enable accurate exposure assessment and targeted mitigation strategies, research is needed to characterize PFAS sources and quantify their relative contributions to DW contamination. Risk-based approaches should be used to prioritize sources for investigation, ensuring sampling focuses on those most likely to significantly impact DW and human exposure. **Methods:** In this study, monitoring data for 29 PFAS from the Environmental Protection Agency's (EPA) Fifth Unregulated Contaminant Monitoring Rule (UCMR5) program were analyzed to identify potential source-related chemical signatures in finished DW. Non-negative matrix factorization (NMF) was applied to resolve distinct PFAS source profiles and corresponding sample contributions. Samples were clustered by profile contributions; cluster geographic locations and profile signatures were examined to generate source hypotheses. PFAS DW concentrations were converted to estimated steady-state plasma concentrations using high-throughput toxicokinetic (HTTK) approaches and an assumption of an average water intake rate. Distributions of mixture bioactivity-to-exposure (BER) ratios in each cluster were developed by comparing, for each sample, the sum of observed sample PFAS concentrations to median modeled mixture points of departure (PODs) derived using a concentration addition model of mixture bioactivity, the sample PFAS composition (component ratios), and available single-component PFAS PODs (activity concentration at the cutoff threshold concentration, ACC) for a PFAS-relevant endpoint (peroxisome proliferator-activated receptor- α activation) from the ToxCast database, invitrodb (v. 4.3). **Results:** A total of 8564 samples contained at least one PFAS concentration greater than the method reporting limit; 16 PFAS were represented. NMF of concentrations identified 5 source profiles that explained 93.4% of the variance in the data. Samples were grouped into 10 clusters reflecting different patterns of source profile contribution. Source 1 was composed of medium chain-length (C5-C7) perfluorinated carboxylic acids; samples with large contributions from this source were co-located with PFAS manufacturers and importers. Source 2 was dominated by 6:2 fluorotelomer sulfonate, a replacement for PFOS in aqueous film-forming foams (AFFF). Source 3 included contributions from several perfluorinated carboxylic and sulfonic acids including perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) and had impacts throughout the U.S.; it may be indicative of historic PFAS contamination. Source 4, dominated by perfluorobutanoic acid and observed in locations along the Mississippi River, may reflect transport of PFAS manufacturing facility emissions. Source 5 contained a high proportion of perfluorobutane sulfonate, a replacement for PFOS in AFFF and other products. ToxCast ACCs and HTTK data were available for 7 PFAS for BER inclusion; 5 other PFAS were inactive for the endpoint. Median BERs for sample clusters ranged from 293 (cluster dominated by Source 2) to 2162 (cluster impacted by Source 1 and Source 4); the 5th percentile of BER in 7 clusters was less than 100. The minimum BER was 5.93, for a sample associated with Source 3 in San Diego County, CA. **Conclusions:** Existing targeted monitoring data from UCMR could be successfully mined for insight into the composition and distribution of PFAS sources in U.S. DW. Future work should aim to further link observed source profiles with available PFAS emission data. Additional work to characterize PFAS contamination with non-targeted analysis can increase the specificity of known source profiles and refine evaluations of risk.

PS 4190 Thinking Zinc: An Intervention study for mitigation of metal toxicity

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Background and Purpose: Zinc is an essential micronutrient with various key functions in human health. Experimental models indicate that zinc protects against genotoxicity through multiple mechanisms including its functions in antioxidant defense and DNA damage repair pathways. Numerous environmental metals share underlying mechanisms of toxicity that contribute to adverse human health outcomes. Studies from our laboratory and others demonstrate that multiple metals disrupt zinc finger motifs altering protein function. In these systems, supplemental zinc confers protection against the metals' effects. Based on this evidence, a community and academic partnership developed an intervention trial called *Thinking Zinc*. *Thinking Zinc* is a mechanism-informed intervention study of zinc supplementation to test the potential benefits of zinc in a mixed metal exposed tribal population living in proximity to more than 500 abandoned uranium mines. **Methods:** The study is a single-arm cohort design with longitudinal collection of biospecimens, specifically blood and urine. We investigated three biomarkers of DNA damage previously reported to be zinc responsive: DNA strand break (Comet assay), oxidative DNA damage (8-hydroxy-2-deoxyguanosine, 8-OHdG) and poly(ADP-ribose) polymerase (PARP) activity. **Results:** Urinary metal analysis finds *Thinking Zinc* study participants with elevated levels of multiple environmental metals compared to the general US population, including some that had at least 10% of the participants above the National Health and Nutrition Examination Survey (NHANES) 95th percentile. Metals concentrations showed substantial fluctuations over time, with greater differences detected

in urinary metals than in serum metals. Zinc supplementation did not appreciably impact urinary levels of measured metals nor significantly increase serum zinc in study participants. However, zinc supplementation did modulate DNA damage markers evaluated by comet assay and PARP activity. **Conclusions:** We conclude that zinc is an important determinant of DNA damage in human populations and that DNA damage biomarkers are not equivalent for detecting the impact of zinc on DNA damage.

PS 4191 A community-based participatory research approach to evaluate toxic metals exposure among agricultural, native communities in the Southern Andes

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Background and Purpose: Elevated exposure to toxic metals is a well-documented risk factor for adverse metabolic, cardiovascular, and developmental outcomes. Copper (Cu) mining releases Cu and co-occurring metals such as arsenic (As), cadmium (Cd), and lead (Pb) into surrounding soil and water, potentially creating multiple exposure pathways for nearby populations. In Peru's Cotabambas province, large-scale Cu extraction from the Las Bambas mine overlaps with local water sources and agricultural lands used by rural Quechua-speaking communities, placing them at elevated risk for environmental contamination. This pilot study investigated potential metal exposure in two such communities, establishing preliminary data to assess human and environmental health risks associated with Cu mining. **Methods:** A mixed-methods approach incorporating participatory research tools was used. Following local community approval, residents guided researchers through their land to identify suspected sites of contamination. Soil, water, dust, and potato samples were collected from potentially contaminated sites. Metals were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) to quantify As, Cd, Cu, and Pb concentrations. Additionally, soil samples were analyzed in the field by x-ray fluorescence (PXRF), and resulting maps of metal distributions were reviewed with community members in public assembly. Participant interviews provided contextual data on environmental practices and perceived exposure risks. All procedures were approved by institutional review boards at the Universidad Peruana Cayetano Heredia (Peru) and Rutgers University (USA). **Results:** A total of 37 soil, 54 water, 33 dust, and 16 potato samples were analyzed by ICP-MS. PXRF estimates of Pb concentrations were strongly correlated with ICP-MS measurements ($R^2 = 0.68$) but consistently underestimated absolute values. Soil metal concentrations exceeded international environmental thresholds for Cu in 6 (16%) samples and As in 1 (3%) sample. No potato samples exceeded international agricultural limits. Pb levels in dust exceeded regulatory U.S. limits in 19 (58%) samples, while water samples exceeded U.S. EPA maximum contaminant levels for As in 4 (7%) and Pb in 2 (4%) samples. Maps created with PXRF facilitated community engagement by visualizing potential contamination sources and helped build trust in the research process. **Conclusions:** PXRF is a feasible and informative tool for rapid, community-engaged assessment of toxic metal contamination. Findings indicate that Pb concentrations in dust frequently exceed regulatory limits, suggesting a potential risk for lead exposure that warrants biomonitoring in future studies. Elevated As levels in dust, though lacking formal standards, also raise concern for possible health effects. Continued community-based monitoring is essential to assess and mitigate toxic metal exposures in mining-affected regions.

PS 4192 Advancing Chemical Exposomics: Untargeted and Targeted LC-MS Approaches for Phase II Metabolite Analyses

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Background and Purpose: Humans are exposed to a vast array of chemicals daily, many of which may impact health. Accurate identification and quantification of these chemicals and their metabolites (e.g., phase II conjugation products) in biospecimens are needed for comprehensive exposure assessment and health outcome evaluation. Liquid chromatography-mass spectrometry (LC-MS) is a powerful tool for chemical exposomic research. Yet, most detected features remain unannotated due to limited reference standard materials and incomplete databases. To help overcome this bottleneck, we utilize and develop untargeted (LC-QTOF/MS) and targeted (LC-MS/MS) approaches for discovery and measurement of prominent phase II metabolites - mercapturic acids (MAs), glucuronic acids (GAs), and sulfates (SAs). Previously, we developed an untargeted workflow using an integrated library-guided analysis (ILGA) and annotated 424 metabolites consisting of 146 MAs, 171 GAs, and 107 SAs derived from ~200 parent chemicals in 844 human urine samples. In this study, we aimed to confirm the chemical identities of some of those metabolites by exposing mice to parent chemicals using LC-QTOF/MS. Then, quantify the confirmed metabolites by LC-MS/MS. **Methods:** Adult male C57BL/6J mice were injected intraperitoneally (IP) with 85 parent organic chemicals prepared in 16 different chemical mixture batches. Urine samples were collected after 24hr exposure and kept frozen at -80 °C until analysis. For LC-QTOF/MS experiment, the urine samples (50 μ L) were

mixed with an internal standard mixture (5 μ L) and 0.1% formic acid in water (195 μ L). The prepared samples (5 μ L) were injected and analyzed using Waters Acquity I-Class UPLC system and Synapt XS HDMS with an electrospray ionization source operated in negative mode. The raw data files were uploaded to UNIFI software for peak picking and metabolite profiling, which was facilitated by in-house curated metabolite libraries. **Results:** LC-QTOF/MS analysis of the mice urine samples have characterized 191 phase II metabolites (62 MAs, 68 GAs, and 61 SAs). These metabolites were derived from 85 parent chemicals consisting of aromatics (e.g., o-catechol and cresol), polycyclic aromatic hydrocarbons (e.g., naphthalene and pyrene), dietary phenolics/flavonoids (e.g., caffeic acid and eugenol), and biogenic volatile organic compounds (e.g., geraniol and citronellol). Currently, we are working on developing a quantitative LC-MS/MS method of all detected metabolites in mice urine samples by using LC-QTOF/MS detection results such as retention times, m/z of molecular ions ([M-H]⁻), and signature fragmentation ions. Afterward, we aim to measure these metabolites in human urine samples to examine prevalence of exposure to their parent chemicals. **Conclusions:** IP exposure of 85 organic chemicals in mice facilitated detection of 191 phase II metabolites in urine samples by LC-QTOF/MS. This is an advancement in untargeted field as there is often a lack of authentic standards available for chemical identity confirmation. In addition, the LC-QTOF/MS results can enable us to establish a LC-MS/MS approach for the quantitative analysis of target metabolites, which will help to expand metabolite list for biomonitoring and exposomic research.

PS 4193 The Application of Non-Targeted, Volatile Organic Compound (VOC) Mobile Air Monitoring in Fenceline Communities

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Background and Purpose: Fenceline communities experience disproportionate exposure to criteria air pollutants, namely PM_{2.5}. However, robust monitoring and assessment of non-criteria air pollutants, such as of the hazardous chemicals, volatile organic compounds (VOCs), is lacking. To investigate if VOCs also contribute to the disproportionate exposure burden in fenceline communities, mobile air monitoring at sites within two fenceline communities and one non-fenceline (background) community in Dallas County, TX was conducted to assess community exposure to VOCs. **Methods:** Mobile air monitoring was carried out using proton transfer reaction mass spectrometry (PTR-MS) using targeted and non-targeted analysis (NTA) to investigate VOC profiles in the community. Statistical clustering analyses of the NTA data assessed the similarity between community VOC profiles, and composite Z-scores of total VOC concentrations within each community were combined with mobile data to visualize the distribution of VOCs within each community. **Results:** Targeted analysis of the VOCs benzene, toluene, and ethylbenzene and/or xylene (BTEX) revealed periodic, significant elevations in their average concentrations in the fenceline communities compared to background. Statistical clustering analyses revealed distinct separation of the communities based on their VOC profiles and identified specific VOCs contributing to these clustering effects. Targeted and NTA revealed that multiple VOCs had periodically large spikes (hotspots) in their concentration throughout the fenceline communities. Notably, hotspots were not detected in the background community. **Conclusions:** Together, our data highlight that VOCs likely contribute to the disproportionate air pollutant exposure burden faced by fenceline communities. These data support the expansion of current fenceline monitoring programs and support the incorporation of VOC monitoring into these programs to protect residential and worker health. Furthermore, our study introduces new strategies for assessing non-targeted VOC datasets that can expand their utilization in ongoing efforts to monitor ambient VOCs and support efforts to regulate and improve air quality.

PS 4194 Air Quality and Respiratory Function among Veterinary Staff: A Pilot Exposure-Response Assessment in Clinical Settings

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Background and Purpose: Veterinary professionals are continually exposed to various airborne particulates and chemical disinfectants, which compromise respiratory health. Fine Particulate matter, specifically PM_{2.5}, penetrate deep into airways reducing airflow and causing inflammation. Limited research fails to explore the direct relationship of PM_{2.5} and pulmonary outcomes in conjunction with inflammatory biomarkers within veterinarian settings. This pilot study aims to investigate the exposure relationships among PM_{2.5} and respiratory performance among staff at a veterinary clinic in Southeast Georgia. **Methods:** Veterinary professionals are continually exposed to various airborne particulates and chemical disinfectants, which compromise respiratory health. Fine Particulate matter, specifically PM_{2.5}, penetrate deep into airways reducing airflow and causing inflammation. Limited research fails to explore the direct relationship of PM_{2.5} and pulmonary outcomes in conjunction

with inflammatory biomarkers within veterinarian settings. This pilot study aims to investigate the exposure relationships among PM_{2.5} and respiratory performance among staff at a veterinary clinic in Southeast Georgia. **Results:** Preliminary results performed from the CEM showed higher PM_{2.5} concentrations in animal handling zones compared to reception areas, with peak levels occurring after cleansing and pest decontamination: 1) Morning PM_{2.5} (1060.18 $\pm$ 1915.30 μ g/m³), 2) Afternoon PM_{2.5} (260.40 $\pm$ 317.72 μ g/m³), (insert total mass (microgram by cubic meter symbol). Early lung function testing indicated a mild reduction in FEV₁ (2.91 $\pm$ 1.45L), FVC (4.37 $\pm$ 1.29L), and elevated eNO levels (17.92 $\pm$ 14.86ppb) post-shift, compared to pre-shift FEV₁ (3.01 $\pm$ 1.50L), FVC (4.48 $\pm$ 1.34L), eNO (16.64 $\pm$ 14.39ppb). Individuals in this preliminary study had consistently below the eNO average compared to the normal range for healthy individuals (eNO <25 ppb), but still showed trends of decreasing eNO at the end of the work day (p < 0.05). Similarly, the preliminary study data for FEV₁ and FVC also remain in standard healthy range, (2.5-4.5L) and (3.0-5.5L) but show no statistical difference between morning and afternoon. This suggests that transient airway inflammation associated with chronic workplace exposure may negatively impact lung function overtime, triggering inflammation in the respiratory tract. **Conclusions:** The preliminary data show differences in PM_{2.5} between working and waiting areas, as well as a greater rate of change in lung function among individuals who frequently work in washing, grooming, and exam rooms, compared with those who work in reception areas. Based on this primary data, further research is needed to closely examine the risk relationship and response to higher particulate matter exposure in veterinary clinics compared to those with less frequent exposure, to inform future interventions to mitigate PM_{2.5} respiratory risks among animal care professionals.

PS 4195 Pinpointing the Pervasive: An Exposome Platform for Subtle Environmental Threats

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Background and Purpose: Exposure to environmental and lifestyle factors is increasingly recognized as a significant, yet under-characterized, driver of numerous emerging health trends, including rising incidences of cancer, neurodegenerative diseases, autoimmune disorders, and metabolic syndromes. The totality of these exposures, termed the "exposome", represents a critical gap in our understanding of disease etiology. Panome Bio developed a novel analytical platform for human exposure profiling, specifically designed to unravel these complex connections. **Methods:** First, we compiled and curated a relational database of small molecule exposure chemicals from public sources (CompTox, FoodDB, The Toxic Exposome Database, DrugBank). Second, we developed an optimized LC/MS-based analytical method. Through comparative evaluation and extensive spike-in experiments on human plasma, we developed a targeted method to quantify >200 compounds with >90% quantitative accuracy. Finally, we developed a robust computational platform for large-scale untargeted exposomics. This platform incorporated memory and compute-efficient peak detection and a machine-learning algorithm for batch effect/technical drift correction, demonstrating >90% coverage of spiked-in compounds and a mean coefficient of variation <20% in technical replicates. **Results:** Next, we applied this methodology to a serum cohort of individuals with Alzheimer's Disease (AD) as well as age/sex matched healthy controls. The results of this analysis identified 28 chemical exposures present in at least one of the serum samples. Comparison between healthy and AD patients resulted in four chemical exposures with differential abundance, including insecticides and pesticides with known neurological effects as well as the forever chemical perfluorooctane sulfonate. **Conclusions:** These findings demonstrate the utility of Panome Bio's exposome profiling platform for sensitively detecting and quantifying environmental chemical exposures in human biofluids, providing a powerful new approach for uncovering links between subtle environmental threats and disease.

PS 4196 Does the Dose Make the Poison? Time Will Tell

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Background and Purpose: Human exposures to environmental and anthropogenic chemical pollutants arrive through several different pathways and at varying rates. In addition to determining exposure pathways, the location of the exposure and its duration are also of key importance for modeling and risk assessment considerations. The GeoTox framework and R package was introduced to provide source-to-outcome modeling of exposures to chemicals and their downstream effects on different biological mechanisms. Users supply geo-spatially resolved steady-state exposure data and study how this affects a given population through a variety of *in vitro* endpoints. However, users of GeoTox are limited by a reliance on steady-state dose-response data, restricting study to longer timescales and less dynamic exposure scenarios. **Methods:** Here, we develop the GeoTox framework to model non-steady state exposure scenarios. The user supplies a time-series of exposure data and simulates a population of a given size, age, and weight classification through functionality provided by the R package htk. Using new GeoTox functions, this data can be analyzed to determine the impact of these exposure scenarios on biological response using traditional dose-response curves characterized by the Hill

equation. We also considered a time resolved dose-response equation derived from a Hill equation model with a dose- and time-dependent decay factor. We developed functionality to model pre-equilibrium chemical dose-response to determine how time-varying exposures impact different endpoints during the exposure. This framework allows users to simulate the biological response to a time-series of exposure values when the exposure does not fit into the steady-state scenario. Functionality is included to determine the magnitude and duration of a response above user-defined response thresholds. **Results:** We completed numeric simulations of three different exposure scenarios (acute, periodic, and constant) on populations of 500 individuals in ten-year age ranges (20-29, 30-39, 40-49, 50-59, 60-69, 70-79) and in "normal" and "obese" weight classifications for 39 chemicals included in the National Air Toxics Assessment list or in the GeoTox package data. Each exposure scenario produced the same total exposure over the same total time but followed different exposure profiles. An examination of distributions of peak blood plasma concentrations and blood plasma concentration area under the curve (AUC) values showed differentiation among the exposure scenarios that held across the various demographic combinations we simulated, with periodic and constant exposures producing similar distributions, distinct from the acute exposure distribution. We also observed a tradeoff between how close the peak plasma concentrations were and the AUC values across exposure and demographic groups for a given chemical. For instance, for 32 chemicals the mean of the ratio of the acute to periodic max plasma values was greater than 2, and for the 7 chemicals for which this did not hold, the mean of the ratio of acute plasma AUC to periodic AUC was at least 1.2. We analyzed the behavior of the pre-equilibrium dose-response functionality of GeoTox. We observed behavior of time-varying dose-response falling between two limiting behaviors, with a tradeoff between peak response values and total response AUC above set response thresholds. We noted that response behaviors depend heavily on the timescales of the exposure and the orders of magnitude of the exposures, binding association constant and dissociation constant (that define the equilibrium dissociation constant) of the molecule with its target receptor. We also determined a rough heuristic relating a decay term in the dose-response model with the dose-response curve behavior. **Conclusions:** To determine downstream biological effects of environmental exposures, it is important to consider not only geospatially resolved exposure data but also temporally resolved data. Expanding the functionality of GeoTox to handle non-steady state exposure time-series provides users with tools to begin exploring more dynamic exposure scenarios aside from low resolution and long-term exposure profiles. Our simulations demonstrate a need for more time-resolved dose-response experimental data as well as time-dependent dose-response models to study more complex exposure scenarios. This is particularly important for understanding spatially and temporally varying exposures across life-course.

PS 4197 Advancing the Systematic Understanding of Quaternary Ammonium Compounds (QACs): Harmonized Nomenclature and Consumer Product Use Patterns

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Background and Purpose: Quaternary ammonium compounds (QACs or quats) are a broad and diverse class of chemicals characterized by at least one positively charged nitrogen bonded to four carbon-based substituents. They are widely used as surfactants and antimicrobial agents in household and personal care products, with significantly increased use during the COVID-19 pandemic, raising concerns about their safety. However, inconsistent nomenclature and limited awareness of the breadth of QAC chemistries and their uses have hindered health and environmental research on this chemical class. As part of an effort to evaluate potential adverse impacts of QACs use in consumer products, we aimed to (1) create a systematic nomenclature for QACs and (2) assess the use of QACs in different consumer product categories. **Methods:** We developed a systematic nomenclature that clearly and concisely describes QACs and applied it to a list of 366 substances on EPA's Distributed Structure-Searchable Toxicity (DSSTox) Database, available through EPA's CompTox Chemicals Dashboard. Using this harmonized list of QACs, we assessed their presence in household and personal care products from the Mintel Global New Products Database (2017-2022), which includes over 140,000 products across 112 categories. **Results:** Using the nomenclature, we accurately categorized structurally diverse QACs by clearly and concisely describing the structure of each QAC or QACs mixture and identified 259 unique QACs in 19,460 consumer products. Of these 259, only 28 belonged to well-studied subclasses such as benzyl alkyl dimethyl ammonium compounds (BACs), alkyl trimethyl ammonium compounds (ATMACs), and dialkyl dimethyl ammonium compounds (DADMACs). The majority were less-studied QACs, including polyquaterniums, cocamidopropyl betaine, betaine, guar gum-derived galactomannans, and organoclays. These compounds were commonly found in hair care, skin care, and soap products, suggesting frequent and potentially chronic human exposure through dermal contact and incidental ingestion. Many of these compounds have little or no environmental fate or hazard data in the peer-reviewed scientific literature, indicating a significant gap between their widespread use and scientific understanding of their safety. These results highlight

the need for hazard testing and human exposure and biomonitoring evaluations for QACs. **Conclusions:** This systematic nomenclature approach strengthens the ability to clearly define, evaluate, and communicate about QACs as a chemical group. Incorporation of this nomenclature into DSSTox facilitates use of this nomenclature by researchers. Further, the incorporation of information from CompTox into other large chemical databases, such as PubChem and ChemSpider will support more widespread adoption of the nomenclature. We demonstrate an example of how this new nomenclature can be used to increase understanding of potential exposure to QACs by characterizing their use in consumer products. Our method of integrating standardized nomenclature with product-use data demonstrates how regulatory science can identify data gaps and inform efforts to design characterize the hazards and potential adverse impacts of chemicals in consumer products.

PS 4198 From Numbers to Mechanisms: A Unified Framework for Interpreting and Predicting Urinary Excretion Fractions

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Background and Purpose: The urinary excretion fraction (FUE), which represents the proportion of an applied chemical dose excreted in urine over time, has been widely used to back-calculate human chemical exposure based on measured chemical or metabolite levels in urine. However, current FUE values suffer from several major limitations. First, FUE can only be obtained through controlled human or animal experiments, and the biological mechanisms underlying measured values are often unclear. This makes it impossible to estimate FUE for most chemicals that have never been tested. Second, even when FUE has been measured, data usually come from very small studies with few participants, meaning that only average values are reported and interindividual variability remains poorly characterized. To overcome these challenges, we developed a mechanism-based, input-parsimonious approach that explains and quantifies how the body processes chemicals and how much appears in urine. This framework provides a scientific basis for interpreting existing FUE measurements and enables prediction of FUE for chemicals lacking human data. In addition, by combining the model with NHANES biomonitoring data, it allows estimation of personalized FUE values rather than relying solely on population averages. **Methods:** We developed a mechanistic model describing the biological processes that govern FUE. The model considers hierarchical and branching metabolism of a parent compounds to 1st-, 2nd-, 3rd-, ... orders of metabolites. It requires only five key parameters: the fraction absorbed into blood (fabs), the fraction metabolized at each metabolic order (M), the fraction undergoing Phase II conjugation (C), the relative yield of each metabolite (Y), and the fraction excreted unchanged in urine (U). Three of these parameters (fabs, M, U) can be estimated directly from chemical properties using the "human exposure and toxicokinetic module" in the PROTEX model (or other similar toxicokinetic models supporting calculating absorption and elimination processes), which has been validated against biokinetic data for over 1,200 chemicals. The remaining parameters, C and Y, can be obtained from experimental data measured in either *in vivo* or *in vitro* observations. Using nicotine biomonitoring data from 1,801 NHANES participants, we further identified how metabolism (M) and urinary elimination (U) vary across individuals. These population patterns allow the model to generate personalized FUE estimates for chemicals that share similar metabolic pathways. **Results:** The mechanistic model showed strong agreement with empirical data for 105 chemicals, with 93 of them exhibiting less than one log-unit difference between predicted and observed FUE values. This level of performance demonstrates that the model successfully captures the essential biological processes that determine FUE. A key insight is that chemicals can have similar overall FUE values but for entirely different biological reasons. For example, the literature reported measured FUE values of ~70% for both monoethyl phthalate (MEP) and monobenzyl phthalate (MBzP). At first glance, this may be interpreted in a counterintuitive manner that humans have a similar urinary excretion capacity for both compounds, despite their markedly different hydrophobicity levels. However, our mechanistic approach reveals that 61.2% of MEHP is eliminated in the unchanged form, whereas only 0.1% of MBzP follows this pathway, with the remaining fraction requiring conjugation before excretion. NHANES data further revealed large interindividual differences in metabolism and urinary elimination, with an approximately 40-fold range in M and a 10-fold range in U. When incorporated into the model, these differences produced nearly a 30-fold variation in personalized FUE for nicotine across the population, highlighting the importance of accounting for individual variability rather than relying on a single average value. **Conclusions:** This study establishes a mechanistic foundation for understanding and predicting FUE. The framework explains why chemicals differ in their FUE values, resolves inconsistencies that arise from small experimental studies, and enables FUE predictions for chemicals without human data. By incorporating population biomonitoring information, the model also provides person specific FUE estimates, supporting more accurate exposure assessment, toxicokinetic modeling, and chemical risk prioritization.

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Background and Purpose: Military personnel are subject to varied and complex chemical exposures with potential to adversely affect health and mission readiness. A common concern for warfighter health is uncharacterized mixtures, such as those produced from thermal degradation during fires, explosions, and jet exhaust. This collaborative effort between the Air Force Research Laboratory and the Johns Hopkins University Applied Physics Laboratory leveraged predictive chemistry models, large language models (LLMs), and targeted burn studies to develop an artificial intelligence (AI)-enabled model for predicting emissions from combustion and pyrolysis at varying conditions. **Methods:** A chemical burn test chamber utilizing a tube furnace was developed to allow precise and automated fuel, air and temperature control. The thermal degradation byproducts were measured with Fourier Transform Infrared (FTIR) spectroscopy and Gas Chromatography/Mass Spectrometry (GC/MS) for direct comparison to the models. The predictive chemistry model was built on a foundation of chemistry and physics, incorporating three modern AI tools to augment chemical kinetics models. The REXGEN AI reaction prediction tool generated candidate products. An LLM-driven literature search workflow was used to identify relevant chemicals, thermochemistry data, and reaction parameters in the literature. The Reaction Mechanism Generator (RMG) software integrated standardized, high-quality thermochemistry and reaction-kinetics databases with custom, literature-derived databases to automatically build chemical kinetics mechanisms. These reaction mechanisms were then used in Cantera model calculations tailored to the experimental conditions in the burn chamber. Results from generated reaction mechanisms were compared with experimental results and published literature. A case study was performed with 1,1,2-trichloroethane (1,1,2-TCA) to demonstrate the utility of this integrated workflow. **Results:** The LLM significantly improved search efficiency with a 10-fold reduction in time compared to manual searches. The REXGEN model accurately predicted relevant decomposition products ~75% of the time on the training dataset. To evaluate to utility of the computational workflow, model predictions were compared to pyrolysis and combustion data for 1,1,2-TCA. The most abundant pyrolysis byproduct was predicted to be hydrogen chloride (HCl), followed by isomers of dichloroethylene (DCE), which were confirmed experimentally. As the parent compound was completely broken down near 650° Celsius, DCE was further degraded to acetylene, chloroethyne and additional HCl. Adding oxygen at 500° C produced carbon dioxide, phosgene and likely carbon tetrachloride, in addition to HCl. There was strong agreement between laboratory and modeling results for 1,1,2-TCA thermal degradation. Modeling and experiments conducted for dichloromethane, cyclohexane and toluene also showed strong agreement between the model and collected data. Success with this process has provided a framework to apply to additional relevant chemistries. **Conclusions:** Together with predictive toxicology models, these thermal degradation models demonstrate a computational approach to identifying, predicting, and mitigating risk from the complex toxic exposures associated with high temperature processes. The project demonstrated a computational workflow for effectively predicting the byproducts of chemical combustion and pyrolysis under varying conditions with experimental validation. The example of 1,1,2-TCA demonstrated how specific byproducts and their relative concentrations can indicate the original parent compound and thermal history. This is a key step in identifying the source of airborne contaminants, prioritizing chemical sensor target development, and estimating health risks associated with operational exposures. Further, this research furthers the goal of creating user-friendly decision aids to ensure a healthy and ready force.

PS 4200 USEtox Modeling of Children's Exposures to Potential Endocrine Disruptors in Personal Care Products

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Background and Purpose: Endocrine disrupting chemicals (EDCs) in personal care products (PCPs) are an important source of exposure, especially for children, who are exposed daily to mixtures of known and potential EDCs through various routes including dermal, oral, and inhalation. Parabens are widely used preservatives found in products marketed to infants and children. Better understanding of children's aggregate and cumulative exposures to different parabens and potential EDCs in children is needed. **Methods:** We applied the USEtox 3.0 tool to systematically evaluate children's aggregate and cumulative exposures to parabens and potential EDCs. To assess the aggregate exposure to parabens, we focused on 6 parabens: methyl- (MeP), ethyl- (EtP), N-propyl- (NPrP), isopropyl- (IPrP), N-butyl- (NBuP), and isobutylparaben (IBuP). We identified 28 PCP categories containing at least one paraben commonly used by children, and collected ingredient list informa-

tion using the Mintel Global New Product database and Clearya. Each PCP category was assigned to appropriate age group(s) of children according to the product's target users and child behaviors, where a total of 8 age groups for children from birth to 11 years old (birth to <1 month, 1 to <3 month, 3 to <6 month, 6 to <12 month, 1 to <2 years, 2 to <3 years, 3 to <6 years, and 6 to <11 years) were considered. The weight fractions of parabens in each PCP category were gathered from the Pharos (Habitable) and the Interstate Chemicals Clearinghouse (IC2) databases. To assess the cumulative exposure to parabens and potential EDCs, we conducted a case study on newborn's (birth to <1 month) body lotions (n=5) and diaper cream (n=1) products. The ingredients of these products were queried in the U.S. EPA's Cheminformatics hazard module to identify potential EDCs other than parabens based on a high hazard score for endocrine disruption. The U.S. EPA's Chemical Product Database was then used to estimate weight fractions of the identified EDCs in these products. **Results: Aggregate exposures to parabens.** Direct dermal exposure contributes to >99% of children's aggregate exposure to parabens. MeP and NPrP result in the highest aggregate exposure doses across all eight age groups, primarily due to their presence in all PCP categories considered. The highest aggregate exposure dose for NPrP was estimated to be 1.57 mg/kg/d in children 3 to <6 years, and for MeP 1.54 mg/kg/d in children 6 to <11 years. The 8 age groups can be divided into three categories: birth to <6 months, 6 months to <3 years, and 3 to <11 years. Paraben exposure is dominated by different products within each category: baby wash (birth to <6 months), sunscreen (6 months to <3 years), body lotion, body wash, and hand cream (3 to <11 years). Within each category, the product use is similar, but the body weight increases with age, so the aggregate exposure dose tends to decrease as the age increases. The paraben exposure estimates are dependent on the model estimated product intake fraction (PiF), the paraben's mass fraction in the product, and the product mass applied. Overall, the paraben's weight fraction displays the largest variation between products, making it the most important factor in determining the exposure. **Cumulative exposure to parabens and potential EDCs.** We identified 6 potential EDCs other than parabens in newborn body lotion and diaper creams that can be modeled by USEtox, including benzoic acid, bronopol, liliol, propylene glycol, stearic acid, and triethanolamine. Parabens found in these products included MeP, EtP, and NPrP. The cumulative exposure doses range from 6.5 to 28 mg/kg/d across the six products. Of the six products studied, one body lotion product resulted in the highest cumulative exposure dose, which was dominated by stearic acid and triethanolamine. The second highest cumulative exposure dose was from the diaper cream product, which was dominated by propylene glycol, MeP, and NPrP. **Conclusions:** This study demonstrates the feasibility of USEtox modeling to assess children's aggregate and cumulative exposures to known and potential EDCs in PCPs. Policymakers can leverage these findings to prioritize chemicals in products for regulation and protect public health.

PS 4201 Oxidative stress, PGE2 and NGF/TrkA/P75^{NTR} dysregulation and neurodegeneration induced by thyroid signaling disruption following PFOS single and repeated treatment

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Background and Purpose: Perfluorooctane sulfonic acid (PFOS), a persistent industrial chemical, has been associated with impairments in cognition. While several studies have attempted to identify the underlying mechanisms, the precise pathways mediating these cognitive deficits remain incompletely understood. PFOS induces cell death in basal forebrain cholinergic neurons (BFCNs), a population critically involved in maintaining cognitive function, through disruption of thyroid hormone (TH) signaling. These neurotoxic effects could be mediated through multiple interconnected pathways, including the generation of oxidative stress, dysregulation of prostaglandin E2 (PGE2) signaling, and disruption of nerve growth factor (NGF) homeostasis, all of which have been independently linked to BFCN degeneration and cognitive dysfunction, and were reported to be induced after PFOS exposure. **Methods:** To systematically evaluate PFOS-induced neurodegeneration in BFCNs, we employed the SN56 cholinergic cell line derived from the basal forebrain. Cells were exposed to PFOS across a concentration range (0.1-40 µM) in combination with various pharmacological agents: thyroxine (T3; 15 nM), recombinant NGF (20 µM), MF-63 (1 µM), and N-acetyl cysteine (1 mM). **Results:** Our experimental results show that PFOS exposure (both single 1-day and repeated 14-day treatments) triggers oxidative stress through reactive oxygen species accumulation coupled with diminished NRF2 pathway activity. Furthermore, PFOS disrupts both PGE2 signaling and the NGF/TrkA/P75^{NTR} neurotrophic pathways, ultimately leading to BFCN cell death. These neurotoxic effects appear to be fundamentally mediated through PFOS-induced interference with TH signaling pathways. **Conclusions:** These findings provide valuable mechanistic insights into PFOS-induced BFCN neurodegeneration and the consequent cognitive decline, while simultaneously suggesting potential therapeutic strategies to counteract these detrimental effects.

PS 4202 **Impact of Developmental Atrazine Exposure on Antioxidant Activity and AD-Related Pathways in Wild-Type and *sorl1* Mutant Zebrafish Embryos**

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Background and Purpose: Dementia is a growing global health concern, affecting around 55.2 million people worldwide. Alzheimer's disease (AD) is the leading cause, responsible for 70-80% of all dementia cases. Despite extensive research, the exact causes of AD remain unclear due to its complex and multifactorial nature. Along with amyloid- β and tau, factors like neuroinflammation, oxidative stress, and mitochondrial dysfunction play major roles in its progression. The recently identified genetic risk factor SORL1 affects APP processing, tau seeding, and interacts with multiple brain metabolites. Lifestyle, environmental, psychosocial factors, and comorbidities can further increase AD risk, although research on gene-environment interactions remains limited. We chose to investigate atrazine (ATZ), which is one of the most widely used herbicides globally. Recent studies reported ATZ disrupts the neuroendocrine and neurotransmitter systems, causes neuronal inflammation, and accelerates brain aging. The purpose of this study was to examine the neurotoxic AD-related outcomes of ATZ exposure on antioxidant enzymes, glutamate and glutamine levels, and the amyloid pathway and tau in wild-type and *sorl1* mutant early development zebrafish model. We investigated the hypothesis that ATZ exposure will induce oxidative stress, disturb the glutamate-glutamine cycle, and interfere with amyloid and tau pathways. In addition, interaction with the *sorl1* mutation is expected to further increase AD-like pathophysiological changes. **Methods:** In this study, experiments were first conducted using wild-type zebrafish (*Danio rerio*). Wild-type fish were bred and embryos were collected 40 minutes post-fertilization. After rinsing, embryos were randomly distributed into Petri dishes in groups of 50. Within 1 hour post-fertilization (hpf), fish were exposed to 20 mL of ATZ at concentrations of 0, 0.3, 3, or 30 ppb ($\mu\text{g/L}$) at 28°C for 1 to 72 hpf, covering the full embryogenesis period. The same experimental setup was applied to the *sorl1* mutants but also included a wild-type control. Each treatment group was replicated at least six times. Antioxidant enzyme activities were determined using colorimetric assays. Glutamine and glutamate concentrations were then measured. Gene expression related to the amyloid and tau pathways was analyzed using qPCR, while amyloid- β peptide and tau protein levels were quantified using ELISA. Data were analyzed in SAS using ANOVA, followed by a post hoc least significant difference (LSD) test when significant differences were detected among treatments ($\alpha = 0.05$). **Results:** Results revealed that in wild-type fish, there were no significant changes in lipid peroxidation in any of the ATZ exposure groups. Similarly, there were no significant alterations in the antioxidant enzyme activity for CAT, CuZnSOD, MnSOD, GST, and GSH. In addition, no differences were observed in the glutamate and glutamine levels in response to ATZ exposure when compared to the negative control group (0 ppb) ($p > 0.05$). In the amyloid pathway, *bace1* gene expression was significantly downregulated in the 0.3 and 30 ppb ATZ-exposed groups ($p < 0.05$), but no significant changes were observed in *sorl1*, *apoea*, *apoeb*, *appa*, *apbb*, *psen1*, and *psen2* gene expression. Likewise, no significant changes were observed in BRI2 protein which is an upstream regulator of *bace1*. On the other hand, the *mapta* and *maptb* gene and the tau protein expression exhibited significant alterations in response to ATZ exposure ($p < 0.05$). *sorl1* mutant embryos also had no significant changes in lipid peroxidation, CAT, and GST activity in any of the exposed groups. However, CuZnSOD, MnSOD, and GSH activity were significantly increased ($p < 0.05$) in all the exposed groups when compared to the wild-type negative control. **Conclusions:** This study suggests ATZ exposure does not increase oxidative stress, does not alter antioxidant enzymes, and resulted in no changes in the glutamate or glutamine levels in the wild-type fish. Alternatively, ATZ exposure did influence expression of specific genes in the amyloid and tau pathways. Furthermore, the *sorl1* mutants showed an elevated antioxidant response, which confirms that the *sorl1* gene and its interaction with ATZ induce oxidative stress in early development, which may have a significant influence on other AD-related mechanisms. However, other AD-related outcomes are yet to be studied.

PS 4203 **Chronic non-cytotoxic manganese exposure disrupts glutamate homeostasis and pathways involved in the integrated stress response in Alzheimer's disease**

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Background and Purpose: Some evidence supports a connection between manganese (Mn) overexposure and Alzheimer's disease (AD). A key pathology shared by Mn neurotoxicity and AD is altered glutamate neurobiology. Neuronal hyperexcitability has been reported in AD patients and studies showed that acute exposure to supraphysiological Mn inhibits synaptic glutamate uptake. Moreover, glutamate is the brain's most abundant excitatory neurotransmitter and free amino acid involved in transcription, translation, and metabolism. How chronic Mn neurotoxicity contributes to AD is unclear. We hypothesized that chronic exposure to pathophysiological Mn levels disrupts glutamate homeostasis as a function of AD genetic risk. **Methods:** Human induced pluripotent stem cells (hiPSCs) derived from neurotypical individuals and AD patients (familial/sporadic) were induced to a neuroectodermal fate via dual SMAD inhibition. Cortical progenitors were maintained for ≈ 100 days to promote neuronal maturation and astrogliogenesis and then exposed to Mn

(0, 0.5 or 5.0 μM) for 4-6 weeks. Following exposure, ¹⁴C-glutamate uptake assay, single cell RNA-sequencing (scRNA-seq), pathway analysis, immunocytochemistry (ICC), and quantitative reverse-transcription PCR (RT-qPCR) were performed. Wild-type (WT) and APP/PSEN1 mice were fed 10 months control (70 ppm) or high Mn diet (2400 ppm) beginning at 3 months of age and were maintained throughout the study period. Liquid chromatography high-resolution mass spectrometry metabolomics was employed to investigate cortical metabolomic profiles. Brain tissue samples from the same animals used for metabolomics analysis were utilized for ribosome profiling (ribo-seq). **Results:** Chronic Mn exposure significantly decreased net glutamate uptake in AD patient-derived cortical cultures compared to neurotypical controls. ICC and RT-qPCR analyses indicated an absence of reactive astrocytes as confirmed by cytoplasmic localization of nuclear factor kappa B/p65, lack of hypertrophic morphology, and unchanged expression of pan-reactive genes. scRNA-seq cluster annotation identified major cortical cell types including glutamatergic and GABAergic neurons, astrocytes, intermediate progenitors, and other cycling progenitors. Qiagen Ingenuity Pathway Analysis and gene ontology (GO) analysis showed, besides alterations in pathways involved in glutamate neurobiology, the most significant enrichment in pathways and GO terms related to translation, mitochondria, and amino acid metabolism, in particular EIF2 signaling, suggesting involvement of the integrated stress response (ISR). *In vivo* metabolomics and ribo-seq showed that WT and AD mice respond differently to Mn as exemplified by clear segregation on PCA plots by treatment/genotype. Pathway analysis via EnrichR using differential translation efficiency genes enriched for similar pathways identified in our hiPSC model. **Conclusions:** Chronic Mn exposure induced alterations in glutamate homeostasis at the functional and transcriptional level, the effect of which seems to be dictated by an individual's genetic risk for AD. This suggests that polygenic AD risk genotypes, at least in some individuals predisposed to AD, confer susceptibility to dysregulated glutamate homeostasis. Absence of astrocyte reactivity suggests that the impaired glutamate uptake phenotype, at least in our model, is likely driven by mechanisms independent of neuroinflammation. Enrichment of biochemical pathways and GO terms associated with translation, cellular energy, and amino acid metabolism suggest dysregulated ISR. Intriguingly, our *in vivo* metabolomics and ribo-seq data suggest presence of a genotype-driven metabolic rewiring and translational response to chronic Mn exposure. Our next experiments will investigate the presence of genotype-dependent susceptibility to chronic Mn by interrogating differentially translated genes and evaluate levels of eIF2 α phosphorylation as a surrogate marker of ISR activation to explore differential chronic ISR activation driven by AD genotype. This work was supported by NIH R01 ES031401 (FEH ABB) and R01 ES035518 (FEH ABB). Acknowledgements: Center for Innovative Technology, Vanderbilt Univ. and TB-Seq, Inc., San Francisco, CA.

PS 4204 **Toxic effects of the obesogenic microbiome metabolite delta-valerobetaine on cognitive function**

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Background and Purpose: Environmental Alzheimer's disease (AD) arises from the interaction of genetic susceptibility with modifiable low-dose toxicities that evolve across the lifespan, with underlying mechanisms associated with mitochondrial dysfunction, oxidative stress, and inflammation. We recently found that the intestinal microbiome produces a metabolite, δ -valerobetaine (VB), that causes obesity in mice and is associated with the abundance of visceral and subcutaneous fat in humans. Epidemiologic data underscore the importance of obesity in heightening the risk of developing dementia and AD. Because VB causes obesity and inhibits mitochondrial fatty acid oxidation, we investigated whether VB causes neurotoxicity measured by a decline in spatial working memory in aged mice. **Methods:** 20-month-old male and female C57BL/6J mice from the National Institute of Aging (NIA) were fed standard mouse food *ad libitum* and water with 1.62 mM VB or without VB (Cont) *ad libitum* for 12 weeks. Body weight and water intake were measured weekly. Prior to the initiation of treatment and after 12 weeks of VB treatment, cognitive function was measured using a Y-maze. At the end of the treatment period, mice were euthanized, and brain tissues were collected. Histological analysis was conducted in the dentate gyrus (DG) and cornu ammonis 1 (CA1) regions of the hippocampus, and brain metabolomics was analyzed using high-resolution mass spectrometry. Human neuroblastoma cells (SH-SY5Y) were used to assess VB's impact on neuronal mitochondrial function. Protocols were approved by Emory University Institutional Animal Care and Use Committee (IACUC). **Results:** Brain VB concentration increased with oral VB intake and showed three to five times higher concentrations in brains from VB-treated mice than control mice, with no apparent differences between sexes. No baseline differences in % alternation during Y-maze were detected for either male or female mice. Following VB exposure, however, VB-treated aged male mice exhibited lower % spontaneous alternation ($58 \pm 3\%$) compared to the controls ($67 \pm 2\%$), indicating an impaired spatial working memory ($p < 0.05$). No effect of VB on cognitive decline was observed in aged female mice, suggesting a male-specific vulnerability to VB-induced cognitive dysfunction. No significant differences in the number of arm entries were observed between groups at baseline or after the treatment period. The hippocampus is critical for spatial learning, navigation, and memory, and histological analysis of the hippocampus revealed marked neuronal degeneration in the DG and CA1 regions of VB-treated aged males. In contrast, only mild neuronal degeneration was observed in VB-treated females, consistent with the behavioral results. Metabolomics results in both males and females showed impact on acyl-carni-

tines, indicating effects on mitochondrial energy metabolism. Mitochondrial effects were further studied in dose-response studies in SH-SY5Y cells. In undifferentiated cells, VB concentrations of 10, 20, and 50 μM significantly decreased MTT reduction compared with untreated controls, whereas 5 μM had no discernible change in MTT reduction. In differentiated SH-SY5Y cells, VB induced a clear dose-dependent decrease in mitochondrial respiration beginning at 5 μM and progressing through 50 μM . These findings reinforce prior findings that VB impairs mitochondrial activities and further show that VB exposure accelerates hippocampal neurodegeneration in aged male mice. **Conclusions:** VB concentration is increased in the mouse brain in response to increased bioavailability through diet. Increased VB in the brain causes a decline in spatial working memory in aging male mice but not in aging female mice, suggesting that VB toxicity contributes to sex-dependent cognitive deficits in aging. VB also had dose-dependent toxic effects on mitochondrial respiratory activity, indicating that VB-induced neurotoxicity parallels VB-induced obesity and is mechanistically linked to mitochondrial dysfunction.

PS 4206 Perfluorooctane Sulfonate (PFOS)-Induced Mitochondrial Dysfunction and Dopaminergic Neurotoxicity: Implications for Parkinson's Disease

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Background and Purpose: Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by dopaminergic (DA) neuron loss and the abnormal accumulation of α -synuclein. While most PD cases are sporadic with multifactorial etiologies, mitochondrial dysfunction and oxidative stress are strongly implicated in its pathogenesis. Environmental factors have gained increasing attention for their potential roles in exacerbating neurodegenerative processes. Beyond intrinsic causes, environmental toxicants with long biological half-lives, such as perfluorooctane sulfonate (PFOS), are likely contributors to both disease initiation and progression. PFOS, one of the most widely used per- and polyfluoroalkyl substances (PFAS), is recognized for its extreme environmental persistence, high bioaccumulation potential, and emerging neurotoxic effects. This study aimed to elucidate how PFOS exposure disrupts neuronal systems, focusing on its impact on mitochondrial function and mechanisms underlying neurodegeneration across multiple experimental models. **Methods:** We employed *Caenorhabditis elegans*, a mouse model, and human iPSC-derived dopaminergic (DA) neurons to investigate PFOS-induced neurotoxicity. In *C. elegans* were exposed to PFOS (0-75 ppm) to assess DA neurodegeneration, α -synuclein aggregation, mitochondrial viability using MitoTracker staining, and total reactive oxygen species (ROS) levels using H_2DCFDA fluorescence. In mice, both acute (15 min, 0-75 ppm) and subchronic (0.2, 2, or 10 mg/kg body weight for 1 month via *ad libitum* exposure) PFOS treatments were conducted to evaluate mitochondrial Complex I-IV activities in brain and liver. Western blotting of cortical tissues examined oxidative phosphorylation (OXPHOS) protein expression, while PFOS accumulation in plasma and cortex was quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Additionally, human iPSC-derived DA neurons (day 26 of differentiation) were exposed to 0-6.25 ppm PFOS for 24 hours and analyzed by immunofluorescence for tyrosine hydroxylase (TH) and beta III-tubulin expression. **Results:** In *C. elegans*, PFOS exposure induced dose-dependent increases in α -synuclein levels, DA neuron loss, decrease in mitochondrial viability, and ROS generation. PFOS exposure resulted in a dose-dependent reduction in DA neurons, while MitoTracker staining showed decreased mitochondrial viability, which correlated with elevated ROS levels. Relative mitochondrial DNA content remained unchanged across PFOS concentrations, suggesting that PFOS primarily impairs mitochondrial function rather than reducing mitochondrial content. Acute mitochondrial exposure assays showed significant inhibition of Complex III and IV activities in mitochondria isolated from liver and brain, whereas subchronic exposure (10 mg/kg BW for 1 month) decreased activities of all mitochondrial complexes (I-IV) in liver mitochondria. Cortical OXPHOS protein analysis revealed significant reductions in Complex III and V subunits. LC-MS/MS confirmed dose-dependent PFOS accumulation in plasma and cortex, indicating systemic bioaccumulation and blood-brain barrier penetration. In human iPSC-derived DA neurons, PFOS exposure caused dose-dependent reductions in TH and beta III-tubulin immunoreactivity, indicating dopaminergic vulnerability. **Conclusions:** Our findings suggest that PFOS exposure is associated with mitochondrial dysfunction and oxidative stress, α -synuclein accumulation, and dopaminergic vulnerability, associating PFOS as a potential health risk relevant to neurodegenerative diseases, particularly PD pathology. This work emphasizes the importance of elucidating PFOS-induced neurotoxicity to better understand its underlying molecular mechanisms. Ongoing studies aim to characterize the dose-dependent effects of subchronic PFOS exposure (0.2 and 2 mg/kg) in brain pathology associated with neurodegenerative disorders.

PS 4207 In Utero Exposure to Ultrafine Particulate Matter Impairs Cognitive Function in APP-KI Mice

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Background and Purpose: Prenatal exposure to particulate matter (PM) has been increasingly recognized as an environmental risk factor for neurodevelopmental and neurodegenerative disorders, including Alzheimer's disease (AD). Epidemiological and experimental studies suggest that maternal PM exposure may alter brain development and long-term cognitive function in offspring. However, the underlying neuropathological mechanisms remain poorly understood. In this study, we investigated whether *in utero* exposure to ultrafine PM affects cognitive performance and AD-related pathology in APP knock-in (APP-KI) mice. We focused on evaluating behavioral outcomes together with amyloid plaque burden and glial activation in the hippocampus and cortex, which are key regions involved in memory processing. **Methods:** Pregnant APP-KI dams were exposed to ultrafine PM or filtered air (Air) during gestation. Adult offspring at 12 months of age were subjected to behavioral testing using the Object Location Memory (OLM) and Object Recognition Memory (ORM) tasks to assess spatial and recognition memory, respectively. Brain tissues were collected and analyzed by ELISA to quantify total Abeta40 or Abeta42 expression levels in hippocampus and cortex. Immunofluorescence staining was performed to visualize amyloid plaques and activated glial cells, including microglia (Iba1, Clec7a) and astrocytes (GFAP, C3). Image analysis was conducted using Fiji/ImageJ for region-based quantification to compare the relative abundance of plaques and activated glia between PM and Air groups. Ongoing analyses are being applied to spatially assess microglial and astrocytic distributions surrounding plaques, as well as synaptic markers, Bassoon and Homer1, to examine potential effects on neuronal connectivity. **Results:** PM-exposed APP-KI offspring exhibited significant impairments in both OLM and ORM performance compared to Air controls, indicating deficits in spatial and recognition memory following prenatal exposure. However, ELISA quantification revealed no significant differences in total Abeta40 or Abeta42 expression in the hippocampus or cortex between the PM and Air groups. Similarly, immunofluorescence analyses showed no significant differences in the total number or area of amyloid plaques, nor in the abundance of activated microglia or astrocytes, in either brain region. These results suggest that the observed cognitive impairments occur independently of gross changes in amyloid deposition or gliosis. **Conclusions:** *In utero* exposure to ultrafine PM resulted in measurable cognitive deficits in APP-KI offspring, as indicated by impaired performance in OLM and ORM tasks. No significant differences were detected in amyloid plaque burden or in the number of activated microglia and astrocytes in the hippocampus or cortex. These findings suggest that prenatal PM exposure may influence neural function through mechanisms not directly reflected by amyloid pathology or glial activation levels. Further ongoing analyses using spatial imaging approaches will help determine whether alterations in plaque-associated gliosis or synaptic abnormality contribute to PM-induced cognitive impairment.

PS 4208 Investigating the Effects of Traffic-Related Air Pollution on Alzheimer's Disease Neuropathology in the Olfactory Bulbs of Transgenic Rat Model of Alzheimer's Disease

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Background and Purpose: Alzheimer's disease (AD) is increasingly recognized to arise via complex gene $\times$ environment interactions, and traffic-related air pollution (TRAP) has emerged as an environmental risk factor for AD. Olfactory dysfunction (hyposmia/anosmia) is one of the earliest clinical indicators of neurodegenerative diseases, including AD, and the olfactory bulb (OB) is the only part of the brain that directly interfaces with the external environment. Ultrafine particulate matter (<100 nm) from TRAP has been shown to reach the brain via the olfactory nerves in both humans and animal models. Together, these observations suggest that the OB may be a particularly vulnerable region for TRAP-induced AD pathology. However, it remains unclear whether chronic TRAP exposure exacerbates AD-related neuropathology and neuroinflammation in the OB. **Methods:** To model near-roadway exposures experienced by humans, we leveraged a unique exposure facility in which animals were housed in a vivarium adjacent to a heavily trafficked tunnel in Northern California and exposed to TRAP collected from the tunnel in real-time and delivered unchanged to the exposure chambers. Control animals were housed in the same facility and exposed to filtered air (FA). AD-relevant pathology and neuroinflammation were quantified in the OB of TgF344-AD rats, which express two human AD susceptibility genes encoding mutant amyloid precursor protein (APP_{sw}) and presenilin 1 (PS1 ΔE9). Male and female TgF344-AD rats were transported to the tunnel at 1 month of age and randomly assigned to either TRAP or FA groups until 15 months of age. Following exposure cessation, animals were euthanized, and the OB were collected for histological analyses. Amyloid- β fibrils were assessed by quantitative immunohistochemical (qIHC) analyses using the OC antibody, which specifically recognizes amyloid- β fibrils. qIHC analyses of Iba1 and GFAP were used to quantify the abundance of microglia and astrocytes, respectively. **Results:** TRAP had sex-specific effects on AD-relevant endpoints in the OB. In females, TRAP increased amyloid- β deposition, as indicated by increased area of OC immunoreactivity compared to FA controls. In contrast, there were no differences in amyloid- β depo-

sition between TRAP-exposed and FA males. TRAP induced microgliosis in males, evidenced as increased area of Iba1+ immunoreactivity, but had no effect on this endpoint in females. TRAP significantly reduced GFAP immunoreactivity in females, but not males. **Conclusions:** These findings indicate that chronic TRAP exposure causes sex-dependent changes in AD-relevant pathology in the OB, with females showing greater amyloid- β accumulation and males showing increased microglial activation. This supports the OB as a brain region vulnerable to TRAP and highlights sex as a key biological variable. Moving forward, darkfield and hyperspectral imaging with the CytoViva system will be used to visualize and quantify ultrafine particulate matter deposition in the OB and compare this to the spatiotemporal patterns of amyloid pathology and neuroinflammation. This work was supported by the NIH (grants RF1 AG074709 to P.J.L. and K.J.B.; T32 GM135741 predoctoral fellowship to A.B.; and T32 HL007013 predoctoral fellowship to H.H.P.).

PS 4209 Developmental MeHg exposure causes persistent changes in hiPSC-derived cortical models reflecting a neurodegenerative disease risk state

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Background and Purpose: The Developmental Origins of Health and Disease (DOHaD) hypothesis posits that developmental exposures can have long-term consequences, increasing the risk for later-life disease. Methylmercury (MeHg) is known to preferentially cross the placental barrier affecting the developing brain, especially glutamatergic (GLUergic) neurons. To understand the mechanisms involved in persistently altered GLUergic function as well as potential increased susceptibility for later-life disease, this study utilized a human induced pluripotent stem cell (hiPSC)-derived cortical model. We hypothesized that developmental MeHg exposure causes persistent transcriptomic changes visible long after cessation of exposure. We further hypothesized that these changes could reflect an age-related neurodegenerative disease risk state. **Methods:** Human iPSCs derived from male and female neurotypical control individuals were differentiated into cortical cultures using dual-SMAD inhibition. Cultures were continuously exposed to *in vivo* environmentally relevant (0.1 or 1 μ M) MeHg concentrations during days *in vitro* (DIV) 4-10 of development, a stage where the main cell types present are neuroepithelial cells and radial glia (RG). To assess transcriptomic effects of exposure, single cell RNA sequencing (scRNA-seq) experiments were performed at DIV 40, 150 and 280. Alterations in pathways were studied using Qiagen Ingenuity Pathway Analysis (IPA) and prediction of upstream chemicals was done with iPathwayguide (Advaita). **Results:** Cluster analysis of scRNA-seq data showed that all major cortical cell types were present with some differences in population size across days, but no notable changes across exposure levels. For both concentrations, significant pathway alterations ($p < 0.05$) were observed across all sampling days indicating a strong persistency of the effects of developmental exposure. In line with clinical observations, no clear concentration dependency was found. Many pathways in the top 25 altered pathways relate to healthy aging (e.g. eIF2, sirtuin, mTOR) and energy metabolism (e.g. oxidative phosphorylation, mitochondrial dysfunction). We then identified "core persistency genes" (CPG): genes that are significantly altered across all 3 time points and in 3 out of 4 main cell types (GLUergic neurons, proliferating or quiescent RG, and intermediate progenitor cells). KEGG analysis on these CPG revealed strong enrichment for neurodegenerative diseases, including Alzheimer's disease (AD). To further explore the connection between developmental MeHg exposure and risk for AD, we utilized scRNA-seq data obtained from a study done in our lab comprising of DIV 150 hiPSC-derived cortical cultures (from neurotypical control and AD patients). We found that all CPG were present in differentially expressed genes (DEGs) derived from this data. Bootstrap analysis revealed significant enrichment of these genes beyond chance. We then searched for predicted upstream chemicals in our AD DEGs. Though these cultures have never been exposed to MeHg, MeHg compounds were predicted ($p < 0.0001$) to be in the top 20 upstream chemicals. **Conclusions:** Effects of developmental MeHg exposure are still present 270 days following cessation of exposure. Cluster annotation of UMAPs derived from scRNA-seq revealed that cells matured as expected, with cell population size changing according to culture maturation stage. No clear effect of exposure on cell population was observed. We identified persistent changes in pathways and gene expression. CPG from this data set showed a link with AD. Interestingly, CPG are statistically enriched in our AD data. Next, our AD vs Control DEG list was enriched for MeHg-like changes despite never being exposed. Overall, these data show that developmental MeHg exposure causes persistent transcriptomic changes. Additionally, we showed a potential link between developmental MeHg exposure and later-life AD risk state. This research was supported by NIH [R01 ES07331 (ABB/MA), R01 ES031401 (ABB), and K99 ES036290 (AMT)].

PS 4210 Investigating the potential impact of FUS phosphorylation-dephosphorylation dynamics on subcellular localization in response to DNA Damage

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Background and Purpose: Frontotemporal dementia (FTD) is a devastating neurodegenerative disease characterized by changes in behavior and language production due to the progressive loss of neurons and glia in frontal and temporal cortical regions. While the neuronal death has been well characterized, the molecular mechanisms driving the initial cellular dysfunction remain poorly understood. Neuropathology from post-mortem tissue suggests that cellular dysfunction may be due to key RNA-binding proteins misfolding, resulting in changes in RNA homeostasis and the accumulation of protein aggregates in affected tissue. Approximately 10% of FTD cases contain cytoplasmic protein aggregates that are positive for the RNA/DNA binding protein fused in sarcoma (FUS). FUS performs various functions, including regulating DNA transcription, mRNA splicing, stress granule formation, and DNA damage repair. While typically localized to the nucleus, FUS can shuttle between the nucleus and the cytoplasm to accomplish its various functions. However, in FTD with FUS pathology, the protein misfolds and mislocalizes into the cytoplasm, exhibiting both toxic gain-of-function and loss-of-function effects. While FUS mutations are the best-characterized case of cytoplasmic aggregation, the majority of FTD patients do not have genetic mutations, suggesting that FTD-FUS may be caused by an environmental trigger. Indeed, double-strand DNA damage can be induced by various environmental triggers, including ultraviolet exposure and oxidative stress. Thus, studying the mechanistic basis for DNA damage may be the key to understanding the pathology of idiopathic FTD. FTD patients show increased γ -H2AX, a marker of double-strand DNA damage. Furthermore, previous work found that double-strand DNA damage induced via calicheamicin γ 1 (CLM) triggers DNA-PK-mediated phosphorylation of FUS (p-FUS) at 12 N-terminal sites. The resulting p-FUS then accumulated in the cytoplasm. However, there are discrepancies in previous findings regarding whether p-FUS becomes mislocalized in response to DNA damage, suggesting context-dependent results and a lack of reproducibility. In this study, we aim to investigate whether phosphorylation-dephosphorylation dynamics regulate the subcellular localization of FUS, and through what mechanisms. By investigating past discrepancies in conjunction with novel variables, we aim to elucidate the mechanisms underlying the molecular basis of FUS FTD pathology. **Methods:** Past reports on p-FUS primarily utilized fixed cell staining and subcellular fractionation to characterize the localization of p-FUS. Even so, each report performed each technique differently. As such, we set out to systematically compare each method used in the various reports. HEK293T or U2OS cells were treated with CLM to induce double-strand DNA damage. Using fixed cell staining, subcellular fractionation, and western blots, we characterized and quantified the amount of FUS in either the nucleus or cytoplasm observed between methods. Lastly, we utilized time-point assays and live-cell imaging to observe the kinetics of FUS phosphorylation and its potential role in FUS localization. **Results:** Overall, we report significant differences in the visualized observation of FUS localization between the tested methods. Namely, we directly compared the three different but previously utilized permeabilization techniques (Methanol, Triton-X-100 and Tween-20) and three different FUS polyclonal antibodies for fixed cell staining. We found that the observed localization of total FUS and p-FUS differed based on the permeabilization and the antibody used. **Conclusions:** Our results demonstrate that visualizing FUS localization is both technique-specific and regulated by phosphorylation states that dynamically respond to environmental and cellular stressors. We find that DNA damage, specifically double-strand breaks, can promote mislocalization. Our findings suggest that FUS phosphorylation may act as a molecular regulator of subcellular distribution and that persistent DNA damage drives a pathogenic shift toward abnormal accumulation. Understanding this modification will not only help elucidate the molecular mechanisms of FUS-related neurodegeneration but also provide insight into therapeutic strategies to restore protein function.

PS 4211 ENVIRONMENTAL CONTRIBUTION AND NEUROANTIBODIES (NAb) BIOMARKERS IN NEONATAL HYPOXIC-ISCHEMIC ENCEPHALOPATHY (HIE)

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Background and Purpose: To date, the etiology of HIE remains equivocal, as does the availability of diagnostic and prognostic biomarkers. We have proposed the use of autoantibodies against nervous system (NS) proteins (i.e., NAb) as potential biomarkers. In addition, the role of heavy metals, with their ability to induce neurotoxic damage and oxidative stress were investigated for their association with clinical outcome measures (e.g., apgar and MRI at 7d). **Methods:** Following IRB approval and parent consent, blood serum of reference at birth and 1d (total serum n=22) and HIE neonates selected for hypothermia therapy at birth, 1, 2 and 7d (total serum n=35) were analyzed by ELISA for serum NAb, IgM and IgG, against neurofilament triplet (NF), glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP), as well as levels of heavy metals (μ g/dl) Al, Cd, Cr, Cu, Fe, Mn, Ni and Pb measured by ICP in 1d samples. Apgar scoring occurred at birth, EEG conducted between 3-4d of birth and MRI at 7d were also measured. **Results:** HIE neonates (n=14) had signifi-

cantly ($p=0.04$) higher levels of Al, Cu and Mn compared to reference neonates ($n=5$). However, Spearman correlation indicated that 1 and 5 min apgar scores inversely ($r=-0.49$ to -0.71) and significantly ($p=0.001-0.04$) associated with levels of Al, Cd and Cu. Prevalence of NAb IgM against NF triplet proteins, GFAP and MBP was consistently higher in HIE neonates ($>50-88\%$ vs $13-35\%$ for reference) based on 6 and 24hr sera, with odds ratios (OD) of 4-9; $95\%CI$ 1-38; $p<0.001-0.01$). For anti-GFAP IgG, prevalence was 70% vs 35% , respectively; $OD=4$; $95\%CI=1.0806$ to 16.3246 . Furthermore, levels of anti-NF triplet were significantly higher in HIE ($p<0.003-0.04$) compared to reference at all time points, as were the GFAP IgG:IgM ratios at 6hr. The latter suggestive of possible maternal contribution, as IgG crosses the placenta. The cumulative and white matter MRI scores directly correlated ($r=0.48-72$; $p<0.00001-0.003$) with levels of Cd, Cu and Al ($n=33$ across all days), strongly suggesting a role of these metals in HIE pathology. Multiple variable regression ($R^2=0.42-0.68$) modeling indicated that low apgar, high MRI scores and heavy metals, including high Al, Cd, Cu, Mn, Ni, Pb and Fe, were major determinants of specific NAb titers. In addition, several metals were determinants of negative outcomes in apgar and MRI scores ($R^2=0.52-0.75$). This included Cd, Cr, Cu and Al. **Conclusions:** This study strongly suggests environmental toxic contribution in the pathogenesis of neonatal HIE. Heavy metals, particularly Cd, Cu, Mn and Al are recognized as risk factors in neurodegenerative conditions. The high prevalence and elevated NAb titers, particularly IgM, in HIE and their association with clinical presentation suggests their potential as biomarkers in HIE. This pilot provides a promising avenue for research into the complexity of HIE and other pediatric neurological conditions and the importance of external and internal environmental considerations, including maternal roles, in the pathogenesis and biomarker validation.

PS 4212 Cannabidiol and Trazadone suppress Amyloid Beta Pathological features in *Caenorhabditis elegans*

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Background and Purpose: Alzheimer's disease (AD) is the most common neurodegenerative disease, which causes shortened lifespan, motor decline, and progressive cognitive impairment. It is characterized by the extracellular accumulation of misfolded proteins, specifically the amyloid beta ($A\beta_{1-42}$) protein. Current treatments available provide limited therapeutic benefit, highlighting the urgent need for new therapeutic interventions. Cannabidiol (CBD) and Trazadone (TRA) have individually demonstrated neuroprotective effects in previous studies through transcriptional regulation of synaptic and inflammatory genes. However, their individual and combined effect to reduce $A\beta_{1-42}$ aggregation at the protein level have not been investigated. This raises the critical question: Will CBD and TRA treatment provide additive or synergistic protection against Alzheimer's disease? The study aimed to evaluate the neuroprotective effect of CBD and TRA, individually and in combination, in a *Caenorhabditis elegans* (*C. elegans*) model expressing human $A\beta_{1-42}$. **Methods:** Synchronized CL4176 worms expressing muscle-specific human $A\beta_{1-42}$ were treated with concentrations of CBD ($1\mu M$, $10\mu M$, $50\mu M$ and $100\mu M$) and trazadone ($1\mu M$, $10\mu M$, $50\mu M$ and $100\mu M$). The $A\beta$ expression was induced by temperature upshift, and paralysis assay were performed. Protein extracts were fractionated into soluble and insoluble fractions and analyzed via Western blot to assess $A\beta$ aggregation profiles. **Results:** CBD ($\chi^2 = 232$, $df = 4$, $p < 2 \times 10^{-16}$) and TRA ($\chi^2 = 34.5$, $df = 4$, $p = 6 \times 10^{-7}$) independently ameliorated the effect of $A\beta$ toxicity, delayed paralysis, and showed improvement in behavior. Notably, CBD significantly delayed paralysis in a dose-dependent manner, with 10 to $100\mu M$ producing strong protective effects, whilst pairwise comparisons between individual TRA concentrations and vehicle reveal no significance. Western blot analysis showed a reduction of $A\beta_{1-42}$ oligomers for the CBD treatments with maximal effects at $50\mu M$ and $100\mu M$. **Conclusions:** In conclusion, these findings suggest CBD's neuroprotective effect against $A\beta$ induced paralysis in *C. elegans*, and TRA's potential to modulate disease progression overall. We predict that the combined treatments will provide synergistic effect against $A\beta$ toxicity. Further studies need to evaluate higher concentrations of trazadone ($>100\mu M$) to determine its full neuroprotective potential and determine the mechanisms underlying this neuroprotection.

PS 4213 Developmental Phthalate Exposure Increases Alzheimer's Disease-Relevant Neuropathological Outcomes

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Background and Purpose: Phthalates are ubiquitous plasticizers present in personal care products, food packaging, and medical devices, resulting in widespread human exposure, including during gestation. This is a significant concern as the developing organism is extremely sensitive to environmental insults. Prenatal phthalate exposure disrupts endocrine signaling, reduces estradiol, accelerates premature ovarian failure and alters steroid receptor activity. Phthalate exposure also increases neuroinflammation and promotes cognitive deficits, effects also seen in Alzheimer's disease (AD) patients and AD mouse models. This led to our hypothesis that developmental exposure to phthalates increases vulnerability to AD by accelerating neuroinflammation that could augment neural hallmarks of the disease later in life.

Methods: Pregnant 3xTg-AD mice, which carry APPSwe, PSEN1, and tauP301L mutations, and B6129SF2/J controls were orally dosed from gestational day 0 through birth with vehicle (oil) or a phthalate mixture at $20\mu g/kg/day$ (environmentally relevant exposure) or $200\mu g/kg/day$ (occupational exposure). The mixture was derived from urinary metabolite concentrations of pregnant women in Illinois and consisted of 35% diethyl phthalate (DEP), 21% di(2-ethylhexyl) phthalate (DEHP), 15% dibutyl phthalate (DBP), 15% diisononyl phthalate (DiNP), 8% diisobutyl phthalate (DiBP), and 5% benzyl butyl phthalate (BzBP). Offspring were aged to 3, 5, and 11 months, then assessed on a battery of cognitive tasks before brain collection. A subset of females underwent ovariectomy at 2 months of age to examine whether estradiol depletion, coupled with developmental phthalate exposure, exacerbates AD pathology compared to intact females and males. The collected tissue underwent immunohistochemistry for Iba-1, a marker of microglia/macrophage neuroinflammation. **Results:** Our initial analysis from 11-month-old animals revealed that the percentage of area occupied by Iba-1 staining in the entorhinal cortex, a cognition relevant region vulnerable to AD pathology, is elevated in males (genotype x treatment interaction: $p < 0.001$). Male 3xTg mice prenatally exposed to both $20\mu g/kg/day$ ($p = 0.05$) and $200\mu g/kg/day$ ($p < 0.001$) had significantly more area covered by Iba-1 staining than control mice exposed to the same phthalate dose. In ovary-intact females, there was a main effect of phthalate treatment at the same 11-month timepoint ($p < 0.05$). Post-hoc analyses show that the $20\mu g/kg/day$ 3xTg animals had a higher percentage of area covered by Iba-1 staining compared to controls exposed to the same dose ($p < 0.05$). Interestingly, ovary-intact 3xTg females exposed to phthalates did not differ from controls, suggesting a potential protective effect of ovarian steroids. Ovariectomized females differed from ovary-intact females and males, as only strain had a significant effect ($p < 0.001$). The wildtype mice exposed to phthalates did not have a significant increase in Iba-1 staining. **Conclusions:** Together these initial results suggest that 3xTg males may be more sensitive to phthalates than females, and phthalate exposure on an AD background may accelerate the onset of pathological changes. We will quantify the markers for amyloid beta, phosphorylated tau, and Iba-1 in earlier timepoints to determine the onset of changes. We will also interrogate additional brain areas relevant to AD. Together, this will provide mechanistic insight into how developmental endocrine disruption interacts with sex hormones and aging to influence AD risk. This work will highlight how prenatal environmental exposures may have lifelong consequences for neurodegenerative disease susceptibility.

PS 4214 Investigating Nanoplastic Effects on Amyloid-Beta Fibril Formation and Neurotoxicity

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Background and Purpose: Plastic pollution has emerged as a global concern, with growing attention to the potential health impacts of nanoplastics. While environmental distribution is well documented, their neurotoxic potential and possible role in neurodegenerative processes remain poorly understood. In particular, links between nanoplastic exposure and Alzheimer's disease pathology have yet to be clarified. This work investigates how nanoplastics influence amyloid- β ($A\beta$) fibrillization kinetics and neuronal health. **Methods:** A cell-free *in vitro* assay was utilized to examine polymer-specific effects of 50 nm polystyrene (PS) and poly(methyl methacrylate) (PMMA) nanoparticles on $A\beta$ aggregation. Increasing concentrations of both polymers shifted the fibrillization profile, altering the time to fibril formation. These findings guided subsequent studies in primary cortical neurons isolated from embryonic day 15.5 mice. Cultures were exposed on *in vitro* day 7 to a nine-point concentration range ($0.8-0.003125\mu g/\mu L$) of PS or PMMA nanoparticles for 24 or 120 hours. Neurotoxicity was assessed via fluorescence-based live/dead cell assays and reactive oxygen species (ROS) detection. **Results:** Exposure to both PS and PMMA does not induce significant changes in percentage of dead cells or ROS at multiple concentrations when compared to the control. Neurons were also co-exposed to synthetic $A\beta$ (1-42) at concentrations of 2.5 or 1.25 μM together with 50 nm polystyrene (PS) nanoplastic particles at 0.05 or 0.00625 $\mu g/\mu L$ for 120 hours, in an effort to reproduce the fibrillization phenomenon observed in the cell-free assay within primary neuronal cultures. The results showed a significant decrease in fluorescent signal labeling $A\beta$ peptide and fibrils in the co-exposure group of 2.5 μM peptide with 0.05 $\mu g/\mu L$ PS when compared to the control. **Conclusions:** These findings suggest that the current *in vitro* neuronal model and endpoints used in this study may not be optimal for detecting subtle or delayed neurotoxic effects of nanoplastics. Further optimization of the $A\beta$ co-exposure model, including refinement of exposure conditions, time windows, and analytical endpoints, will be necessary to more effectively evaluate how nanoplastics may contribute to neurodegenerative disease processes *in vitro*.

PS 4215 Pre-Adsorption of Pesticides onto Nanoplastics Alters Neurotoxic Responses in Primary Cortical Neurons

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Background and Purpose: Nanoplastics are present in virtually all human tissues evaluated. Due to their ability to cross the blood-brain barrier, there is growing concern about neurotoxicity and contributions to neurodegenerative diseases. Importantly, nanoplastics entering the human body are associated with a complex exposome, including chemicals that adsorb to plastic particles. **Methods:** We examined the potential of polystyrene (PS) nanoplastics to modulate the neurotoxic effects of Parkinson's-linked pesticides. We hypothesized that pre-adsorbing polystyrene nanoplastics with fenpyroximate, rotenone, paraquat, or endothal yields varying neurotoxicity, with nanoplastic-induced alterations in the bioavailability of the pesticides based on their relative hydrophobicity. Primary mouse cortical neurons were isolated on embryonic day 15.5 and cultured in 96-well plates. Each of the aforementioned pesticides were incubated for 72-hours with 50 nm PS beads. Two control conditions were incubated for the same 72-hour time period for comparison: 50 nm PS with lab-grade water, and pesticide with lab-grade water. After the 72-hour incubation, each condition was dialyzed against cell culture feeding media for 48 hours using a Slide-A-Lyzer at 37 degrees Celsius. At seven days *in vitro*, neurons were exposed for 24 hours to a five-point concentration range of each PS-pesticide or control condition, based on the post-dialysis PS concentrations (0.78 - 200 µg/ml) or to a four-point dilution series of the corresponding dialysate (12.5%-100% v/v) in cell culture media. Cell viability was assessed using a fluorescent live/dead cell assay and data was normalized to the control. **Results:** Polystyrene nanoparticles were not cytotoxic. However, the pre-adsorption of rotenone or fenpyroximate with PS 50 nm increased neurotoxicity when compared to the pesticide only condition. The dialysate caused cytotoxicity at the highest concentration, suggesting that free pesticide is crossing the dialysis membrane. This toxicity was minimal, which may be due to tendency of rotenone and fenpyroximate to adsorb to the plastic dialysis tubing. The combination of PS 50 nm with paraquat, a pesticide with lower LogP, did not produce significant exacerbation of neurotoxicity. However, the corresponding dialysate exhibited cytotoxicity, suggesting that the amount of paraquat crossing the dialysis membrane is independent of the presence of polystyrene. Results concerning the pesticide endothal are pending. **Conclusions:** Our results suggest that pre-adsorption of highly lipophilic pesticides onto PS 50 nm, followed by dialysis, increased the amount of pesticide available to cells, thereby enhancing overall toxicity. Our work is currently evaluating other endpoints, such as RNA-seq, to better recapitulate human neurological disease processes. Taken together, the regulation and risk assessment of environmental chemicals and plastics must consider additive and increased bioavailability potential to protect human neurological health.

PS 4216 Intranasal infection with Western Equine Encephalitis Virus induces temporally and spatially distinct patterns of gliosis and neurodegeneration in the hippocampus of C57Bl/6 mice

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Background and Purpose: Encephalitic alphaviruses, such as Western Equine Encephalitis Virus (WEEV), are increasingly associated with morbidity and mortality driven by acute viremia and post-encephalitic neurologic dysfunction, including age-related neurodegeneration. Globally, an increase in temperature and annual rainfall has expanded mosquito-occupied territories, increasing infectious risk for previously invulnerable groups and presenting the possibility for large-scale outbreaks capable of drastically altering the health of these populations. Encephalitic alphaviruses have been characterized to possess neurotropic capacity, associated with longitudinal central nervous system dysfunction. Following viral-induced encephalitis, lingering effects such as chronic neuroinflammation and glial-mediated immune modulation are strongly correlated with the onset and progression of neurodegenerative disorders, including Parkinson's disease and related dementias. Our lab has previously demonstrated that sub-lethal infection with WEEV induces robust astrogliosis associated with gait aberrancies, nigrostriatal dopaminergic neurodegeneration, and pathologic protein aggregation in murine models. However, the temporal sequence of glial activation and subsequent neurodegeneration in brain regions relevant to dementia and cognitive decline have yet to be investigated, despite marked neurotropism in these parenchymal structures long after peripheral clearance. Together, this motivated the temporal assessment of glial dynamics, pathologic protein aggregation, and neurodegeneration in the hippocampus of WEEV-infected mice. **Methods:** Six-week-old C57Bl/6 mice were anesthetized and intranasally infected with 20 µL of 1×10^4 PFU/mL McMillan WEEV and subsequently sacrificed at 1-, 2-, and 4-week timepoints. Passive immunotherapy was administered to control the extent of viral replication. Brains were dissected and coronally sectioned for downstream analysis via high-content immunofluorescent microscopy, deep learning-based image analysis, histological examination, and population-level cellular phenotyping. **Results:** We interrogated the complex temporal relationship between gliosis and neuronal loss across time, infection status, and hippocampal subregion. By 4 days post infection, the virus had localized to the brain and was identified within hippocampal neurons. Hematoxylin and eosin staining revealed a sharp increase in neuronal pyknosis at 1-week post-infection (WPI) in the CA1 and CA3 regions of the hippocampus that

was associated with fibrotic-like pathology and confirmed via Masson's trichrome staining. We explored this phenomenon using fluorescence microscopy, reporting a substantial viral-mediated increase in GFAP⁺ and IBA1⁺ cells that peaked at 2 weeks post-infection. Interestingly, this corresponded with the formation of glial-occupied lesions, exclusive to WEEV-infected brains, which inversely correlated with neuronal density and appeared in fibrotic regions. We further investigated the role of these glial inclusions in WEEV-induced neuropathology by querying the proximity to amyloid-B aggregates via nearest-neighbor analysis, observing peak protein aggregation at 1-week post-infection near highly reactive IBA1⁺ cells. Furthermore, we integrated this data with automated rendering of microglial cells to assess morphometric parameters including cellular area, branch length, dendritic arborization, terminal endpoints, and nuclear morphology. This phenomic analysis revealed time- and infection-dependent clustering of cells based on activation status. **Conclusions:** Together, these data highlight robust involvement of glial reactivity in misfolded protein accumulation and neurotoxicity in the pathogenesis of WEEV-induced hippocampal degeneration. Future work will aim to distinguish if modulating glial-mediated inflammatory signaling can protect against these disease-associated molecular sequelae.

PS 4217 Exposure to manganese in juvenile mice primes the hippocampus to glial inflammatory responses and neuronal stress following systemic infection with Influenza A virus subtype H1N1

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Background and Purpose: Neurodegenerative disorders (NDDs) are the fastest-growing cause of disability among older adults. Although the etiology of NDDs is poorly understood, pro-inflammatory immune signaling is implicated in the pathogenesis of these conditions. Additionally, exposure to environmental toxins can increase the risk of developing NDDs. Manganese (Mn) is an essential trace mineral that is a cofactor for numerous enzymes in the body and has immune signaling functions. Excess accumulation of Mn in the basal midbrain can lead to manganism, a neurologic disorder leading to parkinsonian-like symptoms and cognitive impairment. Recent epidemiological studies implicate lifetime systemic viral infection with the risk of developing NDDs. Specifically, infection with RNA viruses can lead to long-lasting neurological sequelae, including cognitive impairment and motor dysfunction. Following the 1918 Spanish Influenza pandemic, there was a marked increase in encephalitis lethargica, but it is not known why only select patients surviving H1N1 infection developed post-encephalitic parkinsonism. **Methods:** Previously, our lab demonstrated that Mn exposure in juvenile C57Bl/6 mice increases glial inflammatory responses in the substantia nigra following infection with H1N1 Influenza virus. In the present study, we employed a dual-hit neurotoxin-pathogen model to investigate how juvenile exposure to Mn alters H1N1-induced pathology in the hippocampus. Mice were given MnCl₂ at 50 mg/kg/day in water at P21 for 30 days, then regular water for a month. Mice were then infected by intranasal administration of phosphate-buffered saline with 10^3 TCID₅₀ A/California/04/2009 H1N1 when the mice were 8 weeks old, and animals were terminated at 21 dpi. Coronal brain sections encompassing the hippocampus were immunostained against IBA1, allowing for assessment of the number and morphology of microglia and invading macrophages across hippocampal subregions. Hematoxylin and eosin (H&E) staining was performed to identify the number of healthy and pyknotic neurons within the dentate gyrus. **Results:** Whole hippocampal analysis reveals a main effect of Mn on IBA1+ microglia number, as seen in the increase of IBA1+ cells in the CA3 subregion. Mn exposure increases the total area occupied by IBA1+ processes in the hippocampus. H&E analysis of the dentate gyrus revealed an increase in pyknotic neurons per mm², as well as the ratio of pyknotic to healthy neurons, with no notable change in healthy neurons per mm². Morphometric profiling of individual microglia was accomplished by measuring the length of IBA1+ processes, the area occupied by IBA1+ cells, the number of branch points, and the number of endpoints. Preliminary morphological characterization of IBA1+ cells reveals that Mn treatment did not result in a more amoeboid cellular phenotype. Rather, IBA1+ cells from Mn-treated animals displayed greater cell volume, process length, number of branching points, and number of end points. **Conclusions:** We conclude that these characteristics represent a novel phenotypic state that we refer to as hyperarborized. These findings highlight the capacity of juvenile Mn exposure to trigger immune priming in the brain that induces a pronounced inflammatory phenotype in hippocampal microglia upon viral infections during adulthood. Such toxin-pathogen interactions may impact the later development of NDDs.

PS 4218 Neuropathological features of Alzheimer's Disease like pathology in aging canines

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Background and Purpose: Alzheimer's disease (AD) is a progressive neurodegenerative disorder and a major global health crisis affecting more than 55 million people worldwide. AD is pathologically defined by the accumulation of amyloid-beta (Aβ) plaques, neurofibrillary tangles (NFTs), and neuronal loss, yet effective disease

modifying therapies remain elusive. While transgenic rodent models have provided valuable mechanistic insights, they fail to replicate show the naturally occurring, age-related progression characteristic of human AD. In contrast, the companion develops spontaneous cognitive decline and neuropathological changes that closely mirrors those seen in humans. Dogs exhibit progressive A β and tau pathology, brain atrophy, and behavioral impairments measurable by the validated cognitive scale, Canine Social and Learned Behavior (CSLB). Despite these parallels, a systematic application of human neuropathological criteria to canine brains remains limited. **Methods:** Postmortem brain tissues from 24 client-owned aged dogs were assessed using Thal, Braak and CERAD criteria. We integrated these assessments alongside antemortem cognitive assessments and plasma biomarkers to evaluate relationship between pathology, owner assessment cognition, and biofluid markers. **Results:** Aged dogs show A β progression consistent with early to intermediate Thal stages, tau pathology following Braak staging, and an absence of neuritic plaques. Quantitative analysis revealed higher A β burden in cognitively impaired dogs, and significant association with age and cognitive scores. **Conclusions:** Aging dogs show progressive A β and tau pathology mirroring early to intermediate AD but lack neuritic plaques. These findings strengthen the role of dogs as a natural aging model for AD, offering a translational bridge for investigating early disease mechanisms and evaluating therapeutic interventions in a naturally aging model.

PS 4219 Clinicopathological Profiles From Aging Canine Cohorts Reveal Changes Associated With Cognitive Dysfunction

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Background and Purpose: Canine cognitive dysfunction (CCD) is a complex, age-associated neurodegenerative syndrome that shares similarities to Alzheimer's disease and related dementias (AD/ADRD) in humans. As canines age, they serve as a naturally occurring model for these diseases, offering valuable insight into disease progression. Because companion dogs age alongside humans and share environmental exposures, they provide a translational model for understanding how systemic aging biology relates to brain health. Our objective was to identify routine systemic clinicopathological biomarkers that may be associated with cognitive status by pairing population-scale clinical data from the Dog Aging Project (DAP) with clinical data from Colorado State University's (CSU) Brain Health Study: Biomarker Research in Aging Dogs (BIORAD), which included matched clinical labs with additional cerebral spinal fluid (CSF) analyses. **Methods:** We performed a cross-sectional analysis of routine clinical biomarkers from dogs enrolled in the DAP and CSU BIORAD cohorts stratified by cognitive status. DAP data included 214 dogs aged ≥ 8 years with hematology and chemistry values aligned to cognitive status using the Canine Social and Learned Behavior survey (CSLB; CCD score: ≥ 50 ; normal cognitive score: ≤ 40). CSU data included 50 dogs aged ≥ 8 years with hematology and chemistry values aligned to cognitive status using the Canine Dementia Scale (CADES; CCD score: ≥ 8 ; normal cognitive score: ≤ 7). Biomarker differences were assessed using Welch's t-tests with FDR correction, including sex-stratified analyses. CSF glucose and CSF protein from the CSU cohort were evaluated using Wilcoxon rank-sum tests. **Results:** Across the DAP cohort, female dogs with CCD showed reduced hematocrit and hemoglobin ($p < 0.01$), higher platelet counts with reduced mean platelet volume ($p < 0.001$), and both sexes showed elevated potassium and serum:potassium ratios. The CSU cohort showed several similar systemic shifts. Dogs with CCD had higher potassium ($p = 0.0148$), higher platelet counts ($p = 0.0396$), higher blood urea nitrogen levels ($p = 0.0343$), and increased anion gap measurements, consistent with metabolic and electrolyte changes also observed in the DAP cohort. Additional changes, although not statistically significant, such as modest increases in alanine transferase values and neutrophil counts, suggest broader systemic metabolic and inflammatory involvement associated with CCD. Unique to the CSU brain health study is the inclusion of CSF biomarkers. CSF glucose was higher in dogs with CCD (87.3 vs 76.9 mg/dL, Wilcoxon $p = 0.027$), indicating dysregulated glucose within the central nervous system. CSF protein was also lower on average in dogs with CCD but was highly variable across groups and not statistically different (Wilcoxon $p = 0.39$). **Conclusions:** Across both independent cohorts, dogs with CCD showed similar systemic hematological and metabolic shifts that include platelet activation, electrolyte imbalance, and metabolic stress. Within the CSU cohort, a central metabolic change reflected by elevated CSF glucose also supports physiological changes related to cognitive decline. Together, these findings support the idea that routine clinical biomarkers, alongside CSF analysis, may capture physiological changes related to neurodegeneration in aging canines. Further work should investigate longitudinal changes in inflammatory and metabolic biomarkers over time in larger cohorts to better understand how biological aging and metabolic dysfunction contribute to neurodegeneration.

PS 4220 Age and Sex Have Significant Impact on Microglia Morphology: Modulation by Acute LPS Challenge

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Background and Purpose: Microglia are the resident innate immune cell of the central nervous system and exhibit functional as well as morphological changes that are influenced by various factors such as age, sex, and inflammatory stimuli. Functionally, microglia can be characterized into three separate states, one unreactive (M0) and two reactive: a pro-inflammatory M1, and an anti-inflammatory M2 state. These functional states are associated with changes in morphology, namely in parameters such as branch length, arborization, density, and soma size. The role that age and sex play in microglia function has been investigated to some extent, functionally, and associated morphological changes have either been evaluated within males (effect of age), or in adults (effects of sex); detailed comparisons between the sexes at different ages are yet to be done. Additionally, morphological responses of microglia to an acute inflammatory challenge have not been investigated. **Methods:** Adult (4-5 months old), middle-aged (14-15 months old), and aged (20-21 months old), C57BL/6J male and mice with GFP-tagged microglia were challenged with LPS (*Escherichia coli* 0111: B4, Sigma Aldrich; 0.3 mg/kg, i.p.) or saline vehicle. Four h post, mice underwent open field behavioral testing; 6 h post brains and plasma were harvested. Brains were fixed, sectioned, and processed for image analyses of hippocampal GFP+ microglia morphology by detailed Sholl, fractal analyses and appropriate statistics. Additionally, selected plasma cytokines were analyzed from a subset of samples from the various age groups; such analysis, together with the open field testing, was also done with "Super-Aged" (25-32-month-old) mice. **Results:** Overall, age exerted strong influence on microglia morphology, with measured reductions in branch complexity and length, soma circularity, and the density of the cell, accompanied by increases to soma size. Adult female microglia also exhibited more complex branches, than adult male microglia; they were also more responsive to LPS, marked by a decrease in these measured areas only in adult females. In the middle-age, branch length was longer in females, though they had less overall intersections and ramifications. Beginning in middle age, microglia of both sexes exhibited age-dependent decreases in overall cell and branch complexity. Soma area, in contrast, was only increased in the aged mice, while cell density was lowest in middle age. Behaviorally, LPS elicited sickness behavior (decreased horizontal and, especially, vertical activity) at all ages; the effects were progressively stronger with age and, females, adults in particular, were affected more than their male counterparts. Super-aged mice exhibited most profound sickness behavior post LPS. In terms of peripheral cytokine levels, in the absence of LPS challenge, there was no observed significance, though there was a nonsignificant age-related increase in pro-inflammatory IL-2. Post LPS, age was a significant factor in the increase of all measured cytokines, including many pro- and some anti-inflammatory. Female response to LPS, in terms of plasma cytokines was greater than the males at all ages. With age, this effect became more exaggerated. Furthermore, there was a non-significant decrease in certain cytokines, namely IL-10 and IL-1B, in Super-Aged mice in comparison to the aged mice, though most other cytokines, saw a numerical increase. **Conclusions:** Collectively, these findings demonstrate that microglia morphology is affected not only by age, but by sex as well, with the middle-age representing a critical transition period. The morphological differences found herein suggest that microglia contribution to brain health and pathology is skewed to pathology by aging, starting at middle age, as characterized by age related loss and retraction of branches and increased soma size. These morphological changes due to age occur with corresponding increases in peripheral cytokine and behavioral responses to acute inflammatory challenge. Additionally, acute inflammatory stimuli might be more consequential in females, especially adults, in terms of microglia morphology, and to all ages in terms of peripheral cytokines. The lack of marked morphological changes post LPS except in adults suggests that at least the early behavioral responses to LPS are driven by peripheral cytokines while central microglia response is not fully engaged at this time.

PS 4221 Neurological Phenotypes and PTSD-Linked Neuroinflammatory Pathways in World Trade Center Responders

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Background and Purpose: World Trade Center (WTC) responders are at heightened risk for neurological impairment due to combined exposure to neurotoxic dust, heavy metals, combustion products, PM_{2.5} and severe psychological trauma. Post-Traumatic Stress Disorder (PTSD) and systemic inflammation have been proposed as shared pathways linking WTC environmental exposures to later-life neurobiological vulnerability, consistent with prior cohort findings of neuroimmune activation, altered neuronal signaling, cortical changes, and early biomarker alterations. However, peripheral neurological patterns, often detectable earlier in the clinical course, have not been systematically examined to determine whether distinct sensory-motor phenotypes may capture early manifestations of these exposure- and trauma-related processes in WTC responders. **Methods:** Standardized neurological examination data from 13,016 visits across 2,671 unique responders were analyzed across five domains: cranial nerves, upper and lower light touch, reflex change (categorized as "normal" vs. "abnormal"), and an overall functioning measure. Neurological findings

were aggregated at the participant level and coded as “ever abnormal” to represent cumulative neurological burden; prospective time-to-event analyses are planned to investigate onset patterns. Participants missing all neurological data were excluded. Partial within-domain missingness was retained, with conservative imputation (Not Available [NA] = 0) applied exclusively for clustering analyses to preserve phenotype structure. Hierarchical clustering (Ward’s D²) identified six sensory-motor phenotypes. Clusters were compared by PTSD status using chi-square tests, and standardized stress-burden z-scores were derived from Mental Health Diagnostic Interview Schedule responses. **Results:** Six neurological phenotypes emerged, ranging from low-abnormality to diffuse cranial-sensory-motor involvement. PTSD prevalence differed significantly across clusters ($\chi^2=17.1$, $p=0.004$), with the highest burden observed in diffuse cranial-sensory phenotypes compared to the low-abnormality group. Stress-burden scores showed no significant group differences ($p=0.33$; $\epsilon^2=0.0003$). Exploratory patterns suggested that reflex asymmetry, reduced strength, and sensory abnormalities were more common in diffuse clusters, indicating broader sensory-motor involvement. **Conclusions:** Distinct neurological phenotypes among WTC responders reveal early, clinically accessible sensory-motor patterns that vary by PTSD prevalence and may align with previously reported exposure-related neurobiological signatures in this cohort. These sensory-motor phenotypes may reflect early downstream effects of previously reported WTC-related toxicant pathways, including microglial activation, axonal injury (NFL), and tau-related alterations. Diffuse cranial-sensory involvement with elevated PTSD burden may represent a potential endophenotype relevant to trauma- and exposure-linked pathways. Integration with ongoing biomarker, neuroimmune, and neuroimaging research is warranted to clarify mechanistic links and advance early detection strategies in disaster-exposed populations.

PS 4222 Systemic murine infection with Western Equine Encephalitis Virus results in age-related Parkinson’s disease-like motor dysfunction *in vivo* and astrocyte-mediated neurodegeneration *in vitro*

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Background and Purpose: Encephalitic arboviruses, such as Western equine encephalitis virus (WEEV), are rapidly emerging in human and animal populations across the globe due to expansion of vector-occupied territories as a result of rising temperatures and increased rainfall. Viral neurotropism, resulting in acute viremia and post-encephalitic neuroinflammation, is a long-understood mechanism of alpha-virus-induced neurodegeneration. Prior epidemiologic work has posited advanced age as a significant risk factor for severity of viral insult and subsequent risk of neurologic dysfunction; however, the consequences of senescence on glial involvement and neuroimmune modulation as contributors to age-related neurodegeneration remain elusive. Our lab has previously demonstrated that intranasal infection with WEEV is sufficient to elicit a robust male-specific astrocyte-mediated complement response associated with disordered movement, dopaminergic neurodegeneration, and nigrostriatal alpha-synuclein aggregation. We have further shown the ability of footpad-administered WEEV to undergo neuroinvasion and result in parenchymal replication. Still, the ability of this arthropod-relevant mode of transmission to induce neurodegenerative sequelae has not been explored. Together, these data motivated the assessment of footpad-delivered WEEV on motor function and dopaminergic neurodegeneration in young vs. old mice, as well as tandem interrogation of astrocytic infection *in vitro* to tease apart molecular consequences on neuronal populations. **Methods:** Ten-week-old or ten-month-old male C57Bl/6 mice ($n = 4-8$ animals/group) were anesthetized and administered saline vehicle or 5×10^3 PFU McMillan WEEV via footpad injection. Baseline behavioral data was collected prior to viral insult and subsequently compared to weekly timepoints assessing all-paw grip strength, pole test descent time, and hindlimb clasping scores. Four weeks post-infection (WPI) all mice were sacrificed and brains were removed and sagittally hemisected. One hemisphere was utilized for downstream striatal neurochemical profiling, basal midbrain bulk RNA-sequencing, and cortical plaque assay. The other hemisphere was post-fixed and sectioned for assessment via robotic multiplex immunofluorescent labeling, high-content microscopy, and deep learning-based image analysis pipelines. We applied spatial phenomics to characterize nigrostriatal reactive gliosis and dopaminergic neurodegeneration in the context of age-related WEEV infection. Murine neonates were simultaneously sacrificed to establish primary neuronal and astrocyte-enriched glial cultures. Subsequent assessment of cell viability, transcriptomic patterns, and inflammatory signaling occurred in low vs. high passage number cells. **Results:** We observed 50% lethality in young group mice in the first two weeks post-infection, despite no old group mice exhibiting signs of moribundity. Interestingly, advanced age mice demonstrated substantial progressive motor dysfunction following WEEV infection. Histopathologic analysis revealed substantial glial reactivity in all viral-infected mice, concurrent with vulnerability of the nigrostriatal projection. To further investigate mechanistic glial involvement underlying the dystonic phenotype, cell viability was assessed across half-log viral titers spanning 0.01 to 10 PFU/cell which revealed that low passage glia are far more susceptible to WEEV-induced cytotoxicity than high passage cells. We additionally examined the effect of WEEV infection on canonical inflammatory signaling by establishing primary glial cultures from NF-kB-GFP reporter mice, which revealed an age- and dose-dependent activation. Glial conditioned media derived from low or high passage cells at viral titers spanning the multiplicity of infection in glia were applied to primary neuronal cultures, which revealed an age- and dose-dependent effect on

neuronal death. Glial-mediated neurotoxicity was blunted by pharmacologic NF-kB inhibition. **Conclusions:** Together, these data highlight the role that age may play in the susceptibility to Parkinsonism secondary to viral neurotropism. Future work will aim to investigate the impact of modulating glial immune signaling *in vivo* on the progression of this pathologic milieu.

PS 4223 A High-Content Neurotoxicity Assay using multiple Neurotransmitter and Synaptogenesis Markers in *C. elegans*

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Background and Purpose: Neurodevelopmental disorders in children are increasing with one important factor being the exposure to potentially neurotoxic chemicals at a young age. Despite widespread human exposure, many chemicals remain untested for developmental toxicity (DNT) endpoints. Conventional DNT studies are performed in rodents, which face growing regulatory restrictions and are slow, costly, and low throughput to address the urgent need for hazard assessment. New approach methodologies (NAMs) including *in silico* tools, *in vitro* methods, and small model organisms are emerging as alternatives for DNT. While *in vitro* models have been instrumental for drug discovery and are being evaluated for DNT test batteries, they suffer with several technical challenges such as inconsistent network formation, high costs for long-duration studies, dependence on serum, and limited ability to measure systemic or behavioral outcomes. To bridge these gaps, we propose using the small model organism *Caenorhabditis elegans* to complement existing *in vitro* assays and support scalable DNT testing. **Methods:** We established a liquid-culture protocol enabling synchronized growth of 80 worms per well in a 24-well plate, using HB101 as a food source and exposing animals to multiple concentrations of reference chemicals for 3-7 days. Adult worms were immobilized in a high-throughput microfluidic device capable of imaging 960 individuals per chip (40 worms $\times$ 24 conditions). Two reporter strains expressing *dat-1::GFP* and *unc-47::mCherry* were used to visualize dopaminergic and GABAergic neurons, respectively. To evaluate synaptic defects, we generated a dual-color strain co-expressing *syd-2::GFP* in the GABAergic model. Using an automated imaging platform, we acquired multi-color z-stack datasets and quantified multiple morphological and synaptic endpoints using semi-automated and machine-learning-assisted pipelines. **Results:** Assay performance was evaluated using methylmercury (MeHg), a well-established DNT reference toxicant. Dopaminergic neurodegeneration was assessed by quantifying blebbing and dendritic breaks in CEP neurons, while GABAergic toxicity was evaluated based on changes in cell-body size, fluorescence intensity, and synaptic morphology (size, localization, and intensity). Both MeHg and bisphenol A (BPA) induced concentration-dependent increases in neuronal damage and aberrant synaptogenesis. All parameters demonstrated strong repeatability, with coefficients of variance ranging from 3-24%. **Conclusions:** We developed a robust and reproducible *C. elegans*-based DNT assay capable of multiplexed imaging and quantitative analysis across two neurotransmitter systems. Validation with two well-characterized neurotoxins confirms the platform’s sensitivity and scalability. Future efforts will expand to additional chemicals and neurotransmitter models to enable more comprehensive, neuron-specific DNT profiling.

PS 4224 Evaluating the protective role of nicotine in pesticide induced Dopaminergic neuron degeneration through mechanosensation, behavioral, and fluorescent assays

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Background and Purpose: Parkinson’s Disease (PD), a prevalent neurodegenerative disorder is characterized by tremors, bradykinesia, and impaired coordination and balance. PD results from the degeneration of the dopaminergic (DA) neurons in the substantia nigra. The complex etiology of PD is understood to be an interaction of genes and the environment, especially pesticide and heavy metal exposure. Mancozeb (MZ), a common fungicide (active ingredient manzate or Mn/Zn ethylene-bis-dithiocarbamate), is known to disrupt neurotransmission and increase oxidative stress. Previous research in our lab has indicated that the fungicide Mancozeb induces PD-like dopaminergic (DAergic) degeneration in the model organism *C. elegans* by inhibiting complex I of the mitochondrial electron transport chain. Increased oxidative stress and mitochondrial dysfunction are hallmarks of the known pathology of PD development. There is no cure for PD and the few available treatments lose efficacy overtime, therefore there is a need to understand the mechanisms leading to PD. Epidemiological studies have shown that PD is less frequent in tobacco users, although the protective effect and potential mechanism are unknown. While there are several potential components of tobacco that may be driving this inverse association, nicotine has a high binding affinity to the nAChRs (nicotinic Acetyl-Choline Receptors) in DAergic neurons and has been shown to modulate DAergic signaling. Nicotine has also been shown to modulate mitochondrial activity and decrease oxidative stress. It is therefore important to investigate the protective effects of nicotine as well as the potential interaction with mitochon-

drial function associated with DAergic degeneration. **Methods:** To investigate the role of nicotine in pesticide-induced DAergic degeneration, N2 (wild type *C. elegans*) and BY250 (GFP tagged dopamine transporter) were treated with either nicotine or MZ individually, a dual treatment of nicotine followed by MZ and control. DAergic neuron function was monitored by two behavioral based assays. A mechanosensation assay was performed, with the nose touch assay targeting DAergic neurons, soft touch targeting Touch Receptor Neurons (TRNs) and harsh touch serving as a general response control. The DAergic system also controls movement and feeding behavior. The basal slowing response was observed to monitor their locomotion with and without the food source (*E.coli*). The BY250 fluorescence of the GFP-dat-1 was measured as an indicator of DA function. Mitochondrial function was monitored with the MitoToxGlo assay kit to measure membrane integrity and ATP production. **Results:** A significantly decreased response was only observed in the nose touch response for MZ treated worms (p-value <0.5) in both the N2 and BY250 nematodes, indicating that MZ induces DA degeneration and nicotine provides protection on behavioral assays. Neither the harsh or soft touch showed any deviation from control, indicating the DAergic neurons were targeted. The basal slowing response showed a significantly decreased locomotion rate after exposure to food for all treatment groups except MZ, demonstrating an inability to adapt to their surroundings, indicating DAergic degeneration. Supporting previous work, preliminary fluorescent data indicate a decrease in mitochondrial function from both BY250 and MitoToxGlo assays following exposure to MZ. The investigation of the effect of nicotine and nicotine pre-treatment on mitochondrial function is ongoing. **Conclusions:** Taken together, the combination of these assays provides important insight into the protective effect and potential mechanism of nicotine on pesticide induced DAergic neuron degeneration, leading to a better understanding of how nicotine may decrease the risk of developing PD and open potential avenues for understanding underlying mechanisms and finding new treatments.

PS 4225 Methanol Enhances Vascular K_{ATP} Channel Activity: A Potential Mechanism for Methanol-Induced Hypotension

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Background and Purpose: Methanol poisoning causes severe metabolic acidosis, visual impairment, and often fatal cardiovascular collapse. Profound hypotension is a frequent but poorly understood complication. Vascular ATP-sensitive potassium (K_{ATP}) channels (Kir6.1/SUR2B) regulate vascular tone through smooth muscle hyperpolarization and vasodilation when activated. Because hypotension and hyperglycemia - both common in methanol toxicity - are expected consequences of K_{ATP} channel activation, we hypothesized that methanol enhances vascular K_{ATP} channel activity, thereby contributing to its toxic cardiovascular effects. **Methods:** Fluorescence-based thallium (Tl^+) flux assays were performed in HEK293 cell lines stably expressing vascular Kir6.1/SUR2B channels. Channel activity was measured under basal conditions and following pharmacological activation with the K_{ATP} opener pinacidil (0.1-300 μ M), in the absence or presence of methanol at concentrations consistent with toxic exposures (1 M and 5% v/v). Glibenclamide (10 μ M) was used to confirm K_{ATP} -specific effects. Flux rates were extracted from fluorescence traces using exponential fitting, and statistical significance was assessed by ANOVA with appropriate post-hoc testing. **Results:** Methanol significantly enhanced Kir6.1/SUR2B activity when preactivated by pinacidil. At 1 M methanol, augmentation was observed across multiple concentrations of pinacidil, with the most robust effect at 100 μ M ($p < 0.001$); these effects were fully reversed by glibenclamide. At 5% methanol, channel activity was enhanced at both 30 μ M ($p < 0.01$) and 100 μ M pinacidil ($p < 0.001$). In a small pilot dataset, 5% methanol alone increased basal activity ($p < 0.01$), suggesting possible direct activation. **Conclusions:** These findings provide the first direct electrophysiological evidence that methanol enhances vascular K_{ATP} channel activity at toxicologically relevant concentrations and might suggest a plausible mechanism for the profound hypotension seen in methanol poisoning. Given pancreatic K_{ATP} channels inhibit insulin secretion when activated, similar effects on Kir6.2/SUR1 channels could explain the associated hyperglycemia. Notably, during methanol toxicity, severe metabolic derangements and ATP depletion may leave K_{ATP} channels in preactivated states, amplifying methanol's potentiating effects. Ongoing studies aim to assess the effects of methanol on these processes. Collectively, these data promise to advance mechanistic understanding of methanol toxicity.

PS 4226 The Effects of Carbon Nanodots on Ultrasonic Vocalizations and Other Behaviors of C57BL/6J and LDLr^{-/-} mice.

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Background and Purpose: Carbon nanodots (CNDs) are new nanomaterials widely used in biomedicine for their cost-effectiveness and low toxicity compared with other nanomaterials and applications such as bioimaging and drug delivery. While multiple *in-vitro* and *in-vivo* studies elucidated the low toxicity of CNDs during a short

period of time (7 days or less), limited information exists regarding their long-term effects on *in-vivo* models. Specifically, we do not understand the potential toxicity of CNDs on the observable appearance, development, and behavior of an individual. **Methods:** We hypothesized that long-term administration of CNDs alters behavior and physiology in laboratory mice. We tested our hypothesis using C57BL/6J mice and LDLr^{-/-} mice and evaluated behavioral and physiological responses to 2.5 mg/kg CNDs over 8 weeks. Specifically, we focused on evaluating the effects of CNDs on Ultrasonic Vocalizations (USVs) as a measure of communicative behavior. We also utilized two standardized behavioral tests, the Open Field Test and the Elevated Plus Maze Test, to analyze alterations in specific behavioral patterns induced by CNDs. Finally, we examined the influence of CNDs on the force strength of mice through a neuromuscular test. **Results:** We found that CND-treated C57BL/6J mice produced fewer USVs than control mice and that CND-treated mice produced USVs with lower-end frequency and maximum frequencies. However, in LDLr^{-/-} mice, we found no difference in calling rate between treatment groups, but CNDs treated mice produced vocalizations with different spectral characteristics. Furthermore, CNDs treated LDLr^{-/-} mice produced calls with lower end frequency, maximum frequency, start frequency, the frequency at maximum amplitude, and minimum frequency. CNDs increased the total floor velocity in the Open Field Test in C57BL/6J mice. We found no differences between treatment groups using the open-field or Neuromuscular tests. **Conclusions:** Together, these results suggest that the difference in behavioral tests, especially in calling rate and changes in spectral characteristics of USVs, may reveal that CNDs induce a potential stress response to C57BL/6J mice and LDLr^{-/-} mice.

PS 4227 The Influence of Isoflurane on the Gut Microbiome in a Rat Model of Diet-Induced Hypertension

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Background and Purpose: Dysbiosis can elicit systemic inflammation and disrupt intestinal mechanotransduction, a process involved in blood pressure regulation. Isoflurane, a commonly used anesthetic agent for maintaining general anesthesia, has known effects on BP regulation. Isoflurane may also alter the gut microbiome, which can lead to systemic inflammation and metabolic changes that contribute to hypertension. In this study, we evaluate the effects of diet and repeated isoflurane exposure on the gut microbiome and associated BP changes. **Methods:** 36 Dahl salt-sensitive (SS) rats were used and divided into two cohorts (n=18): control diet (CD; 10% kcal from fat) and high fat diet (60% kcal from fat). Additionally, half of the rats (n=18) were exposed to isoflurane every two weeks for ultrasound imaging procedures, resulting in 12 isoflurane exposures per rat. Arterial BP was measured continuously by implanted radiotelemetry or tail cuff plethysmography. Fecal pellets were collected and analyzed by shallow shotgun whole genome sequencing using the Transnetyx microbiome sequencing service. Fecal pellet collection took place at baseline, and 10, 17, and 24 weeks on diet. **Results:** Our findings will demonstrate that diet plays a role in driving changes in the gut microbiome, which is associated with the development of hypertension. Rats fed a HFD will exhibit altered microbial composition and diversity compared to a CD, associated with elevated BP. Repeated exposure to isoflurane is expected to modulate the diet-associated microbiome changes and potentially the hypertensive response. **Conclusions:** Understanding the impact of diet on the microbiome is crucial for hypertension research. Diet-induced changes in microbial composition can directly impact BP regulation, and repeated exposure to isoflurane can alter the gut microbiome, influencing the development of hypertension. This data could shift how we approach the management of hypertensive patients by encouraging more personalized dietary interventions and reconsidering the type or frequency of anesthetic use in individuals at risk.

PS 4228 Metabolic Remodeling and Mitochondrial Impairment in HFpEF: Insights from a Murine Model

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Background and Purpose: Heart failure (HF) is a highly morbid condition affecting over 60 million people worldwide. Importantly, heart failure with preserved ejection fraction (HFpEF) accounts for approximately 50% of all HF cases, and continues to rise, especially in young and aging populations. Despite recent advances, the pathophysiology of HFpEF remains incompletely understood, hindering early diagnosis and effective treatments. Emerging evidence highlights metabolic remodeling and mitochondrial dysfunction of the heart as central pathophysiological processes in HFpEF development. However, the mechanistic link between altered cardiac metabolism and mitochondrial impairment remains poorly defined. **Methods:** In this study, we investigated the role of cardiac metabolic remodeling in regulating mitochondrial function during HFpEF progression, using an established angiotensin-II (AngII)/phenylephrine (PE) infusion murine HFpEF model without the confounding effects of multi-stressors, such as obesity and aging. HFpEF was induced in 8-10-week-old mice by implanting osmotic pumps subcutaneously to infuse AngII (1.5 μ g/g/day) and PE

(50 µg/g/day) for 28 days, while Control mice received saline infusion only. **Results:** Mice infused with AngII/PE exhibited key HFpEF characteristics, including preserved ejection fraction, elevated E/E' ratio, prolonged isovolumetric contraction time, and increased myocardial performance index. Compared to Control, AngII/PE-infused mice showed significantly reduced mitochondrial respiration, diminished complex II protein, and impaired mitochondrial supercomplexes formation. Furthermore, AngII/PE-infused mice demonstrated decreased mitochondrial calcium retention capacity compared to Saline. Metabolomic analysis of mitochondria revealed increased metabolites of alternative glucose utilization pathways, and reduced fatty acid metabolites, suggesting a metabolic shift from fatty acid oxidation toward reliance on alternative glucose metabolism pathways. Methylglyoxal, a biomarker of dicarbonyl stress, was elevated in the heart, whereas advanced glycation end products (AGEs), biomarkers of glycation stress, were elevated in both heart homogenates and mitochondria of AngII/PE-infused mice compared to Saline control, suggesting increased glucose metabolite-adducts. Proteomic analysis of mitochondrial proteins co-immunoprecipitated using an anti-AGEs antibody revealed glycation of mitochondrial proteins essential for maintaining electron transport chain integrity. **Conclusions:** Collectively, our findings suggest that metabolic remodeling critically contributes to the increase of dicarbonyl/glycation stress, impairing mitochondrial supercomplexes formation and respiration, as well as mitochondrial calcium retention capacity in the HFpEF heart. The present findings yield novel insights into future promising therapies targeting anti-dicarbonyl/glycation stress and mitochondrial function to improve disease prognosis in HFpEF patients.

PS 4230 Transcriptomic Profiling Reveals Enhanced Cardiomyocyte Maturation in 3D Culture with Fibroblast Co-culture

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Background and Purpose: Cardiomyocyte (CM) maturation is essential for modeling adult heart physiology and ensuring cardiac safety during drug development. While human induced pluripotent stem cell-derived CMs (hiPSC-CMs) offer a promising tool, they often remain immature *in vitro*. **Methods:** To identify conditions that enhance maturation, we evaluated the effects of culture format (2D vs. 3D), mechanical environment (static vs mechanical stimulation) and cellular composition (100% CMs vs. 75% CMs + 25% fibroblasts). **Results:** Principal component analysis of RNA-Seq data revealed distinct clustering based on culture format and cell composition. 2D monocultures showed tight clustering over time, indicating minimal transcriptomic divergence, while 3D co-cultures occupied unique PCA space, reflecting intermediate transcriptional profiles and enhanced biological activity. These patterns suggest that both physical environment and cellular diversity drive transcriptional variance and maturation. Differential gene expression analysis showed that 3D co-culture significantly upregulated maturation markers (e.g., MYH6, MLC2v, RYR2, SERCA2, PPARα, PGC1α, TFAM) and downregulated immature markers (e.g., MYH7, MLC2a, HCN4). Pathway enrichment analysis revealed that 3D cultures activated oxidative phosphorylation, calcium signaling, and cardiac conduction pathways, while 2D cultures enriched cholesterol biosynthesis and inflammatory signaling. Mechanical stimulation in 3D cultures further promoted sarcomeric organization and functional responsiveness to inotropic agents. **Conclusions:** These findings demonstrate that optimized 3D co-culture systems provide a more physiologically relevant model for CM maturation. This platform may improve early-stage drug screening and cardiovascular safety evaluation by generating preclinical cardiac models better recapitulating human adult myocardium.

PS 4231 Comparative Assessment of Inhalation Exposure to Military Burn Pit and Wildland Urban Interface Fire Emissions on Systemic Microvascular Function

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Background and Purpose: Globally, air pollution is the second greatest mortality risk. Complex combustion emissions are some of many contributing factors to air pollution. Military burn pits (BP) and wildland urban interface (WUI) fires are examples of open-air burning that result in complex combustion emissions and lead to worsening air quality. Over 3.5 million military Veterans have been exposed to BP emissions, with many reporting chronic multisymptom illness following deployment. WUI fire and wildfire emissions have been linked to adverse pulmonary outcomes, cardiovascular health, and increased hospitalizations during periods of increased wildfire prevalence. To date, the systemic microcirculation has not been studied after these complex combustion emission exposures. This project's purpose is to assess and compare systemic microvascular function after BP and WUI fire emission inhalation exposures. **Methods:** A computer-controlled modified pellet stove served as a surrogate combustion emissions generator for these experiments. Burn pit pellets composed of 80% wood, 10% plastic, and 10% rubber burned with the presence of jet fuel with additives (JAA) in the modified pellet stove. Alternatively, WUI pellets were

composed of 55% pine, 25% oriented strand board, 17% vinyl, 3% green foam board, and burned without JAA. 7-8 week-old male Sprague Dawley rats were exposed in a whole-body inhalation exposure chamber for a minimum of 4 hours for 2-6 days. 24 hours following exposure, the spinotrapezius muscle was surgically prepared for intravital microscopy. The spinotrapezius muscle was superfused with acetylcholine (ACh, 10⁻⁶-10⁻⁴ M) to assess endothelium-dependent arteriolar vasodilation. Vasoconstriction was evaluated with either phenylephrine (PE, 10⁻⁵-10⁻³ M) or angiotensin II (ANG II, 10⁻⁶-10⁻⁴ M) superfusion. **Results:** Following the whole-body inhalation exposure to BP emissions, there were no impairments to percent change from control in endothelium-dependent vasodilation from the sham control group (-3.92 - 128.67 ± 22.35%) to the 2-day exposed group (-5.24 - 63.49 ± 14.18%). There were no impairments to percent change from control in vasoconstriction from the sham control group (-19.76 - -70.33 ± 5.03%) to the 2-day exposed group (-7.85 - -31.11 ± 8.01%). Compared to the sham control group, significant impairment in endothelium-dependent vasodilation was observed in the 3-day exposure group (1.27 - 24.02 ± 9.80%) and 6-day exposure group (2.69 - 8.42 ± 7.91%). Likewise, vasoconstriction was significantly impaired in the 3-day exposure group (-14.99 - -42.26 ± 8.38%) and 6-day exposure group (-20.01 - -48.73 ± 9.17%). **Conclusions:** This is the first study to conduct a direct comparison of microvascular function between sham control, BP exposed, and WUI exposed rats. Significant microvascular dysfunction was observed through the minimal response in endothelium-dependent vasodilation and vasoconstriction. Future experiments will expose rats to WUI fire emissions by burning WUI pellets in the surrogate combustion emissions generator and making comparisons of the health effects from BP exposures compared to WUI exposed animals. **Support:** UL Research Institutes, NIH P20-GM103434 (WVINBRE), U54-GM104942 (WVCTSI), R21-ES034942 (TRN), T32 ES032920 (TRN, VTN)

PS 4232 High-content phenotypic profiling of human aortic endothelial cells reveals class-specific lipid and organelle perturbations by environmental chemicals

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Background and Purpose: Environmental exposures have been associated with cardiovascular disease risk, the leading cause of death worldwide. Endothelial cells are critical sentinels of vascular health and may serve as sensitive targets for toxicant-induced cardio-metabolic stress. To investigate class-specific effects of environmentally relevant compounds, we applied a high-content phenotypic profiling strategy using primary human aortic endothelial cells (HAECs). **Methods:** HAECs were exposed for 72 hours to vehicle (DMSO) or a curated library of 48 compounds consisting of phthalate, bisphenol, pesticide, flame-retardant, liquid crystal monomer (LCM), and per- and polyfluoroalkyl substance (PFAS) classes across a 50 nM-100 µM range. Following exposure, cells were stained using Cell Painting (CPA) and a metabolic CPA (mCPA) variant and imaged on an automated confocal platform. The SPACe image analysis pipeline extracted region-specific metrics encompassing nuclear, endoplasmic reticulum (ER), lipid, and mitochondrial features. Phenotypic distance from DMSO controls and unsupervised UMAP clustering were used to assess concentration- and class-dependent responses. **Results:** Most compounds induced measurable, concentration-dependent morphological phenotypes. Pyrethroid pesticides and LCM emerged as the most active classes, strongly altering lipid-related features by increasing total BODIPY intensity per cell with altered lipid droplet number and size. Flame retardants and phthalates produced weaker, more variable responses, whereas PFAS primarily induced nuclear and ER-localized phenotypes. UMAP-based clustering demonstrated a reproducible progression from inactive to intermediate and strong phenotype states as concentration increased, capturing both potency and mechanistic diversity across chemical classes. **Conclusions:** High-content morphological profiling of endothelial cells can sensitively resolve compound-class-specific activity patterns and identify early indicators of lipid and organelle stress relevant to cardiovascular toxicity. Ongoing work is focused on defining the functional impact of these phenotypic changes, including effects on lipid uptake, activation of pro-inflammatory pathways, atherosclerotic potential, and regulation of endothelial nitric oxide synthase (eNOS)-dependent vascular physiology including vasomotor function and angiogenesis

PS 4233 Loss of PPARα in Murine Hearts Alters Female Sex-Specific Cardioprotection to Doxorubicin

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Background and Purpose: Doxorubicin (DOX), a chemotherapeutic agent, causes severe cardiotoxicity, oxidative stress, and mitochondrial dysfunction specifically in FA metabolism. PPARα (Peroxisome Proliferator Activated Receptor alpha) is essential for cardiac FA uptake and mitochondrial β-oxidation. The literature shows that loss of PPARα has no effect on DOX-induced cardiotoxicity or is even cardioprotective, however, these studies are not investigating sex differences and only report male data. Thus, we tested the hypothesis that loss of PPARα would eliminate sex-specific cardioprotection in females during DOX treatment. **Methods:** To test this, we used

quadruple-parametric optical mapping to simultaneously image transmembrane potential (V_m), calcium handling (Ca), NADH, and FAD intensity in male and female murine hearts. Male and female mice were randomly assigned to three groups; 1. Wild type (WT) (n=6) 2. WT+DOX (n=6) and 3. PPAR α KO+DOX (n=3-6). DOX (20mg/kg) was intraperitoneally injected 5 days before echocardiography, electrocardiography and optical mapping were performed in each mouse. **Results:** DOX therapy alone increased ejection fraction (62.37 ± 4.98 vs 54.83 ± 3.32) and fractional shortening (33.02 ± 3.55 , vs 28.03 ± 2.14) in female WT mice but not males, $p < 0.05$. On the other hand, in PPAR α KO+DOX mice vs WT, reduced stroke volume (Male: 24.48 ± 4.599 vs 43.95 ± 7.20 , Female: 23.94 ± 6.96 vs 36.60 ± 4.20) and cardiac output (Male: 12.03 ± 2.93 vs 18.66 ± 4.35 , Female: 10.13 ± 4.40 vs 16.28 ± 2.57) were observed in both sexes, $p < 0.05$. ECG analysis revealed that QTc interval was reduced in both male and female PPAR α KO+DOX mice vs WT (Male: 17.50 ± 2.93 vs 36.22 ± 2.67 , Female: 24.31 ± 3.30 vs 33.61 ± 1.05) and this was associated with increased arrhythmia incidences (Male: 3.52 ± 0.89 vs 0.54 ± 1.17 , Female: 5.45 ± 1.78 vs 1.68 ± 0.94) in these mice. Lastly, optical mapping studies revealed that, in male WT+DOX mice increased V_m rise-time (7.33 ± 0.59 , vs. 5.19 ± 0.64), Ca rise time (10.39 ± 1.43 , vs. 7.16 ± 0.76), and shortened V_m -Ca delay (-3.55 ± 2.56 , vs. -10.59 ± 4.13) were observed. Interestingly, in the male PPAR α KO+DOX mice, these depolarization/calcium release parameters were restored back to control levels contrary to the repolarization parameters recorded from the ECG. No significant electrical remodeling was observed in female WT+DOX or PPAR α KO+DOX mice. There were also no significant changes in NADH, FAD, or redox ratio (FAD/NADH). **Conclusions:** DOX treatment in WT mice, altered female mechanical function and male electrophysiology. On the other hand, DOX treatment in PPAR α ablated mice impaired mechanical and electrical function in both males and females and increased arrhythmia incidences. Notably, DOX-induced arrhythmogenic remodeling in cardiac depolarization observed in WT males was prevented in the PPAR α ablated mice. These findings suggest that 1. PPAR α is cardioprotective during DOX therapy and 2. females have different compensatory mechanisms than males with DOX exposure. This preliminary data adds to the long-term goal of developing sex-specific cardioprotective strategies for patients undergoing cancer chemotherapy.

PS 4234 Comparing Predictability of Drug-Induced Proarrhythmia Using hiPSC-Cardiomyocytes, Ion Channel Inhibition, and APD Simulation

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Background and Purpose: Cardiotoxicity, particularly proarrhythmia, is a leading cause of attrition in drug development. Therefore, early assessment of a drug compound's proarrhythmic potential is critical to mitigate the risk of encountering such issues in later stages. Proarrhythmic potential is typically evaluated by screening drug-induced inhibition of key ion channels, including hERG, NaV, and CaV, followed by phenotypic assays using hiPSC-derived cardiomyocytes. However, limited data exist comparing the predictive accuracy of these different methods in identifying proarrhythmic risk. In this study, we developed a phenotypic assay using hiPSC-derived cardiomyocytes combined with a multi-electrode array (MEA) system to evaluate the proarrhythmic potential of compounds. The assay outcomes were compared against predictions based on hERG inhibition and computational simulations of action potential duration incorporating effects on hERG, NaV, and CaV channels. **Methods:** iCell® Cardiomyocytes2 (FUJIFILM Cellular Dynamics, Inc.) were cultured in 96-well Cytoview MEA plates (Axion Biosystems, Inc.). A dose-escalation protocol was established to assess dose-response effects across up to four concentration levels. Extracellular field potentials were recorded at each dose, and field potential duration (FPD) was analyzed. We evaluated 27 compounds with known proarrhythmic risk, assessing predictability by identifying compounds that prolonged FPD or induced proarrhythmic events at concentrations up to 30-fold the clinical C_{max}. Ion channel inhibition data were obtained from published literature, and computational simulations of action potential duration were performed using the ORD model as described in prior studies. The same 30-fold C_{max} threshold was applied to evaluate proarrhythmia predictability across methods. **Results:** The hiPSC-cardiomyocyte assay detected 15 true positives and 9 true negatives among the 27 compounds. False negatives (terfenadine) and false positives (ranolazine and mexiletine) were consistent with previous reports. The same misclassifications were observed in assessments based on hERG inhibition and computational simulation. Compounds that inhibit hERG channels are not always associated with a high proarrhythmic risk due to compensatory effects from multi-channel inhibition. Notably, the hiPSC-CM assay effectively predicts risk by capturing this balance, whereas hERG inhibition assays and computational simulations alone may be insufficient for fully accurate risk prediction, as seen with compounds such as clozapine and verapamil. **Conclusions:** In conclusion, we have established a robust *in vitro* proarrhythmia assay using hiPSC-derived cardiomyocytes combined with an MEA system. This assay demonstrated higher predictive accuracy for identifying the proarrhythmic potential of drug compounds compared to the conventional ion channel inhibition assays and computational simulations of action potential duration.

PS 4235 Ready-to-use Human Cardiac Organoids for Chronic Contractility-Based Toxicity Profiling of Small Molecules and Biologics

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Background and Purpose: Human cardiac organoids (hCOs) serve as a human-based alternative for functional cardiotoxicity evaluation, offering complex three-dimensional tissue architecture that mirrors native cardiac cellular composition and cell-cell communication. Their ready-to-use availability enables steady workflow integration, supporting rapid assay initiation and improved experimental consistency. Here, we present a detailed functional characterization of hCOs using a high-throughput label-free system designed to assess cardiac contractility over time. **Methods:** Cryopreserved, ready-to-use cardiac organoids were plated onto flexible membranes resembling human heart mechanical conditions in a 96-well assay format (FLEXcyte 96). Contractile function was monitored using non-invasive capacitive distance sensing analysis. Contraction force was continuously monitored to determine the establishment of a functional steady state. Subsequently, organoids were exposed chronically to cardiotoxic agents doxorubicin (small-molecule) and trastuzumab (monoclonal antibody). Contraction force was measured for three days to evaluate concentration-dependent changes over time. **Results:** Cardiac organoid contraction force increased steadily during pre-culture and reached a stable functional plateau after approximately 14 days, indicating successful establishment of a mature contractile phenotype. Subsequent chronic exposure to 500 nM doxorubicin or 687 nM trastuzumab led to a pronounced reduction in contractile force to 55% and 70% of baseline, respectively. Combined treatment resulted in an additional decline to 50%, consistent with the recognized cardiotoxic profiles of these agents. **Conclusions:** This proof-of-concept study demonstrates that cryopreserved human cardiac organoids plated on flexible membranes provide a sensitive and scalable system for chronic functional cardiotoxicity assessment of both small molecules and monoclonal antibodies. These findings support broader adoption of human-relevant 3D *in vitro* models in cardiovascular safety and toxicology, aligning with the growing regulatory momentum towards acceptance of NAMs in preclinical drug development.

PS 4236 Non-invasive assessment of electromechanical coupling in the study of cardiomyocytes

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Background and Purpose: Quantitatively assessing cardiomyocyte contractility, in combination with electrophysiological measures, is crucial for understanding cardiac function, cardiovascular diseases, and pharmacological responses. Drug-induced cardiotoxicity remains a significant concern, contributing to nearly one-third of drug failures in late-stage development and causing approximately 10% of global drug withdrawals. Many compounds affect both ion channel activity and mechanical contraction, highlighting the need for integrated, non-invasive approaches to evaluate electromechanical behavior *in vitro*. **Methods:** This poster presents an innovative approach based on the use of the IntraCell system, developed by Foresee Biosystems, to simultaneously monitor the mechanical contractility at a high frame rate (150 frames per second) and electrical activity of human iPSC-derived cardiomyocytes *in vitro* by integrating non-invasive optical and electrophysiological techniques. Contractility and displacement dynamics were quantified using Farneback's dense optical flow applied to high-resolution videos acquired through an integrated camera. In parallel, intracellular action potentials (APs) were recorded using a micro-electrode array (MEA) system combined with laser-based optoporation technology, which preserves cell integrity and viability. A tailored trigger allowed precise synchronization of mechanical and electrical signals. **Results:** We compared control and dofetilide-treated cells, highlighting drug-induced AP prolongation and its effects on the electromechanical behavior of cardiomyocytes. Specifically, dofetilide treatment led to a slower repolarization phase and delayed relaxation, confirming the platform's ability to detect and correlate pharmacologically induced changes in both electrical and mechanical cardiac function. The system successfully recorded high-quality, intracellular-like action potentials using an entirely non-invasive technique, capturing hallmark features such as early afterdepolarizations (EADs) induced by dofetilide. Moreover, the proposed methodology enabled precise tracking of the time evolution of these signals across contraction-relaxation cycles in both control and treated cells, demonstrating its reliability for dynamic, long-term analysis. **Conclusions:** In conclusion, this methodology enables both acute and long-term monitoring, offering significant potential for drug screening and evaluation of cardiotoxicity. The ability to correlate electrical and mechanical signals offers an innovative perspective on the electromechanical coupling of cardiomyocytes, thereby enhancing the understanding of their physiological and pathological dynamics and filling a critical gap in cardiac research.

PS 4237 **Lead (Pb) Exposure During Differentiation Induces Cell Line-Specific Transcriptional and Functional Alterations in Human iPSC-Derived Ventricular Cardiomyocytes**

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Background and Purpose: Cardiovascular disease (CVD) etiology is known to be strongly influenced by lead (Pb) exposure and highly sexually dimorphic, but the underlying sex specific molecular and functional mechanisms are unclear. Further, the dose-response effects of Pb exposure during heart development are yet to be fully understood. Using human induced pluripotent stem cell (iPSC) derived ventricular cardiomyocytes from one male and one female donor, we characterized how Pb exposure during cardiomyocyte differentiation impacts transcriptional as well as voltage and calcium dynamics. **Methods:** We used an established small molecule protocol that temporally modulates Wnt signaling to differentiate iPSCs from one male and one female donor into ventricular cardiomyocytes. During the first 8 days of differentiation, we treated with 0.5 or 5 μ M Pb acetate. We collected samples on days 0-15, extracted RNA, conducted RNA-Seq using a plexwell protocol for library preparation in the female-derived line, and did benchmark dose modeling using BMDExpress 3 on this data. Between days 10-20 of differentiation, the male and female derived iPSC-cardiomyocytes were purified using a magnetic bead protocol to selectively deplete non-cardiomyocyte populations, and after recovery of purified samples, optical mapping of action potentials and calcium (Ca) transients was conducted using a Nautilus instrument and fluorescent dyes (FluoVolt for action potentials and Calbryte for Ca transients). **Results:** RNA-Seq of the Pb-exposed female-derived cell line revealed significant changes in expression of genes throughout differentiation (p -adj < 0.05) and dysregulation of pathways related to heart development and function throughout differentiation in response to Pb, including epigenetic and mitochondrial machinery (FDR < 0.05). Upon benchmark dose modeling of this data, we found unique dose-response gene expression changes induced by Pb on different days of differentiation. While the expression changes were unique, they corresponded to a similar median benchmark concentration of 0.2 μ M for all days assessed. Gene ontology analysis of benchmark modeled genes showed changes in pathways related to heart development (Fisher's exact two-tailed p -value < 0.05). Further, we characterized functional changes in female- and male-derived iPSC-cardiomyocytes exposed to Pb during differentiation using optical mapping of Ca transients and action potentials. We found cell line-specific effects of Pb, including an increase in beating rate in both cell lines with Pb exposure (p -values < 0.0001 for both cell lines at 5 μ M Pb), and a decrease in the change in fluorescence and upstroke slope in the male cell line (p -values < 0.0001 and = 0.0001 for 5 μ M Pb, respectively). Interestingly, in the male cell line, median conduction velocity decreased when measuring voltage and increased when measuring Ca dynamics (p -values 0.0087 for 0.5 μ M Pb and < 0.0001 for 5 μ M Pb, respectively). **Conclusions:** We characterized the transcriptional and electrophysiological changes induced by Pb in ventricular cardiomyocytes derived from iPSCs. Transcriptional changes assessed in the female-derived line showed a dose-response effect correlating to human-relevant exposure ranges. Optical mapping iPSC-cardiomyocytes from the female and male patient donors revealed Pb-induced changes in beat characteristics in both cell lines and disruption of voltage and Ca coupling in the male cell line. Importantly, these changes were present 9-17 days after cessation of exposure to Pb. A limitation of this work is the use of only one cell line for transcriptional studies and two for optical mapping; further transcriptomic and functional studies should use a representative sample of numerous male and female donors to draw conclusions about potential sex differences. Through a combination of transcriptomic and functional analyses, we found that Pb exposure during cardiomyocyte differentiation induces changes related to the development of cardiac pathology, opening the door to future mechanistic studies examining how Pb exposure during differentiation may impact heart function.

PS 4238 **A comprehensive assessment of *in vitro* heart safety evaluation for Tyrosine kinase inhibitors**

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Background and Purpose: Tyrosine kinase inhibitors (TKIs) and other small-molecule anticancer drugs have significantly improved the treatment and prognosis of various cancer types. However, unpredicted cardiac toxicity has been observed in patients undergoing these therapies. This toxicity can manifest as arrhythmias, heart failure, or myocardial infarction, posing severe risks to patient safety. The current study aims to reassess the cardiac safety of anticancer drugs by evaluating their effects on isolated heart electrocardiograph, cardiac action potential, related ion channels, and cytotoxicity. This approach seeks to establish an early cardiac safety assessment method for anticancer drugs, facilitating the safe development of new therapeutic agents. **Methods:** *In vitro* heart perfusion assay was conducted on healthy male New Zealand White rabbits. The hearts were extracted and perfused using the Langendorff system, with Sunitinib (0.3, 3, 10 μ M/L), Crizotinib (0.3, 1, 3 μ M/L), and Doxorubicin (1, 30 μ M/L) administered sequentially for 120 minutes. A control group without drug perfusion was also included for comparison. ECG parameters

and left ventricular pressure were recorded. Additionally, manual patch clamping was used to assess the effects of these drugs on ion channel currents (hERG, Nav1.5, and Cav1.2) and action potentials in human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). Cell viability was evaluated by measuring ATP levels using the CellTiter-Glo luminescent cell viability assay. **Results:** *In vitro* heart perfusion experiments revealed that Sunitinib and Crizotinib at concentrations ≥ 3 μ M/L significantly decreased heart rate (P < 0.01), prolonged QT/QTc intervals (P < 0.01), and reduced left ventricular pressure compared to the control group. Manual patch clamping demonstrated that Sunitinib and Crizotinib at 3 μ M/L inhibited the activities of hERG, Nav1.5 and Cav1.2 channels and significantly prolonged the duration of action potential (P < 0.01). Furthermore, Sunitinib, Doxorubicin, and Crizotinib significantly reduced ATP levels and cell viability in hiPSC-CMs at their respective IC50 values (Sunitinib: IC50 = 4.64 μ M/L; Doxorubicin: IC50 = 4.21 μ M/L; Crizotinib: IC50 = 2.87 μ M/L; P < 0.01). **Conclusions:** This study successfully established an early cardiac safety assessment method for anticancer drugs, incorporating *in vitro* heart perfusion assay and electrophysiological patch clamping technique. The results indicate that Sunitinib, Crizotinib, and Doxorubicin exhibit concentration-dependent cardiac toxicity, affecting ECG parameters, ion channel function, and cardiomyocyte viability. This comprehensive evaluation approach provides valuable insights into the cardiac safety of anticancer drugs and supports the safe development of new therapeutic agents. Future studies should further validate this method in larger animal models and clinical trials to ensure its applicability and reliability in predicting cardiac toxicity in humans.

PS 4239 **Complementary cardiotoxicity screening pipeline from 2D hiPSC-cardiomyocytes towards complex 3D cardiac organoids and living myocardial slices**

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Background and Purpose: Cardiotoxic side events represent one of the major concerns within pharmaceutical drug development and remain one of the leading causes for market withdrawal. The prevailing preclinical testing strategies rely on the use of cell lines (e.g. transfected HEK293, CHO) that express hERG artificially or iPSC-derived cardiomyocytes. However, these models lack the structural and functional complexity when compared to 3D mature myocardium and intrinsic cardiomyocyte action potentials. To circumvent these experimental limitations, we established a screening pipeline of diverse cardiac models, namely 2D hiPSC-derived cardiomyocytes, multicellular cardiac organoids and a microphysiological system (MPS) for 3D *ex vivo* living myocardial slice (LMS) cultivation. The complementary usage of these platforms enables a multifaceted screening for cardiotoxic events *in vitro* and *ex vivo*. **Methods:** First, dose-dependent effects of known cardiotoxic compounds were investigated using multi electrode array (MEA) measurements of hiPSC-derived cardiomyocytes in adherent cultivation. Second, multicellular cardiac organoids (consistent of cardiomyocytes, fibroblasts, endothelial cells and mesenchymal cells) were utilized for contractility assessment and global characterization of compound effects. Lastly, MPS was applied by combining left ventricular porcine LMS (300 μ m) on a heart-on-chip approach. Here, slices were paced using a dual (S1S2) pacing protocol to monitor action potential duration in functional cardiac tissue upon compound stimulation. **Results:** Initially, adherent hiPSC-cardiomyocytes were treated with known cardiotoxic compounds to perform dose-finding studies. Tolerable concentrations were further investigated in MEA analysis of cardiac beating behavior, revealing altered contractility for fenoterol and bosentan administration, respectively. Cisapride, a known hERG channel blocker, did not display clear impacts on contractile function within this model system. However, reevaluation of these compounds in the multicellular cardiac organoid platform displayed concentration-dependent impairment of contractility, especially with regard to beating frequency and relaxation time for all three mentioned compounds. Lastly, cisapride and the specific hERG channel blocker dofetilide were confirmed to prolong the action potential duration of 3D porcine myocardium within the MPS system in a dose dependent manner. Importantly, these observations were detectable within minutes after drug administration. **Conclusions:** The combination of well-established adherent hiPSC-cardiomyocytes with the complex multicellular organoid and 3D LMS heart-on-a-chip model, presents a complementary approach for the evaluation of drug cardiotoxicity. By integration of a diverse cellular environment and usage of mature porcine cardiac tissue, translatability of drug safety was enhanced complying the need for more reliable testing platforms to reduce animal studies. The herein described approach can be universally applied in pre-clinical drug development to screen for potential cardiac off-target effects.

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Background and Purpose: Minoxidil, a potent arteriolar vasodilator, is known to induce reflex tachycardia and conduction abnormalities. However, noninvasive techniques for monitoring vascular toxicity in repeated-dose toxicity studies remain limited. This study aimed to evaluate an integrated approach combining vascular ultrasound and ambulatory electrocardiography under isoflurane anesthesia to characterize cardiovascular toxicity profile in beagle dogs following repeated oral administration of minoxidil for 15 days. **Methods:** Three male beagle dogs received minoxidil (3 mg/kg/day) via oral capsule for 15 consecutive days. Evaluations included clinical observations, body weight, hematology, blood chemistry, 24-hour ambulatory ECG (pre-dose and at multiple timepoints on Day 1 and 14), and vascular ultrasound of the abdominal aorta and renal arteries (Day -10, 2, and 15). Cardiac ultrasound was performed on Day -10, 2, and 15. Histopathological examination of the heart and kidneys was conducted at study termination. Data were compared to individual pre-dose baselines. **Results:** Minoxidil was generally well tolerated, with transient gastrointestinal signs and body weight gain. ECG revealed marked tachycardia shortly after dosing, with shortened PR and QT intervals shortening. Mild atrioventricular block (AV block) and premature ventricular contraction were intermittently observed. Vascular ultrasound showed increased aortic flow velocity and shear stress early in the dosing period, with partial normalization by Day 15. Renal blood flow and resistive indices increased, indicating altered regional hemodynamics. Cardiac ultrasound showed increased cardiac output and transient improvement in myocardial performance, with preserved ejection fraction. Mild valvular regurgitation and pericardial effusion were noted in individual animals. Histopathological examination revealed mild hemorrhage and degeneration in the right atrial wall and papillary muscles of the heart, consistent with previously reported minoxidil-induced cardiac lesions. In the kidneys, minimal tubular dilation and congestion were observed, without overt nephrotoxicity. Hematology revealed increased reticulocyte counts, while coagulation parameters remained stable. Blood chemistry showed decreased lipid levels and transient electrolyte changes; liver enzymes remained within expected variability. These findings are consistent with previous reports indicating that minoxidil-induced cardiac output elevation and atrial overstimulation may contribute to right atrial hemorrhagic lesions and papillary muscle necrosis in dogs. The observed hemodynamic shifts and arrhythmogenic potential align with established mechanisms of minoxidil cardiotoxicity. **Conclusions:** Oral administration of minoxidil for 15 days induced acute hyperdynamic circulation and reflex cardiac changes in beagle dogs. Vascular ultrasound sensitively detected systemic and regional hemodynamic shifts, while ambulatory ECG captured conduction changes and arrhythmias. Histopathological findings supported the functional observations, confirming mild cardiac lesions and minimal renal changes. This combined noninvasive platform offers translational value for detecting vascular toxicity in repeated-dose toxicity studies of vasodilators. Further studies with larger cohorts and controls are warranted to refine thresholds and mechanistic interpretation.

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Background and Purpose: The myocardium enables powerful contractions essential for circulation and oxygen delivery. Cardiac contractility is a critical parameter for diagnosing cardiac conditions and guiding therapeutic development. Engineered Heart Tissues (EHTs) derived from human induced pluripotent stem cells (iPSCs) have become indispensable for cardiac research, yet most platforms remain low-throughput, costly, and lack reproducibility. **Methods:** We present a high-throughput 96-well EHT platform featuring a PDMS-free, bioinert consumable manufactured via high-resolution 3D printing, ensuring reproducible pharmacological testing and reduced cell requirements. **Results:** The EHTs displayed spontaneous contractions for approximately 30 days in culture. Over this period, they responded to electrical pacing and exhibited progressive functional maturation, evidenced by increased contraction amplitude and the development of a positive force-frequency relationship. Electrical pacing enhanced contractile strength while simultaneously reducing the spontaneous beating rate, suggesting adaptive remodeling of the tissue. Pharmacological profiling confirmed physiological responsiveness: Isoproterenol induced positive inotropic and chronotropic effects; Nifedipine caused negative inotropy; E4031 prolonged beating pulse width without affecting force; Cisapride slowed relaxation and extended contraction duration. **Conclusions:** These results validate the platform's ability to replicate clinically relevant drug effects and cardiac physiology *in vitro*, offering a scalable, cost-efficient solution for preclinical cardiotoxicity screening and drug discovery.

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Background and Purpose: Natural products, including botanical dietary supplements, are widely used but have variable chemical compositions due to cultivation, extraction, and processing factors. Ensuring their safety is critical, yet no routine *in vitro* methods exist for testing these complex mixtures. Consequently, safety data are limited, and animal studies remain the primary source despite translatability and animal welfare concerns and high costs for routine use. An international effort led by the US Food and Drug Administration (FDA), National Institute of Environmental Health Sciences (NIEHS), and Health and Environmental Sciences Institute (HESI) is evaluating New Approach Methodologies (NAMs) for their applicability to botanical mixtures. **Methods:** For the safety endpoints, a battery of well-established cardiotoxicity assays for single chemicals was selected to evaluate their suitability for botanical mixtures. The assay parameters included contraction force, beat rate, duration, upstroke/downstroke slopes, and arrhythmic events, complemented by metabolic and calcium transient analyses. The assays were performed in human iPSC-derived cardiomyocytes (hiPSC-CMs) cultured in a 96-well microphysiological system mimicking native heart tissues and its vasculature, enabling physiologically relevant contractile measurements. **Results:** Botanicals with known cardiotoxicity were chemically profiled and tested, while milk thistle and Asian ginseng served as negative controls. Among 16 botanicals, aconite, oleander, goldenseal, and yohimbe showed significant effects on contraction force, beat rate, and duration, aligning with their arrhythmogenic potential. Ashwagandha, kratom, comfrey, and thunder god vine had minimal or no impact. Ephedra transiently increased the beat rate by ~40%, resolving within 24 hours. Decaffeinated green tea showed dose-dependent effects after 24 hours, possibly due to higher concentrations than typical consumption levels. The findings were substantiated by calcium transient and metabolic analyses. The botanicals with high cardiotoxic risk showed significant impact on the calcium handling of hiPSC-CMs both under acute and chronic administration, while chronic oxygen consumption analysis revealed impact of a broad range of botanicals, indicating the need for refinement of the concentration ranges and other assay parameters. **Conclusions:** The results from these assays based on hiPSC-CMs show that microphysiological systems are useful for assessing potential for cardiotoxicity of botanical mixtures.

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Background and Purpose: Cardiotoxicity remains a leading cause of drug attrition, highlighting the need for predictive non-clinical models. We developed a cardiac safety database of 163 reference compounds, including the CiPA panel, spanning diverse mechanisms of action. This work validates a multi-endpoint strategy incorporating New Approach Methodologies (NAMs) for early cardiotoxicity risk assessment. **Methods:** Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) as monocultures or as 3D microtissues in co-culture with fibroblasts and endothelial cells. Our integrated NAM-based approach combined high-content imaging (HCI), calcium transient kinetics (CaT), lactate dehydrogenase (LDH) release, and whole-genome transcriptomics (ScreenSeq™). A cardiotox framework of predictive and mechanistic gene signatures was constructed. Differential gene expression, pathway enrichment, and benchmark dose modeling (BMD Express 2) of gene expression and cardiotox signature regulation were performed. Cardiotoxicity prediction metrics were derived from minimal effective concentrations (MEC) relative to clinical Cmax. AI/ML models were applied to transcriptomic profiles for enhanced prediction. **Results:** Cardiotoxants elicited responses at lower concentrations than non-cardiotoxants. Combined HCI, CaT, and transcriptomics provided the strongest predictive performance in 2D hiPSC-CM monocultures, with sensitivity, specificity, and accuracy of 83.5%, 84.1%, and 83.7%, respectively; AI/ML models improved these to 86.7%, 84.1%, and 85.9%. Dose-response analysis of cardiotoxicity signatures confirmed lower points of departure for cardiotoxants. Clustering and transcriptional signatures revealed mechanistic insights, distinguishing target-related and off-target effects across key cardiotoxicity characteristics. 3D microtissues extended the spectrum of detectable cardiotox characteristics by fibroblast- and endothelial-specific responses. **Conclusions:** This NAM-based, multi-endpoint approach enables robust detection and prediction of diverse cardiotoxicity mechanisms, offering mechanistic insight and early de-risking of new chemical entities in drug discovery.

PS 4244 Developmental exposure to Trifluoroacetic Acid cause vascular toxicity via lactate dehydrogenase-dependent glycolytic disturbance

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are globally persistent contaminants, yet ultrashort-chain PFAS have been inadequately investigated in current research. Trifluoroacetic acid (TFA), a two-carbon (C2) PFAS, is a terminal degradation product frequently detected in the environmental matrices. This study sought to determine the vascular effects and the underlying mechanism by developmental exposure to environmentally relevant concentrations of TFA. **Methods:** Zebrafish larvae [Tg(fli1a:EGFP)] were exposed to TFA (800 ng/L-2 mg/L) from 2hpf to 72hpf. High-resolution images of the vasculature were obtained using a high-content imaging platform for morphological phenomics. Human Umbilical Vein Endothelial Cells (HUVEC) were treated with TFA and the viability, tube formation, migration, and F-actin assays were measured at 72h. Larvae samples were collected for the RNA-seq and analyze the transcriptome. Glycolytic function was quantified by lactate, pyruvate, ATP production. Lactate dehydrogenase (LDH) inhibition was validated by Lineweaver-Burk kinetics and molecular docking. **Results:** TFA exposure induced a dose-dependent developmental toxicity in zebrafish, increasing malformation rates and reducing body length, while hatching and survival remained largely unaffected. The morphological phenomics exhibited a pronounced impairment of the subintestinal vein plexus (SIVP) as compared to the alteration of intersegmental vessels (ISV), common cardinal vein (CCV), and posterior cardinal vein (PCV). *In vitro* study showed a reduction in cell viability, tube formation capacity, migration, and F-actin disruption. RNA-seq analysis identified the gene expression pattern related to the suppressed glycolysis and the impaired angiogenesis. Consistent with transcriptomic signatures, TFA suppressed glycolytic metabolism, decreasing lactate and ATP while elevating pyruvate levels in a dose-response fashion. Enzyme kinetics demonstrated a competitive inhibition pattern on LDH. Molecular docking confirmed interactions of TFA within the LDH catalytic site. **Conclusions:** Our findings revealed that developmental exposure to environmentally relevant concentrations of TFA impairs vascular growth by disturbing LDH-dependent glycolytic process. This study deepens current understanding of the ultrashort-chain PFAS' toxicity and help establishes a mechanistic framework for identifying sensitive biomarkers of early exposure.

PS 4245 Chemical, Drug, and Stress induced Cardiovascular Dysfunctions: A Comprehensive Bioinformatics and Bench Studies in Gulf War Illness

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Background and Purpose: Cardiovascular diseases (CVD) are an emerging problem amongst the different health issues underlying Gulf War Illness (GWI), a multi-symptom illness affecting the Gulf War veterans. Despite emerging evidence suggesting significant impacts of GWI on the cardiovascular systems, research in these areas remain limited. While the exact causes of these symptoms are unknown, they are thought to be a consequence of exposures to multiple chemicals, drug the veterans consumed, and the stress they endured during the war. In this project, we investigated the cardiovascular connection linked to GWI using computational analysis in clinical cohort of healthy and GWI along with well-established GWI rat model *in vivo* and cardiomyoblast cell *in vitro*. **Methods:** We carried out a multi-level computational analysis using transcriptome data from GWI and healthy human cohort's blood samples at baseline (T0) and maximal exertion (T1) timepoints. Core analysis was performed using Ingenuity Pathway Analysis (IPA) to understand the disease end point due to chemical exposure in Gulf War Veterans. To investigate the effect of GWI chemicals *in vivo*, eight-week-old male Sprague Dawley rats were dosed with an acetylcholinesterase inhibitor pyridostigmine bromide (PB), orally daily; N,N-diethyl-m-toluamide (DEET) and Permethrin (PER) dermally, and 15-min restraint stress for four weeks as per our established GWI model. Naïve control rats were maintained in parallel to GWI rats, which were not subjected to chemical or stress exposure. Serum was collected at 7 months and 13 months post-exposure from both naïve as well as GWI groups. To examine the effect of GWI serum on cardiac health, H9C2 (rat cardiomyoblast cell line) was treated with naïve and GWI serum for 48h. Additionally, to confirm the effect of GWI chemicals on cardiotoxicity, H9C2 was treated with PB, DEET and PER individually or in combination at low, medium, and high concentrations. We then investigated a series of inflammation markers, apoptosis markers, and oxidative stress markers in both serum as well as chemical treated cells. **Results:** In GWI veterans, gene ontology analyses showed dysregulation of several processes involving cardiac muscle cell repolarization, action potential, as well as anatomy and physiology compared to healthy controls. For example, ZDHHC12, involved in regulating cardiac function, stability, and responses to injury; was differentially regulated in male and female under stress condition (T0 vs T1). In addition, tox function analysis using IPA revealed the association of molecular alterations in GWI patients to cardiotoxicity such as, cardiac death/ necrosis, cardiac enlargement, and cardiac dilation compared to the healthy ones. Furthermore, IPA also indicated that GWI chemicals can induce apoptosis and mitochondrial dysfunction via various intermediate pro-inflammatory molecules. Serum treatment on H9C2

showed increased Namp1 expression and interleukin-6 (IL-6) in GWI-serum treated group compared to the naïve group indicating inflammatory milieu due to GWI serum components. In addition, we observed a significant increase in apoptosis markers, Caspase-3 and Caspase-8 in both GWI-serum treated timepoints. Furthermore, we observed a significant increase in Sod1 and Gpx which are the key regulators of oxidative stress and known to be induced by inflammation and apoptosis which can further induce mitochondrial dysfunction which is the key contributor to the cardiometabolic disease. Chemical treated cardiomyoblast showed a decreasing trend in mitochondrial marker, Nrf1 across all the treatment groups and an increasing trend of Caspase-3 under all the treatment groups, which is a crucial protein that executes the apoptosis pathway. **Conclusions:** In a nutshell, we showed sex-specific differences in cardiovascular dysfunction of GWI veterans. Our *in vitro* and *in vivo* analysis confirmed dysregulation of functional end points which are the hallmark of CVD. Future work will investigate in-depth *in vivo* characterization at both whole-body and tissue-specific levels in male and female GWI rat model.

PS 4246 Sensitivity of Latin Square and Ascending Dose Design for Cardiovascular Safety Pharmacology Studies in Telemetry Implanted dogs

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Background and Purpose: The latest ICH E14/S7B Q&As permit the inclusion of high-quality nonclinical QTc data in integrated risk assessments to support waivers for Thorough QT (TQT) studies, provided that rigorous validation criteria are met. Given that Latin Square and ascending dose designs are widely utilized in cardiovascular safety pharmacology studies, we conducted a systematic statistical power analysis of the standard cardiovascular safety study models routinely employed at our facility. This analysis aimed to ensure the generation of high-quality preclinical data and to demonstrate that these models are capable of producing data that meet regulatory requirements. **Methods:** We retrospectively analyzed telemetry data from Latin Square cardiovascular safety pharmacology studies in beagle dogs (n=308) to evaluate the statistical power of these experimental models. The studies typically employed a 4x4 Latin Square crossover design or an ascending dose design with N=3 or 4 dogs. Data were collected over approximately 24 hours, and results from the pre-dose and each subsequent 1-hour interval were analyzed using ANOVA and mixed models, respectively. Minimum detectable differences (MDDs) with 80% statistical power were calculated for standard parameters, such as heart rate (HR) and ECG intervals. **Results:** For the Latin Square crossover design (N=4 or 8), the MDDs were: QRS interval (3.3 ms in males, 3.1 ms in females), PR interval (7.6 ms in males, 7.7 ms in females), and QTc interval (10.1 ms in males, 10.3 ms in females). For the ascending dose design (N=3 or 6), the MDDs were: QRS interval (4.8 ms in males, 3.7 ms in females), PR interval (7.3 ms in males, 7.4 ms in females), and QTc interval (10.5 ms in males, 11.5 ms in females). In addition, we report reference intervals for the root mean square error (RMSE) and its 2.5th and 97.5th percentiles as measures of variability within the studies. **Conclusions:** These findings demonstrate that the Latin Square cardiovascular safety pharmacology model in beagle dogs provides high statistical power and is capable of detecting subtle changes in electrocardiogram parameters, thereby offering a robust experimental foundation for cardiovascular safety evaluation of drug candidates. Nonrodent cardiovascular models are sensitive tools for early detection of cardiovascular risk. Furthermore, the results confirm the assay sensitivity of functional endpoints and support the use of these data within the framework of the ICH E14/S7B Q&As.

PS 4247 Sex Matters: Differences in Cardiac Phenotype and Responses to Inhaled Nano-TiO₂

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Background and Purpose: Epidemiological studies identify particulate matter (PM) exposure as a risk factor for adverse cardiovascular events. There is growing evidence demonstrating sex-dependent effects in cardiac pathophysiological responses to airborne toxicants in humans. Despite this, few studies have assessed sex-specific mechanical and structural outcomes associated with PM inhalation. Therefore, the purpose of this study was to evaluate differences in cardiac responses to inhaled nano-TiO₂ between male and female rats. **Methods:** Age matched male (n=6) and female (n=6) Sprague-Dawley rats were exposed to nano titanium dioxide (nano-TiO₂) aerosols, a surrogate for fine and ultrafine PM, for an average of 9.2 ± 0.04 mg/m³ per day. Animals were exposed in a whole body inhalation chamber 4 hours a day, 5 days a weeks for 2 weeks. Naïve, unexposed rats (n=6/sex) served as controls. Twenty-four hours post final exposure, cardiac ultrasound was conducted, followed by mean arterial pressure (MAP) measurement, sacrifice, and tissue collection. Histopathological and morphometric analyses were conducted on cardiac samples collected at time of euthanasia. **Results:** Data analysis revealed significant exposure/sex interactions, indicating that sex modifies cardiac responses to inhaled nano-TiO₂. Notably, LV mass and volume, key markers of adverse remodeling, exhibited sex-dependent outcomes. Exposure in females resulted in an 11% elevation in LV mass, while males exhibited a 15% loss. Similarly, LV volume was increased by 3% in females and reduced by 11% in males after nano-TiO₂ inhalation. Stroke volume also

presented sexually dimorphic responses, with a 7% increase in females and an 11% decrease in males after exposure. However, nano-TiO₂ increased MAP regardless of sex. Histopathological and morphometric outcomes are pending. **Conclusions:** These findings demonstrate that nano-TiO₂ disrupts cardiac anatomy and function differentially in males and females, which may contribute to sex-dependent pathophysiological responses to PM exposure. Future work will investigate mechanisms underlying these sexually dimorphic responses. Supported by: R01-ES-031285, P30-ES-005022, T32-ES-007148, Grover Foundation, and BMS fellowship

PS 4248 Organ-on-a-chip for cardio- and neurotoxicity studies on spheroids

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Background and Purpose: Human cardiac and neuronal stem cell-derived spheroids or organoids are emerging models for drug discovery and drug safety studies. Investigations of their electrical properties are frequently carried out by means of multielectrode arrays (MEAs). The round shape of these 3D models restricts, however, electrode contacts with planar MEAs. Contractions of cardiac 3D models and movement artefacts during the application of test solutions can interfere with the measurements. Here we propose an organ-on-a-chip that attaches free floating spherical cell models such as cardiospheres and neurospheres to conventional planar MEAs. The integrated microfluidics device stabilizes the surface contacts with the spherical cell models, reduces motion artifacts and permits complete solution exchange in pharmacological studies. **Methods:** Cardio- and neurospheres were generated according to the manufacturers instructions. Using the organ-on-a-chip the spheroids could be recorded on planar MEA electrodes. By fixing the spheroids on the electrodes motion artefacts could be reduced throughout the experiment, including fluid exchange for compound application. In addition, this system enables simultaneous recordings of field potentials with MEA and action potentials or calcium signals using fluorescent probes. As proof-of-concept cardiac reference compounds E4031 and dofetilide were cumulatively applied in increasing concentrations every 5 minutes. For neurospheres epilepsy-like bursting was induced using 4-aminopyridine and blocked by ML213. **Results:** The chip was successfully used for field potential measurements and simultaneous imaging using fluorescent probes on small cardio- and neurospheres (diameter 300 to 500 µm). We observed concentration-dependent prolongation of action- and field potentials of cardiospheres by the potassium channel (Kv1.11) inhibitors E4031 and dofetilide. In neurospheres application of the potassium channel (Kv7.2/7.3) activator ML213 reduced spiking and bursting. **Conclusions:** In summary, we present a novel organ-on-a-chip with microfluidics that enables field potential measurements on cardio- and neurospheres using conventional planar MEAs and simultaneous imaging studies. Proof-of-concept studies demonstrate that this technology is suitable for drug testing and toxicity studies.

PS 4249 Investigation of adverse effects of long-term exposure to e-cigarette compounds in human cardiomyocytes

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Background and Purpose: Electronic cigarettes (e-cigarettes) have garnered popularity as a “healthy” alternative to traditional cigarette smoking, though evidence suggests that e-cigarettes pose their own unique risks to the cardiovascular system. As one of the major compounds in e-cigarettes, nicotine has been shown to induce oxidative stress, impair mitochondria, and promote apoptosis, all of which contribute to the pathology of cardiovascular diseases. However, there remains a knowledge gap in the understanding of e-cigarette toxicity in cardiomyocytes (CMs) due to methodological shortcomings in the existing literature that do not reflect real-world e-cigarette use. Notable deficiencies include the use of supraphysiological chemical concentrations, unrealistic and unrepresentative chemical compositions, short-term exposure regimes, and non-human models. This study aimed to address these shortcomings by investigating the individual and synergistic cardiotoxic effects of mainstream e-cigarette chemicals on human stem cell-derived CMs under clinically relevant exposure conditions. Human embryonic (hESCs) and induced pluripotent stem cells (hiPSCs) provide a robust platform for disease modeling and chemical screening, and they can be considered as a new approach methodology (NAM) for predictive toxicology. **Methods:** The hESC-derived CMs (hESC-CMs) were exposed to physiologically relevant combinations and concentrations of the most prevalent chemical constituents found in modern e-cigarettes, including freebase nicotine (L-nicotine), salt nicotine (nicotine benzoate), and propylene glycol (PG). After 2 weeks of exposure, mitochondrial and cardiac functions were evaluated, and genome-wide transcriptome was analyzed in the CMs. **Results:** We found that nicotine-derivative or PG treatment alone did not induce obvious adverse effects, however, when CMs were exposed to nicotine and PG in combination, they exhibited more adverse effects on both mitochondrial and cardiac functions, including lower ATP production, decreased oxidative phosphorylation (OXPHOS) and mitochondrial network branching, altered contractility, and sarcomere disorganization. In addition, salt nicotine exhibited more cardiotoxic effects than freebase nicotine, and the mixture of nicotine salt and PG induced the greatest dysfunction of all treatment groups. **Conclusions:** Modern-era, pod-based e-cigarettes formulated with nicotine salts and PG induce greater cellular

dysfunction in CMs than tank-based electronic smoking devices formulated with freebase nicotine and PG. Ultimately, this work addresses a critical gap in the literature by employing clinically relevant exposure conditions and human cell models, providing evidence that the combined effects of modern e-cigarette constituents pose a cardiotoxic risk.

PS 4250 Effects of Titanium Dioxide Nanoparticles on Human Red Blood Integrity and Function: Implications for Anemia and Occupational Health

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Background and Purpose: Over the past decade, concern has grown about occupational inhalation of titanium dioxide nanoparticles (TiO₂ NPs) and their potential systemic health effects. While research has focused on pulmonary and inflammatory responses, red blood cells (RBCs) have received little attention despite being the first and most abundant cell type that TiO₂ NPs encounter in circulation. Workers in industries such as pigment manufacturing, coatings, and additive production may experience repeated low-level exposures to TiO₂ NPs, underscoring the need to understand systemic targets beyond the lung. RBCs are essential for oxygen transport and maintaining cardiovascular and metabolic homeostasis, and their dysfunction has been linked to anemia, thrombosis, and cardiovascular disease. This study investigates the effects of TiO₂ NP exposure on RBC integrity, premature cell death (eryptosis), and hemoglobin function. We hypothesize that occupationally relevant TiO₂ NP compromise RBC membrane stability and oxygen-carrying capacity, leading to functional impairment and increased vulnerability to secondary stressors. **Methods:** Deidentified human RBCs from healthy donors were exposed *in vitro* to rutile TiO₂ NPs (15-20 nm; MSE Supplies LLC; 5-200 µg/mL) for up to 24 h. Eryptosis, a programmed form of RBC death characterized by cell shrinkage, membrane blebbing and phosphatidylserine (PS) externalization was assessed by Annexin V-FITC staining and RBC size and membrane complexity (blebbing) quantified by forward and side scatter (FSC/SSC) both on a BD Fortessa flow cytometer. Ionomycin (1 µM) served as a positive control, and donor-matched non-exposed RBCs provided baseline signals. Hemolysis was quantified by free hemoglobin absorbance at 415 nm on a Tecan microplate reader and normalized to 0.5% Triton X-100 (100% lysis). Osmotic fragility was tested by incubating post-exposure RBCs in graded NaCl solutions (0.0-0.9%) for 30 min, followed by hemoglobin release measurement. All endpoints were normalized to paired non-exposed RBCs to account for interindividual variability. Flow cytometry data were analyzed using FlowJo, and statistical and dose-response analyses using GraphPad Prism. **Results:** Exposure to TiO₂ NPs induced dose-dependent eryptosis (scored by PS Annexin V-FITC staining) increase in RBCs relative to donor-matched controls, 6.3%, 15.7%, 20.6%, 44.4% and 68.3% at 5, 10, 20, 50 and 200 µg/mL respectively (mean of five donors). Dose-response modeling (4-parameter logistic regression) yielded an inhibitory concentration 50 (IC50) of 34.7 µg/mL (95% CI 19.1-526.9) with a Hill slope of 1.65 (0.61-3.85). Side scatter, reflecting membrane blebbing, increased by 14.9%, 29.2%, 67.3%, 143.9% and 354.4% at the same dose range of 5-200 µg/mL. Linear regression suggested a strong correlation between TiO₂ dose and SSC increase (Y = 1.78X+15.33, R² = 0.90, slope P < 0.0001). Low doses 5-50 µg/mL caused minor cell shrinkage (-0.57% to -2.74%), whereas 200 µg/mL produced slight size gain (+2.78%), suggesting nanoparticle attachment or osmotic effects. TiO₂ exposure induced concentration-dependent hemolysis, 5.2%, 5.5%, 9.0%, 12.5% and 16.6% at 5-200 µg/mL, respectively, confirmed by medium and particle controls. Osmotic fragility assays showed 35.1-60.2% greater hemoglobin release in RBCs exposed to 200 µg/mL TiO₂, indicating compromised membrane integrity after 24 h. **Conclusions:** Our results showed 24 h exposure to TiO₂ NP induces dose-dependent eryptosis, hemolysis, and increased osmotic fragility in human RBCs. Such RBC dysfunction may contribute to systemic effects including anemia, thrombosis and inflammation, and increase the physiological burden on RBC clearance organs (spleen, liver and bone marrow). These findings highlight the need to support further studies to establish occupational exposure thresholds (e.g. LOAEL) to better inform health risk assessment for TiO₂ nanoparticles.

PS 4251 Divergent functional responses in 2D and 3D human iPSC-derived cardiomyocytes to doxorubicin-induced cardiotoxicity and prevention

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Background and Purpose: Despite major improvements in anticancer treatment, chemotherapy-induced cardiotoxicity continues to present an ongoing challenge. Anthracyclines are a commonly prescribed class of anti-cancer drugs with a dose-dependent risk of heart failure after treatment. As a result, there is substantial interest in developing strategies for mitigating anthracycline-induced cardiotoxicity, especially through cardioprotective cotreatments. Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) can serve as human-specific preclinical

models of cardiotoxicity, but the effects of culture parameters on toxicity metrics are still not fully understood. In this study, we investigated the interplay of culture format (2D vs. 3D) and media composition (standard vs. maturation medium) in ventricular hiPSC-CM-based models of chronic doxorubicin (DOX) toxicity and cardioprotection from the iron chelator and topoisomerase II β inhibitor dexrazoxane (DEX). **Methods:** Ventricular hiPSC-CMs were generated using established protocols before being seeded into 2D monolayers or formed into 3D microtissues with 5% human cardiac fibroblasts. HiPSC-CMs were treated with DOX alone, DOX with DEX, or vehicle control (DMSO). Concentration and exposure time are noted with corresponding results. DOX toxicity was assessed by measuring concentration-dependent changes in cell viability via the metabolic MTT assay, functional contractile changes via video tracking, electrophysiological changes via high-speed optical mapping, and metabolic shifts via Seahorse Assay. The influence of medium-based maturation on both toxicity and cardioprotection was assessed by culturing hiPSC-CMs in a low-glucose, high-fatty acid, high-calcium maturation medium for one week prior to DOX and DEX treatment and downstream analysis. **Results:** As expected, 2D hiPSC-CMs showed multi-functional changes reflecting concentration- and time-dependent DOX toxicity. Loss of relative cell viability was strongly associated with treatment time (1 μ M DOX: 45.0 $\pm$ 4.7% after 72 hours vs. 86.0 $\pm$ 4.8% after 24 hours, p < 0.05). After treatment with 1 μ M DOX for 72 hours, hiPSC-CMs also showed functional declines in basal respiration compared to vehicle controls (2.4 $\pm$ 0.4 vs. 62.4 $\pm$ 5.7 pmol/min/10,000 cells, p < 0.05). Similarly, in 3D microtissues, there was substantial shortening in action potential duration (332.8 $\pm$ 17.8 vs. 498.8 $\pm$ 30.4 ms, p < 0.05) and reduction in contractility (0.6 $\pm$ 0.5 μ m vs. 4.5 $\pm$ 2.3 μ m, p < 0.05) compared to vehicle controls. Cotreatment with DEX was used to examine the ability of hiPSC-CM models to detect cardioprotection *in vitro* and the effects of culture format. In 2D, cotreatment with up to 30 μ M DEX for 72 hours did not affect viability of hiPSC-CMs treated with 1 μ M DOX (control: 62.3 $\pm$ 19.0% vs. 30 μ M DEX: 62.4 $\pm$ 12.9%). Similarly, in 3D microtissues, treatment with 10 μ M DEX and 1 μ M DOX did not significantly alter DEAD stain intensity (0.092 $\pm$ 0.022 AU) compared 1 μ M DOX alone (0.110 $\pm$ 0.33 AU), suggesting no cardioprotection in standard culture media. With an additional week in maturation medium prior to 1 μ M DOX treatment, 2D hiPSC-CMs did not show significant differences in viability. In contrast, maturation media-treated 3D cardiac microtissues showed 2.1-fold higher DEAD stain intensities (p < 0.05) in response to 1 μ M DOX compared to standard medium. However, we did not observe a significant difference in matured tissues cotreated with DEX and DOX compared to DOX alone, suggesting minimal or no rescue of viability by DEX even with culture in maturation media. **Conclusions:** Together, these data highlight the critical interplay of culture conditions on DOX toxicity in hiPSC-CM with a combination of 3D culture and maturation media driving an increase in observed DOX toxicity, but no DEX cardioprotection. Moreover, they suggest a broader role for 3D systems to represent adult cardiac function as mature culture platforms, especially with supplemental medium-based maturation.

PS 4252 Heart to Heart. Maternal and fetal cardiovascular consequences of gestational nano-titanium dioxide exposure

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Background and Purpose: Nano-titanium dioxide (nano-TiO₂) is an established vascular toxicant and is also known to impair fetal growth and development when exposed *in-utero*. Deficits in placental and fetal growth and development due to exposure occur in a sex-dependent manner, however these responses remain poorly characterized. Utilizing longitudinal echocardiography, both maternal and fetal cardiac function was tracked through a gestational model of nano-TiO₂ inhalation. **Methods:** Timed-pregnant dams received six nonconsecutive days of whole-body aerosol inhalation after gestational day (GD) 10 at a targeted concentration of 12mg/m³ nano-TiO₂ or sham air (25 mL/m) as controls. Particulate aerosolization was accomplished with an acoustic generator, with concentrations being held constant with the aid of a dataRAM™ aerosol monitor. Immediately following exposure on GD 13,15,17, and 20, animals were assessed for cardiovascular function via ultrasound assessment on a Vevo F2 preclinical ultrasound system using a 29MHz transducer. Motion analysis was used for maternal and fetal cardiac function, with brightness mode acquisitions supplementing maternal cardiac function and speckle-tracking analysis for assessment of cardiac strain. Uterine artery flow data was also assessed using doppler assessment. Additionally, the placental vascular network was assessed via placental explant perfusion with silicone vascular contrast perfusion and imaged via micro-computed tomography. Placentae of the proximal uterine horn were removed immediately following euthanasia of the dam, and the umbilical artery and vein surgically separated. The umbilical artery was cannulated and perfused with heparinized PBS (~500 μ l) immediately followed by Vascupaint® contrast media (~800 μ l) until perfusate exited the umbilical vein indicating complete perfusion throughout the placental vasculature. Male and female placentae (n = 3-4 per group) from exposed and control dams were analyzed with Imaris imaging software to assess total area, vascular length and diameter, as well as branch hierarchy within the placental vasculature. **Results:** Exposed dams presented with significantly decreased cardiac output (62.05 $\pm$ 3.79 mL/min vs. 75.36 $\pm$ 6.37 mL/min controls) beginning at GD 17 and further declining (49.64 $\pm$ 4.50 mL/min vs. 70.92 $\pm$ 2.52 mL/min) near term at GD 20. Fetal cardiac output in exposed animals (3.69 $\pm$ 0.42 mL/min) was also decreased at GD 20 when compared to sham air controls (4.73 $\pm$ 0.32 mL/min). Uterine artery resistance index was significantly decreased in exposed (0.54 $\pm$ 0.02 RI) versus control (0.67 $\pm$ 0.10 RI) dams at GD 20. **Conclusions:** The ability to monitor cardiovascular

adaptations of pregnancy during maternal toxicological stress, through the placenta and into the developing fetus provides a powerful tool that may be advantageous for limiting inter-subject variability within cardiovascular health during gestation and beyond.

PS 4253 β -Adrenergic Inhibition Prevents Gestational E-cig Exposure-Induced Sympathetic Dominance and Ventricular Arrhythmias throughout Pregnancy and Postpartum

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Background and Purpose: Perceptions that e-cigarettes (e-cigs) are safer than traditional cigarettes may contribute to the popularity of vaping during pregnancy among smokers and ex-smokers. Pregnancy is inherently associated with increased risk for arrhythmias, and the prevalence of pregnancy-associated arrhythmias has recently surged. We previously reported that e-cig exposure accentuates pregnancy-induced sympathetic dominance while evoking ventricular premature beats (VPBs, a predominant arrhythmia in pregnancy), persisting weeks after exposure cessation and labor. Sympathetic activation can evoke VPBs; however, the role of sympathetic dominance in the persistent arrhythmogenesis induced by gestational e-cig exposures remains unknown. Thus, we assessed the role of β -adrenoceptor activation in the arrhythmogenicity of gestational e-cig exposure by administering the non-selective β -adrenergic blocker, propranolol. **Methods:** Pregnant 11-week-old C57BL/6J mice received normal drinking water (n =4-7/group) or propranolol (0.5 g/L; n =4/group), and were exposed to e-cig aerosols or air daily throughout gestation, with exposures ceasing upon parturition. Electrocardiogram (ECG)-derived heart rate (HR) and VPBs were compared between normal water and propranolol groups matched by inhalation exposure. Home-cage ECGs were collected for 21 hours during labor and for 48 hours at 3 weeks postpartum. Propranolol was gradually withdrawn by halving concentrations weekly over the first two weeks postpartum. **Results:** E-cig exposure alone increased maternal HR on gestational day (GD) 1-15 (p <0.05 vs. Air) and depressed maternal RMSSD on GD 1-5 (p <0.05 vs. Air), suggesting sympathetic dominance. Concurrently, e-cig alone also increased the incidence rate of VPBs (p <0.05 vs. Air). Gestational β -adrenergic inhibition opposed these effects, decreasing HR on GD 1-15, increasing SDNN and RMSSD on GD 1-18, and, importantly, abolishing the arrhythmogenic effects of e-cigs (all p <0.05 vs. e-cig alone). During 21-h overnight monitoring of the parturition phase, gestational exposure to e-cig alone increased HR and depressed SDNN and RMSSD for 1 h each (p <0.05 vs. Air) while also increasing VPB incidence rate (p <0.05 vs. Air). In contrast, β -adrenergic inhibition in e-cig-exposed dams opposed these effects over the 21-h parturition phase, reducing HR for 4 h, increasing SDNN for 6 h, increasing RMSSD for 9 h, and abolishing VPBs relative to e-cig alone (all p <0.05). Three weeks after parturition and cessation of e-cig exposures (one week after discontinuing propranolol), there were no group differences in HR or HRV. However, gestational e-cig exposure alone increased VPBs (p <0.05 vs. Air), and prior β -adrenergic inhibition continued to abolish this effect (p <0.05 vs. e-cig alone) despite the discontinuation of propranolol. **Conclusions:** Gestational exposure to e-cigarette aerosols induces maternal sympathetic dominance and ventricular arrhythmias that persist well after parturition and exposures. β -adrenergic inhibition during gestation abolishes the immediate and persistent arrhythmogenic effects of gestational e-cigarette exposures. Thus, vaping during pregnancy may persistently induce arrhythmias and increase maternal peripartum cardiac risk through gestational sympathetic dominance.

PS 4254 Chronic E-Cigarette Exposure Induces Pulmonary Artery Remodeling in Apoe^{-/-} mice

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Background and Purpose: Electronic cigarettes (e-cigarettes) have gained popularity during the recent decade and are often considered to be a safer alternative compared to traditional combustible cigarettes. However, the chronic impact of e-cigarette vapor on cardiopulmonary structure and function remains poorly understood. The pulmonary arteries are a critical site of vascular regulation, and their structural remodeling is known to elevate pulmonary pressures, impair hemodynamics, and contribute to pulmonary hypertension. In pulmonary arterial hypertension (PAH), arterial stiffening worsens vascular remodeling and elevates pressure within the pulmonary circulation by placing strain on the right ventricle, which may lead to heart failure (PMID: 30073166). Increased arterial collagen content, particularly Type I collagen, has been shown to contribute to pulmonary artery stiffening (PMID: 21538012). This study aims to investigate the long-term effects of e-cigarette use on pulmonary artery remodeling, fibrosis, and changes in extracellular matrix (ECM) composition. **Methods:** Adult female Apoe^{-/-} mice (8 $\pm$ 1 weeks of age) were randomly assigned to either the e-cigarette aerosol or the air control exposure groups. The mice were exposed for 95 minutes/day, 5 days/week, over 24 weeks through a nose-only aerosol delivery (SCIREQ). Aerosol originated from virginia tobacco flavored JUUL pods containing 3% (35 mg/mL) nicotine and delivered at a rate of 2 puffs/

min, a puff volume of 55 mL/puff (DOI: 10.1093/ntr/ntaf084). Histology samples were collected at the 8 week, 16 week, and 24 week time points. Lung sections were stained using Picro-Sirius Red (PSR) and MOVAT Pentachrome, and imaged under polarized light or brightfield microscopy, respectively. Polarized light images were segmented and analyzed using ImageJ to determine the percentage area of mature (Type I, orange-red birefringence) and immature (Type III, green-yellow birefringence) collagen fibers in the adventitia layer of the pulmonary artery. MOVAT Pentachrome stained samples were used to quantify elastin and fibrin content, as well as artery thickness. Immunofluorescent and chromogenic histology staining (for CD31, vWF, VEGFR3, Fibrinogen, and CCL21 markers) was utilized to investigate fibrosis of pulmonary arteries and infiltration of immune cells into the arterial tissue. **Results:** Polarized light imaging revealed a time-dependent shift toward collagen maturation in the e-cigarette exposed pulmonary arteries exhibited by a gradual increase of Type I collagen relative to Type III collagen in the adventitia (8 week $p = 0.4697$; 12 week $p = 0.0923$; 24 week $p = 0.0002$). Elevated levels of Type I collagen ($p = 0.0235$) and decreased levels of Type III collagen ($p = 0.0490$) were observed in the e-cigarette arteries at the 24 week time point. Collagen maturation relative to controls indicates early fibrotic remodeling of the pulmonary artery wall. Preliminary analysis of MOVAT images showed increased arterial thickness in the e-cigarette exposed samples. Immunostaining analyses characterize the molecular basis of these structural changes, including changes in endothelial and lymphatic markers mentioned above. **Conclusions:** Arterial stiffening, fibrosis, and inflammation in response to chronic e-cigarette exposure suggest potential impairment of pulmonary artery function. This study highlights a correlation between chronic e-cigarette use and arterial remodeling that may predispose users to PAH and other heart diseases. Future research must explore the long-lasting effects of e-cigarettes on the structural remodeling of the pulmonary artery.

PS 4255 Immune Dysregulation in E-Cigarette Users: Contribution of Volatile Organic Compounds and Nicotine

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Background and Purpose: Electronic cigarette (e-cig) use is increasing rapidly, particularly among adolescents and young adults, yet its systemic immunological consequences remain poorly understood. We employed high-dimensional mass cytometry for deep phenotyping of peripheral blood and the functional profiling of peripheral blood mononuclear cells and examined their association with the urinary metabolites of volatile organic compounds (VOCs) and nicotine. **Methods:** We recruited 62 healthy young adults (18-30 years old), including 37 non-users and 25 e-cig users. Circulating immune cell populations were characterized by complete blood counts (CBCs) and high-dimensional mass cytometry (CyTOF). Immune cell activation and intracellular cytokines were assessed *ex vivo* by CyTOF following stimulation of peripheral blood mononuclear cells with lipopolysaccharides and phytohaemagglutinin. Urinary metabolites of VOCs and nicotine were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Multivariate analysis was used to evaluate associations between biomarkers of exposure and immune cell phenotypes and functions. **Results:** We observed that nicotine and its metabolites were positively associated with basophils, memory B cells, and plasmacytoid dendritic cells; and has inverse association with CD3⁺, CD4⁺ effector memory (EM), and total CD8⁺ T cells. Functionally, nicotine suppressed CD8⁺ T cell activation (TNF α , PD-1, CD69) but increased CD69, PD-1, and TNF α expression in early, late, and total NK cells. Epoxide metabolites, such as propylene glycol and glycerol showed divergent effects: glycidol was associated with reduced naive and effector T cells and decreased CD69, IL-6, and TNF α responses, whereas propylene oxide increased monocyte activation (CD40, IL-1 β , IL-6, IL-10, GM-CSF) and enhanced PD-1 and CD69 expression in NK and CD8⁺ T cells. Aldehydes and α,β -unsaturated carbonyls (acrolein, crotonaldehyde) were strongly associated with myeloid activation, including increased dendritic cells, multiple monocyte subsets, eosinophils, and basophils. Acrolein showed negative associations with naive T cells and positive associations with terminal effector and central memory (CM) and Th17-like T cells. Functionally, acrolein suppressed monocyte cytokines (IL-1 β , IL-6, IL-10, GM-CSF) while enhancing CD69, PD-1, and TNF α in CD8⁺ T cells. Acrylamide metabolites (AAMA, GAMA) were linked to higher dendritic cells, monocytes, NK cells, and basophils, but showed broad functional suppression across T and B cell activation markers (CD69, PD-1, CD80, TNF α). Alkenes metabolites, such as 1,3-butadiene (DHBMA, MHBMA3) and isoprene (IPM1) were associated with increased monocytes, dendritic cells, effector T cells, memory B cells, and eosinophils and reduced $\gamma\delta$ T cells and neutrophils. Functionally, both compounds enhanced CD69, PD-1, and TNF α in effector T cells while suppressing monocyte cytokine responses. Aromatic hydrocarbons metabolites such as benzene (PPMA), toluene (BMA), ethylbenzene (PGA), styrene (PGA, MA), and xylenes (2MHA, 34MHA) exhibited strong innate activation (monocytes, dendritic cells, NK cells, basophils, plasmablasts) with reduced naive T and B cell activation. Functionally, they increased IL-10, IL-1 β , and GM-CSF in monocytes but suppressed B cell activation markers. Metabolites of nitriles and halogenated VOCs Acrylonitrile (CYMA), 1-Bromopropane (BPMA) displayed mixed effects. Acrylonitrile increased dendritic cells, monocytes, NK cells, memory B cells, and CD8⁺ CM T cells and elevated monocyte cytokines, whereas 1-bromopropane reduced monocyte cytokines and B-cell activation but increased PD-1 and CD69 on CD4⁺ and CD8⁺ TE/CM T cells and decreased neutrophils and MAIT/NKT cells. **Conclusions:** These find-

ings demonstrate that e-cig derived VOCs and nicotine metabolites are associated with phenotypic and functional immune dysregulation across multiple innate and adaptive cell types.

PS 4256 Mice Exposed to Chronic Cadmium Display Pulmonary Arterial Hypertension-like Pathogenesis

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Background and Purpose: Pulmonary arterial hypertension (PAH) is a disease wherein chronic high pulmonary arterial pressure causes right ventricular (RV) dysfunction, resulting in global heart failure, eventually leading to patient mortality. Our previous clinical studies demonstrated that patients diagnosed with PAH had increased blood and urine cadmium (Cd) concentrations compared to non-PAH controls. Therefore, we hypothesized that chronic environmental exposure to Cd may novelly contribute to PAH pathogenesis. **Methods:** Recently, we published two articles where we observed the pathological features of PAH in a PAH-induced mouse model through the combination of Semaxinib (SU5416) and hypoxia (SuHx). Right ventricle (RV) diastolic and systolic function, systolic pressure, cardiomyocyte size, collagen deposition, and prominent fibrotic markers were significantly altered within this model. Additionally, pulmonary collagen deposition, arterial remodeling, and fibrotic markers were accentuated as well. Based on these previous studies, we focused on the role chronic Cd-exposure plays in fibrosis, cardiac hypertrophy, and arterial remodeling, to see whether it may contribute to PAH pathogenesis. Twenty male C57BL/6J mice were split into two groups; a control group (CTRL, $n = 10$) who received tap water, and a Cd group (Cd, $n = 10$) who received 5ppm Cd CdCl₂ in their drinking water. The mice were exposed to this experimental design for a total of 12 weeks (wks). To analyze cardiac function, transthoracic echocardiography (echo) was performed on the left and right ventricles (LV and RV) for all mice after 12 wks of Cd-exposure. Then, three mice per group underwent right ventricular catheterization, measuring their diastolic, systolic, and atrial pressures, respectively. At the end of the exposure duration, the mice were euthanized wherein plasma, heart, and lung tissue were collected to undergo: (1) Heavy metal analyses with Inductively Coupled Plasma Mass Spectrometry (ICP-MS), (2) Immunohistochemistry (IHC) staining and western blotting for specific target protein abundance and location, and (3) Reverse transcriptase quantitative polymerase chain reactions (RT-qPCR) of target gene expression. GraphPad Prism 10 software was used regarding statistical analysis. After normality was established, either the parametric or nonparametric equivalence of the student's t-test or TWO-WAY ANOVA was used; with a p -value < 0.05 being considered significant. **Results:** After 12 wks, the mice who underwent chronic Cd-exposure showed significant alterations in both RV diastolic and systolic function, reflected by the increased E/E' ratios and decreased tricuspid annulus plane systolic excursion (TAPSE) values. Exposure to Cd for 12 wks significantly increased heart and lung Cd levels, although this was not the case in plasma. Mice body weight and lung weights were significantly increased after 12 wks of Cd-exposure, which was not seen when comparing heart weights. Pathological examination determined increased lung and RV collagen deposition, alongside LV cardiomyocyte size when compared to the CTRL mice. Additionally, the number of abnormal pulmonary arteries, pulmonary arterial medial thickness, and wall area were all significantly increased when compared to the CTRL mice. These results indicate significant RV dysfunction, alongside pulmonary vascular and cardiac ventricular remodeling. Furthermore, western blot and RT-qPCR analysis on lung tissue determined that 12 wks of Cd-exposure alone significantly upregulated genes and proteins involved in pulmonary arterial remodeling including alpha smooth muscle actin and elastin, further confirming our histological results, all which were seen in the mice with PAH induced by SuHx in our recent published work. **Conclusions:** This concept-proof study confirms our hypothesis: chronic Cd-exposure via 5 ppm drinking water for 12 wks indeed induced a PAH-like phenotype, with further studies currently being conducted to investigate its underlying mechanism. This work is supported by the National Institute of Environmental Health Sciences (T32-ES011564), the American Heart Association (25DIVSUP1477259), and the Kentucky Pediatric Cancer Research Trust Fund (PON2 728 2300001625).

PS 4257 Cardiac Transcriptomics Uncovers Safety Markers for Potentially Cardiotoxic Kinase Inhibitors

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Background and Purpose: Tyrosine kinase inhibitor drugs (TKIs) are highly effective anti-cancer drugs, yet many TKIs are associated with various forms of cardiotoxicity. The mechanisms underlying these drug-induced adverse events remain poorly understood. The analysis of gene expression profiles could help identify potential mechanisms of toxicity. The aim of the study was to identify safety markers that might be indicative of TKI-induced cardiotoxicity. We analyzed transcriptional profiles of 6 different human induced pluripotent stem cells (hiPSCs)-derived cardiomyocyte cell lines treated for 48h in quadruplicate with one of 8 TKIs exhibiting cardiotoxic potential (dabrafenib, lapatinib, pazopanib, ponatinib, sorafenib, sunitinib, trametinib

and vandetanib) (GSE217421). We identified their effects on subcellular pathways and processes. **Methods:** We used our proprietary database and software for data interpretation and visualization, generated to include internally curated and various publicly available data on proteins, biologically-active chemicals, their interactions, pathways and pathologies. Over 30 million references were catalogued, supporting each database entry. Molecule interactions were hyperlinked to appropriate PubMed articles as support, whereas proteins were hyperlinked to corresponding NCBI gene and UniProt protein pages, and chemicals to corresponding PubChem pages. In order to support data integration and capture different levels of histopathological observations, we developed toxicology ontology with over 9,000 disease and 800 pathology endpoints. The system is also supported by 6.5 million interactions between genes/proteins, chemicals, diseases, pathologies, Gene ontology (GO) clusters and Signaling and metabolic pathways. **Results:** The analysis of gene expression data following each of 8 TKI treatments, using the cut off criteria of log2 fold change $\geq \pm 1$, and $\text{padj} < 0.05$, identified 181 - 909 differentially expressed genes (DEGs). Our system recognized 73-87% of DEGs. We analyzed each set of DEGs and used the compare analysis results function within our system to identify common pathways and pathologies to all 8 TKI treatments. We identified GO processes that indicated potential mechanism behind cardiotoxicity (extracellular matrix organization, collagen fibril organization, Wnt signaling pathway, regulation of blood pressure, response to hypoxia, regulation of heart contraction, and blood coagulation). In addition, enriched Signaling and metabolic pathways were identified providing further explanation of cardiotoxicity potential of these TKIs (platelet aggregation, cell surface interaction at the vascular wall and VEGFR2 mediated vascular permeability). We identified enriched Organ and tissue pathologies that also imply potential cardiovascular pathologies (fibrosis, vascular smooth muscle hyperplasia and vascular endothelial cell apoptosis). In order to find gene expression signatures common to all 8 TKI treatments, we used the compare search results function within our system and identified 13 DEGs (upregulated: SLC6A9, ALDH1L2, NUPR1, CRISPLD2, GLT8D2, FIBIN, SERPINF1, BMP4, CHST2, RSPO3, NHERF1, COL8A2, and downregulated: APLN) common to all 8 TKI treatments. Analysis of these genes showed association with angiogenesis, regulation of heart contraction, and regulation of systemic arterial blood pressure GO processes within our system, indicating indeed involvement in cardiovascular relevant biological pathways. To verify the obtained cardiotoxicity gene expression signature, we analyzed the gene expression data sets from hiPSCs-derived cardiomyocyte cell lines treated with cabozantinib (TKI that induces cardiotoxicity as a recognized adverse effect with potentially reversible outcomes) as well as afatinib (TKI with no evidence of direct primary cardiovascular toxicity). Indeed, all 13 genes were found among the DEGs from cabozantinib treated cells, whereas only 10 out of 13 genes were identified among DEGs from afatinib treated cells. **Conclusions:** By interrogating our proprietary database and software we showed their potential in identifying dysregulated pathways, pathologies and gene signatures of drug cardiotoxicity.

PS 4258 Toxicity of evaluation of R-1132(E) using human cardiomyocytes

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Background and Purpose: R-1132(E), trans-1,2-Difluoroethylene, is an Hydrofluorolefin (HFO) refrigerant with a global warming potential of 1 or less, very low environmental impact, and excellent energy efficiency. R-1132(E) belongs to a chemical family, called haloalkenes, which also includes R-1234yf (2,3,3,3-Tetrafluoropropene) and HFO-1123 (1,1,2-Trifluoroethene). Both R-1234yf and HFO-1123 have shown cardiac effects following inhalation exposure in rabbit studies. Exposure to R-1132(E) also induced similar cardiac effects in the rabbit, but not in the rat and dog. However, R-1132(E) cardiac effects on humans remain unclear. Therefore, this study evaluated the cardiotoxicity of R-1132(E) using human cardiomyocytes. **Methods:** We seeded iCell Cardiomyocytes 2.0 onto 0.1% gelatin-coated plastic plates and fibronectin-coated Microelectrode array (MEA) plates. Seven days after cell seeding, culture plates were exposed to either 0 ppm or 15,000 ppm of R-1132(E) in Tedlar bags. These bags are gas sampling bags made from polyvinyl fluoride, which provides excellent chemical resistance and low adsorption properties. Five days after exposure, we evaluated cell viability using the CCK-8 assay and recorded the beat rate using MEA (Maestro Edge, Axion Biosystems, Atlanta, GA). Furthermore, we assessed the predictivity of proarrhythmic risk potential based on changes in field potential duration with Fridericia's correction (FPDcF). FPDcF, an indicator used to predict arrhythmia risk when fluctuations exceed 10%, was calculated from the FPD and Inter-spike interval obtained from MEA measurement of human iPS-derived cardiomyocytes. **Results:** CCK-8 levels were significantly elevated in the 15,000 ppm R-1132(E) exposure group (0 ppm: 0.1239 ± 0.0013 , 15,000 ppm: 0.1334 ± 0.0022 , $p < 0.001$). In contrast, heart rate showed no significant change due to R-1132(E) exposure (0 ppm: 33.2 ± 2.0 , 15,000 ppm: 34.5 ± 1.1 , $p = 0.595$). Similarly, the relative FPDcF value showed no change more than 10% in the 15,000 ppm R-1132(E) exposure group (0 ppm: 1.00 ± 0.02 , 15,000 ppm: 1.02 ± 0.01 , $p = 0.403$). **Conclusions:** These results suggest that R-1132(E) may not directly induce cell death and arrhythmia risk in human cardiomyocytes.

PS 4259 Integrating qHTS and QSAR Models to Identify Safe GPCR-targeted Compounds: A Focus on hERG-dependent Cardiotoxicity

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Background and Purpose: G protein-coupled receptors (GPCRs) are a diverse family of seven-transmembrane domain receptors that play pivotal roles in various physiological and neurological processes by mediating extracellular signals through G proteins. Notable GPCRs such as ADRB2, CHRM1, DRD2, and HTR2A are important therapeutic targets, linked to conditions ranging from asthma to schizophrenia. The human ether-a-go-go-related gene (hERG), encoding the Kv11.1 potassium channel, is critical for cardiac repolarization, the inhibition of which can lead to prolonged QT intervals and increased risk of arrhythmias. Consequently, assessing hERG-GPCR interactions is essential during drug development to enhance safety and ensure regulatory compliance. **Methods:** In this study, we utilized quantitative high-throughput screening (qHTS) to identify GPCR agonists and inhibitors in the Tox21 10K compound library. We applied machine learning (ML)-based quantitative structure-activity relationship (QSAR) models to predict selective GPCR-targeting compounds with reduced hERG liability, employing different data processing sequences. Our models trained on the Tox21 10K library screening data were subsequently validated using the Library of Pharmacologically Active Compounds (LOPAC). Furthermore, the models were applied to virtually screen approximately 360K diverse compounds with the top predictions experimentally validated. **Results:** Validated compounds revealed new GPCR modulators with minimal hERG liability. **Conclusions:** The findings provide efficient strategies for the development of lead compounds targeting GPCRs while minimizing cardiac risks associated with hERG inhibition.

PS 4260 Impact of Ephedra on vascular function

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Background and Purpose: Cardiovascular disease (CV/CVD) remains the leading cause of death worldwide, accounting for a third of global deaths in 2021. Central to cardiovascular health are the endothelial cells (ECs) that line the inside of all vasculature. Adverse effects of air pollution, smoking, or harmful drugs often result in endothelial dysfunction which can result in CVD such as thrombosis or hypertension, leading to heart attack or stroke. The continued use of over-the-counter supplements containing Ephedra or ephedrine alkaloids, are of particular concern due to their documented history of toxicity. While Ephedra's ability to cause tachycardia is well established, the specific effects of Ephedra on ECs remain unknown. **Methods:** Supernatant samples from primary human umbilical vein endothelial cells (HUVEC) cultures treated with or without 100 $\mu\text{g/mL}$ Ephedra were first analyzed using a large immunoassay panel (42 cytokines). Subsequent cytokine analysis of supernatant samples was performed using a smaller, targeted panel informed by the larger panel. Thrombin assays on HUVEC cultures were performed using platelet-rich plasma (PRP) pre-treated with 10% CaCl₂ and 20 μM ADP; a fluorogenic substrate was then added and thrombin formation was measured in 30 seconds intervals for 1 hour. Fibrinolysis assays were conducted by adding biopsy-punched fibrin discs onto HUVEC cultures for 1 hour and measuring supernatant samples with an immunoassay. These assays were conducted using both cell media and PRP samples. In some cases, a plasminogen activator inhibitor-1 (PAI-1) inhibitor (Tiplaxtinin or TM5441) was added to isolate the coagulation pathway impacted by Ephedra. **Results:** Cytokine analysis of HUVEC treated with ephedra showed a significant decrease in tissue plasminogen activator (tPA) which is important in regulating coagulation. Additionally, increased thrombin activity was observed when HUVEC were treated with Ephedra. This would suggest a higher rate of clot formation via fibrinogen conversion, although additional studies are needed. Last, preliminary results from the fibrinolysis assays showed a decrease in D-dimer when HUVEC were treated with Ephedra. D-dimer is a byproduct of the clot breakdown process, meaning Ephedra is also slowing down clot breakdown which could result in thrombosis. **Conclusions:** Endothelial cells maintain vascular homeostasis by regulating blood flow via blood vessel contractility, delivering oxygen and nutrients to supporting cells, and monitoring immune responses. Additionally, ECs contribute significantly to hemostasis by secreting coagulation factors, coagulation inhibitors, and clot dissolution mediators. Because clinical coagulation assays use whole blood without ECs, it is important to examine effects of xenobiotics on ECs and to develop coagulation models that include ECs. Further investigation will clarify whether Ephedra-containing supplements disrupt hemostasis and contribute to CV adverse effects. These results may improve our understanding of how such supplements affect CVD.

PS 4261 Paradoxical Effects of an Anti-Inflammatory Drug, Anakinra, on Endothelial Cells

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Background and Purpose: Anakinra is an anti-inflammatory drug used to treat a variety of autoimmune and inflammatory diseases. Anakinra is comprised of a recombinant form of the endogenous IL1 Receptor Antagonist (IL1RA). By blocking IL1beta binding to IL1 Receptor 1, anakinra suppresses production of inflamma-

tory cytokines by macrophages, T-cells, neutrophils and epithelial cells. **Methods:** Primary human endothelial cells were cultured from umbilical, aortic, and microvascular beds and treated with anakinra or etanercept for 15 minutes or 24 hours. Cytokines were quantified in the media using Luminex bead-based kits representing 42 secreted proteins, or by RNA sequencing of poly-adenylated mRNA. **Results:** Surprisingly, cultured HUVEC endothelial cells responded to anakinra by secreting a variety of predominantly pro-inflammatory cytokines including TNF, IL1beta, LIF, MIP1alpha, IL4, IL5, IL10, IL12, IL13, IL15, IL16, IL17A, IL20, IL21, IFN-alpha, IFN-gamma, GM-CSF, Eotaxin, P-selectin, OPG, and TSLP. Human Aortic endothelial cells (HAEC), or Human Microvascular Endothelial Cells (HMVEC), as well as HUVEC cells from other subjects, also responded by secreting the same inflammatory cytokines. Thus, this proinflammatory response to anakinra is likely a general feature of human Endothelial Cells (ECs). Puzzlingly, a similar profile of cytokine secretion was observed following treatment of HUVEC with etanercept, an anti-inflammatory recombinant peptide drug that blocks TNF from binding its receptors. However, we observed that etanercept caused secretion of the endogenous cytokine IL1RA by ECs, suggesting the similar profile of cytokine secretion by ECs in response to etanercept is redundant with the response of ECs to the recombinant IL1RA, anakinra. Mechanistic studies showed that cytokine mRNAs did not increase upon anakinra or etanercept treatment, and release of cytokines by ECs occurred within 15 minutes of anakinra treatment, indicating that cytokines were released from storage rather than *de novo* transcribed and translated. **Conclusions:** Anakinra and etanercept caused a rapid release of stored proinflammatory cytokines by cultured primary ECs, with etanercept acting indirectly via release of IL1RA. Recognition that anakinra or etanercept exert a proinflammatory response from ECs seems paradoxical, but does not contradict clinical successes treating a variety of inflammatory diseases. Instead, understanding these responses should help to explain which diseases are likely to respond to anakinra or etanercept.

PS 4262 Toxicological Agents in Self Poisoning Cases at Mzuzu Central Hospital: Trends and Treatment Approaches

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Background and Purpose: There is a dearth of knowledge around mechanisms of self-poisoning, prevalence or patterns, the toxicological agents involved and their concentrations, in Malawi. Thus this study aimed to characterize the trends around toxicological agents in self-poisoning cases and their treatment approaches at Mzuzu Central Hospital. **Methods:** This study employed a quantitative retrospective chart review of registered self-poisoning cases managed between January 2022 and December 2023 at the emergency department of Mzuzu Central Hospital using purposive sampling. STATA version 17 was used to analyze the data, create descriptive analysis tables and figures, and to run a multinomial logistic regression. **Results:** Self-poisoning cases (n=313) were higher in males (n=265, 84.66%) than females (n=48, 15.34%). Unintentional self-poisoning (77.96%) was reported in 211 (67.41%) males and 33 (10.54%) females. Intentional self-poisoning (20.77%) was recorded in 50 (15.97%) males and 15 (4.79%) females. Self-harm poisoning was reported in 4 (1.28%) males. The common toxicological agents involved in intentional self-poisoning were pesticides (10% in 2022 vs 8.28% in 2023), prescription medications (8.13% in 2022 vs 4.14% in 2023), alcohol (3.13% in 2022 vs 2.96% in 2023), and acids (1.25% in 2022 vs 1.18% in 2023). Unintentional self-poisoning agents involved alcohol (56.80% in 2023 vs 47.50% in 2022), foods (15.00% in 2022 vs 1.18% in 2023), snake bites (8.28% in 2023 vs 8.13% in 2022), unknown agents (1.88% in 2022 vs 1.78% in 2023), pesticides (1.78% in 2023 vs 0.63% in 2022), prescription medications (1.25% in 2022 vs 1.18% in 2023), and traditional herbs (1.78% in 2023). Where pharmacological treatment was administered, this comprised: ceftriaxone (11.82%), metronidazole (11.35%), paracetamol (8.85%), thiamine (7.75%), intravenous fluids (5.87%), ringers lactate (5.41%), dextrose (5.09%), vitamin b complex (4.93%), diazepam (3.52%), diclofenac (3.37%), omeprazole (3.21%), normal saline (2.82%), atropine (2.35%), ciprofloxacin (2.19%), tramadol (1.41%), activated charcoal (1.17%), and tetanus vaccination (0.86%). Non-pharmacological treatments comprised: respiratory support (supplemental oxygen, non-invasive ventilation, intubation) (33.05%), catheterization (20.91%), nasogastric tube (12.45%), oral rehydration solution (10.42%), limb elevation (6.11%), suction (5.79%), surgery (2.66%), milk (1.25%), chest physiotherapy (1.17%), and water (1.17%). Psychological interventions recorded comprised: dialectical behavioural therapy (34.94%), cognitive behavioural therapy for suicide prevention (5.37%), and improving mood-promoting access to collaborative treatment (0.78%). Regarding outcomes: 276 (88.18%) were discharged with approval of a physician, 24 (7.67%) cases ended in mortality, 10 (3.19%) absconded, and 3 (0.96%) were referred to another tertiary hospital. Exposed individuals that died were at (Relative Risk Ratio: 0.795435) 80% greater risk of dying without molecular toxicological laboratory analysis (p=0.01). **Conclusions:** Self-poisoning is prevalent among males, though often unintentional/accidental. Pesticides, prescription medications, and alcohols are the most common toxicological agents involved. This study demonstrates the need to characterize concentrations of toxicological agents involved, clinical outcomes, and molecular toxicology units to minimize the risk of treatment or death and reduce the burden of poison treatment and management in hospitals. This knowledge is essential in expanding and improving prehospital toxicological and pharmacological treatment measures.

PS 4263 The Importance of Monitoring Indoor Air Quality

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Background and Purpose: The importance of indoor air quality (IAQ) is often trivialized in the human health equation. Many pollutants cannot be seen and people are not always aware of the risks. This study focuses on using simple air quality sensors as an education tool to make invisible pollutants visible. The main purpose is to increase environmental health literacy and help people understand toxic exposures in their homes. **Methods:** This research was completed as an independent study project with faculty guidance. I reviewed scientific literature, technical reports, and public health resources. I examined evidence about the health effects of common indoor pollutants and analyzed how real-time sensor feedback can shape behaviors. I interpreted all findings in the context of health education. **Results:** The literature shows that using low-cost indoor air quality (IAQ) sensors as educational tools can increase public awareness and make invisible environmental risks visible. Scientific evidence demonstrates that IAQ pollutants have measurable health effects and the role of real-time feedback from sensors in shaping behaviors. This approach combines environmental health and toxicology with community-based education strategies by making them understandable for the public. The central hypothesis is actually that through sensor feedback, environmental health literacy can be increased, public knowledge about pollutants in the air can be improved, and people can be motivated to take protective actions such as avoiding products that release pollutants. **Conclusions:** This work suggests that using IAQ sensors as an educational tool can support toxicology and environmental health. Making invisible pollutants visible can increase public knowledge, support informed choices, and help communities reduce toxic exposures.

PS 4264 Eucalyptus Oil Ingestion: A Retrospective Descriptive Analysis of Exposures Reported to a Poison Information Helpline in South Africa over five years.

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Background and Purpose: Eucalyptus Oil is a widely available household product in South Africa, often used for its perceived therapeutic benefits in managing respiratory symptoms. However, ingestion of Eucalyptus Oil can result in significant toxicity including seizures, respiratory depression and even death (Ittyachen et al, 2019). Despite its potential for harm, there is limited local epidemiological data available. This study aims to describe the incidence and characteristics of eucalyptus oil exposures reported to a Poison Information Helpline, South Africa. **Methods:** A retrospective, descriptive analysis was conducted using data from the Poison Information Helpline, Western Cape (PIHWC) encompassing all cases of Eucalyptus Oil exposures reported from 2018 to 2023. Non-patient-related and animal enquiries were excluded. Data was analysed descriptively to summarize demographic variables, exposure circumstances, clinical severity using the Poison Severity Score (PSS) and seasonal trends. Basic statistical comparisons were made to evaluate differences in clinical presentation and severity. **Results:** A total of 510 cases of *Eucalyptus Oil* exposures were identified over the five-year period. Most calls were received from the public (287, 56.3%) involving 261 females (51%) and 247 males (49%). Overall, most of the exposures were accidental (371; 72.8%), including 156 (30.6%) in the 1-3 year age group. Intentional self-harm exposures (7, 1.4%) were most common among adults ages >19. A Therapeutic error was recorded in (128; 25.1%) of the exposures. At the time of the call, most patients were asymptomatic, PSS 0 (268; 52.5%) or experienced mild symptoms, PSS1 (204; 40.0%). Only 0.2% presented with severe symptoms, (PSS 3) and no deaths were recorded. While no seasonal trend was observed, a spike in cases occurred in 2021 (286, 56.1%) in comparison to the other years. Most exposures involved liquid formulations (94.7%). Only two of the patients presented with seizures over the 5-year period. **Conclusions:** Eucalyptus oil ingestion remains a frequent cause for Poison Information Helpline consultation, though most cases are of low severity. Young children, especially those <3 years are most vulnerable and highlight the need for targeted caregiver education on safe storage and use, improved product labelling and continued surveillance. The study reinforces the role of Poison Information Helpline in identifying preventable toxic exposures, their respective presentations and outcomes.

PS 4265 Cannabis Abuse, Intoxication, and Vomiting in Mayo Clinic Patients in Arizona, Florida, and Minnesota Between 2017 and 2024

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Background and Purpose: As more states legalize medical and recreational cannabis in the U.S., there is an increase in emergency room reports of cannabis hyperemesis syndrome (CHS), which is marked by recurring vomiting, nausea, and abdominal

pain in patients with prolonged use of cannabis. The symptoms can be alleviated with hot water baths and abate with prolonged cessation of cannabis use. The occurrence of CHS have been hypothesized to link to genetic and environmental factors, such as differences in individual metabolism and contaminants in cannabis products (e.g., *Fusarium* mycotoxins). **Methods:** This study aims to characterize adult CHS patients from 2017 to 2024 in the Mayo Clinic patient populations in three states with legalized medical cannabis programs - Arizona, Florida, and Minnesota - using the Informatics for Integrating Biology and the Bedside (I2B2) tool, which allows queries of patient population size using different inclusion and exclusion criteria and anonymized electronic health records (EHRs). While no code in the International Classification of Diseases, Tenth Revision, Clinical Modification (ICD-10-CM) specifically indicates CHS, the co-occurrence of cannabis use, abuse, or dependence (ICD-10-CM codes F12.*) or cannabis intoxication (T40.7) with a vomiting syndrome (ICD-10-CM codes G43.A or R11.1) was used as a proxy to estimate the size of the patient populations with CHS. The aggregate data for patient population sizes with cannabis abuse, intoxication, and vomiting was pulled from I2B2, including the yearly breakdown in each state. Two of the states (Arizona and Minnesota) legalized recreational cannabis during the 2017 - 2024 period. **Results:** From January 1, 2017 to December 31, 2024, Mayo Clinic had a total of 2,204,590 adult patients, with 465,851 in Arizona, 497,353 in Florida, and 1,336,253 in Minnesota. The number of patients with cannabis abuse or intoxication for each state in that time was 6,079 in Arizona (1.31%), 3,530 in Florida (0.71%), and 15,796 in Minnesota (1.18%). The time-series of cannabis abuse or intoxication rates in Arizona for each year show increasing rates, while Minnesota rates have generally decreased from 2017. The vomiting syndrome was reported in 33,696 patients (1.5% of the total patient population) and at a higher percentage (3.7%; 949 patients) in patients with cannabis abuse or intoxication. **Conclusions:** This study highlights the number of patients that were affected by cannabis abuse, intoxication, and vomiting. Since only a portion of patients using cannabis were annotated with ICD-10-CM codes F12.*, the number reported here was not representative of patients who could be affected by CHS. Further studies using large language models and other information from EHRs can provide a more accurate estimate of the prevalence of patients affected by CHS.

PS 4266 Integrating wastewater-derived fentanyl consumption rates and public health data: A triangulation approach for community surveillance

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Background and Purpose: Drug-involved overdoses have caused nearly one million deaths in the United States over the past two decades, with synthetic opioids—especially illicit fentanyl—driving the increase since the mid-2010s. Although recent reports show that fentanyl-related deaths appeared to decline for the first time in ten years, tracking these trends remains difficult due to long reporting delays. Wastewater-based epidemiology (WBE) offers a promising complementary surveillance tool by detecting community-level drug use patterns in near real time. Despite growing WBE reports on opioids, few studies have compared these findings with public health outcomes across multiple regions. This study tracked wastewater-derived fentanyl consumption rates (FCR) across several states in the US and assessed whether it was associated with multiple indicators of opioid burden, including fentanyl-related deaths and emergency medical services data, at both the state and county levels. **Methods:** Between March 2024 and April 2025, weekly 24-hour composite wastewater samples were collected from eight catchments across four anonymized U.S. cities. The samples were processed using solid-phase extraction, and norfentanyl concentrations (ng/L) were measured via liquid chromatography-tandem mass spectrometry (LC-MS/MS). Fentanyl consumption rates (FCR, mg/day/1,000 people) were then estimated from the excretion rates by applying the urinary excretion fraction of fentanyl, adjusted for the molecular weight ratio of fentanyl to norfentanyl and then normalized to flow rate and population. FCR were compared with CDC-reported synthetic opioid deaths, and EMS-reported overdose cases and naloxone administrations. Monthly FCR medians from three anonymized cities—West, Midwest 1, and Midwest 2—were merged for comparison at the multi-state level; Southeast was excluded due to suppressed data. County-level FCR were compared individually with monthly naloxone administrations. Spearman rank correlation (ρ) tested monotonic associations. Trend and change-point detection methods, including Mann-Kendall, Theil-Sen slope, and Pettitt tests, characterized temporal changes ($p < 0.05$). **Results:** At the multi-state level, FCR showed moderate-to-strong positive correlations with all opioid burden indicators ($\rho \approx 0.68$ - 0.71 , $p < 0.001$), indicating that higher FCR coincided with increased overdose and mortality indicators. Overdose outcomes were highly intercorrelated ($\rho > 0.93$). At the county level, strong positive associations appeared in Midwest 1 and West ($\rho \approx 0.78$ - 0.80 , $p \approx 0.002$), while Midwest 2 showed weaker, non-significant trends ($\rho \approx 0.39$, $p = 0.26$), and Southeast showed none ($\rho \approx -0.04$, $p = 0.89$). Trend analyses indicated gradual upward drift in Southeast, decline then renewed increase in West, and significant change-points ($p < 0.05$) marking declines followed by stabilization in Midwest 1 and 2 during mid to late 2024. **Conclusions:** FCR showed meaningful alignment with indicators of opioid burden, particularly overdose and mortality, at both state and county levels. Strong correlations were seen in West and Midwest 1, while weaker, non-significant patterns

appeared in Midwest 2 and Southeast, likely due to limited data. Temporal analyses identified regime shifts in fentanyl consumption during 2024 in both Midwest and West. However, these results show associations, not causation, and limitations such as small sample size, unaccounted confounders, and lack of lag analysis must be acknowledged. Overall, the findings support WBE as a timely, complementary surveillance tool for monitoring fentanyl consumption and overdose risk. Expanding datasets, extending time coverage, and including lag/confounder analyses are key next steps. This project was supported by a supplement from the National Institutes of Health, NIDA, to the parent award U01DA051126, the National Drug Early Warning System (NDEWS). We also thank our many partners and stakeholders who helped with this project.

PS 4267 An Epidemiologic Evaluation of Inorganic Arsenic Exposure and Teratogenic Effects

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Background and Purpose: Inorganic arsenic is considered a developmental toxicant but its potential for teratogenicity in humans remains uncertain. In its 2024 review, the European Food Safety Authority (EFSA) stated that maternal exposure to inorganic arsenic is associated with congenital heart defects. In contrast, the US EPA determined that the available evidence was insufficient to support dose-response analyses for teratogenic outcomes in its 2025 Integrated Risk Information System (IRIS) toxicological review of inorganic arsenic. This divergence in scientific interpretation underscores the importance of critically evaluating the evidence base on inorganic arsenic exposure and potential human teratogenicity. The present work describes a review and quality assessment of epidemiologic studies to determine whether inorganic arsenic exposure during pregnancy is causally linked to teratogenic outcomes in humans. **Methods:** We conducted a targeted review, drawing on major agency assessments (ATSDR, 2007, 2016; EFSA, 2024; US EPA, 2025) and an independent search of the peer-reviewed literature on epidemiologic studies of maternal inorganic arsenic exposure and birth defects. Literature searches were conducted in PubMed and Scopus using combinations of terms related to inorganic arsenic, pregnancy, and birth defects. After summarizing results for each identified study, all studies were evaluated for methodological quality based on (but not limited to) the following: (1) study design and sample size; (2) exposure assessment methods; (3) outcome ascertainment methods; (4) consideration and control of potential confounders (e.g., folate status, alcohol use, maternal age); and (5) temporal alignment between exposure and critical developmental windows. Studies with poor quality were considered of limited utility for causal inference. **Results:** We identified over 14 epidemiology studies of inorganic arsenic exposure and birth defects. Epidemiologic studies generally reported inconsistent associations between inorganic arsenic exposure and endpoints, including neural tube defects, orofacial clefts, spina bifida, congenital heart disease, and “any” birth defects. Several studies reported null findings, while others suggested inconsistent exposure-response associations. Across the studies, common limitations included exposure misclassification (e.g., biomarker measurements post-diagnosis, reliance on total arsenic rather than speciated inorganic arsenic), small sample sizes, and inadequate adjustment for key confounders (e.g., folate, alcohol intake). Notably, studies cited by EFSA (e.g., Richter *et al.*, 2022; Rudnai *et al.*, 2014) that suggested associations with congenital heart anomalies contained several limitations (e.g., exposure assessment and covariate control), undermining their interpretability. **Conclusions:** The current body of evidence does not provide reliable or consistent evidence for a causal association between inorganic arsenic exposure and teratogenic effects in humans. Methodological limitations - particularly in the form of small sample sizes, exposure misclassification, and inadequate adjustment for key confounders - introduced uncertainty into reported associations from individual studies. Future research on inorganic arsenic exposure and teratogenicity should strive to address the study limitations discussed above to better inform agency hazard assessments.

PS 4268 Age and Sex Specific Associations of Arsenic Exposure with Hypertension and Elevated Blood Pressure Measures in African-Caribbean Adults

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Background and Purpose: Arsenic (As) is among the most important environmental toxicants that contribute to the global prevalence of cardiovascular diseases and hypertension, although the impacts of arsenic vary with populations and geographical location. Notably, there is a greater burden of hypertension and cardiovascular diseases among people of African ancestry including in the Caribbean, yet the etiology for this increased burden is largely unexplored. **Hypothesis:** We investigated the hypothesis that environmental factors, such as arsenic exposure, are associated with a greater risk of hypertension and high blood pressures among a cohort of Afro-Caribbean men and women enrolled in the Tobago Health Study (n= 965,

age 40-87). **Methods:** Arsenic exposure was measured as the sum of inorganic and methylated As (Σ As = inorganic As + dimethylarsinic acid + monomethylarsonic acid) in spot urine samples using Liquid Chromatography - Inductively Coupled Plasma Mass Spectrometry. We used multiple linear regression to examine cross-sectional associations of urine Σ As with systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure (PP) & mean arterial pressure (MAP). We used logistic regression to examine odds of hypertension (HTN) defined as systolic BP $\geq$ 140 mmHg, diastolic BP $\geq$ 90 mmHg, or use of antihypertensive medication. The models were adjusted for age, sex, waist circumference, urinary creatinine, physical activity, smoking. In secondary analyses, we additionally adjusted the blood pressure models for antihypertensive medication use. We conducted sex-stratified analyses separately alongside the total cohort. In separate analyses, we further stratified men and women by median age (58 years). We tested for effect measure modification (EMM) by sex across all outcomes and calculated p-values for interactions with likelihood ratio tests to observe whether the associations between arsenic exposure and these outcomes differ significantly by sex. **Results:** Each unit (ug/L) increase in Σ As was associated with 2% higher odds of HTN (OR: 1.02, 95% CI: 1.01-1.04, p: 0.008) in the total cohort and 4% higher odds (OR: 1.04, 95% CI: 1.01- 1.08, p: 0.027) among women. Sex-stratified analyses indicated that higher urinary Σ As was associated with elevated DBP (β = 0.08, 95% CI: 0.001-0.15, p = 0.048) and MAP (β = 0.08, 95% CI: 0.01-0.15, p = 0.029) among men whereas women trended towards higher PP (β = 0.087, 95% CI: -0.003-0.177, p = 0.057). The association of higher Σ As and HTN among women was significant in younger (OR = 1.05, 95% CI: 1.01-1.09, p = 0.017), but not older women, alone. Similarly, the association of higher Σ As with higher MAP among men was significant in younger (β = 0.10, 95% CI: 0.01-0.20, p = 0.037), but not older men, alone. The data suggest evidence of effect modification by sex for the relationship between Σ As and MAP (p-interaction = 0.048) with men having higher MAP, including adjustment for medication effect. **Conclusions:** These findings suggest that exposure to inorganic arsenic is cross-sectionally associated with age and sex specific patterns of greater burden of hypertension and high blood pressures among Afro-Caribbean adults in Tobago. The results may suggest different sex-dependent mechanisms for arsenic effects on blood pressure and response to anti-hypertensive medication. *Supported by NIH grants R01ES033519-01, R01AG074956, R01HL143793, R01-AR049747, R01-DK097084 and U2CES026542.*

PS 4269 An Evaluation of the Epidemiologic and Experimental Animal Evidence of Oral Cadmium Exposure and Teratogenic Effects

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Background and Purpose: For the non-smoking general population, the primary source of cadmium exposure is through the diet. In 2012, the Agency for Toxic Substances and Disease Registry (ATSDR) stated in its "Toxicological Profile for Cadmium" that it is not known whether cadmium can cause birth defects in people. Since that review, additional studies have been published regarding the relationship between exposure to cadmium and teratogenic effects. We performed an updated review, quality assessment, and integration of epidemiologic and toxicologic studies to determine whether oral exposure to cadmium during pregnancy is causally associated with human teratogenicity. **Methods:** We searched for epidemiologic and experimental animal studies from government agency reviews (e.g., ATSDR, United States Environmental Protection Agency [US EPA], California Office of Environmental Health Hazard Assessment [CalOEHHA]) and performed an independent literature search in PubMed and Scopus for relevant studies published since ATSDR's 2012 review. For epidemiologic studies, study quality was evaluated based on, but not limited to, the following: (1) study design and sample size; (2) exposure assessment methods; (3) outcome ascertainment methods; (4) consideration and control of potential confounders (e.g., alcohol use, maternal age); and (5) temporal alignment between exposure and critical developmental windows. For animal studies, we focused on studies conducted in mammals that used oral exposures (e.g., food, water, oral gavage), as these exposures are the most relevant to typical human exposures. Study quality in animal studies was evaluated based on several considerations, including but not limited to: (1) study design and sample size; (2) number of dose levels, such that a dose-response curve could be detected; (3) and consideration of maternal toxicity. Studies with poor quality were considered of limited utility for causal inference. **Results:** We identified over 20 epidemiologic studies of cadmium and birth defects. Overall, epidemiologic studies of birth defects reported inconsistent associations with cadmium exposure. In addition, most studies had several limitations, such as cross-sectional study design, potential confounding bias, and unreliable measures of cadmium exposure, which made reported associations uncertain. With respect to animal studies, we identified over 23 studies of oral cadmium exposure in mammals that evaluated potential malformations. Across these studies, exposure to cadmium generally was not associated with an increased incidence of malformations even, in some cases, at levels associated with maternal toxicity (e.g., maternal weight loss). **Conclusions:** Overall, integration of the evidence from studies in humans and experimental animals does not support a causal association between oral cadmium exposure and teratogenicity. In general, epidemiologic studies had several methodological limitations that should be addressed in future research. Evidence from experimental animal studies supports the conclusion that human-relevant oral exposures to cadmium do not result in teratogenic effects.

PS 4270 Disparities in Arsenic Exposure: Evidence from Public Water Systems and Human Biomonitoring

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Background and Purpose: Arsenic is a naturally occurring element found in groundwater and a recognized human toxicant linked to various adverse health outcomes. While regulatory efforts have reduced arsenic levels in drinking water, it is unclear whether these improvements have equitably reduced human body burden across populations. This study integrates environmental monitoring data from Texas public water systems (PWS) and biomonitoring data from the U.S. population to assess temporal trends and disparities in arsenic exposure from source to body. **Methods:** Arsenic concentration data from 4,681 residential public water systems in Texas from 2007 to 2023 were obtained from the Texas Commission on Environmental Quality. Descriptive statistics, geospatial mapping, and logistic regression were applied to assess temporal changes and disparities based on system size, water source, rurality, and Social Vulnerability Index (SVI). Separately, urinary inorganic arsenic concentrations in U.S. women aged 20 to 44 years were evaluated using six cycles of the National Health and Nutrition Examination Survey from 2007 to 2020. Survey-weighted linear regression estimated adjusted geometric means (aGMs) and percent change per cycle while adjusting for sociodemographic, dietary, and behavioral factors, including urinary creatinine and serum cotinine. **Results:** In Texas, arsenic violations declined from 9.04% of PWS in 2007-2011 to 2.76% in 2020-2023, with mean arsenic concentrations decreasing from 3.20 μ g/L to 1.75 μ g/L. However, violations remained concentrated in small, groundwater-reliant, and high-SVI systems, with increasing odds ratios for non-metro (OR = 2.15) and high-SVI (OR = 4.82) areas. In NHANES, urinary inorganic arsenic declined by approximately 3% per cycle overall. Yet, Mexican American women and low-income groups exhibited smaller reductions (2.36% and 1.78%, respectively) compared to their higher-SES counterparts. Consumers of arsenic-associated foods had higher aGMs and slower declines than non-consumers. Community water users saw modest reductions, while trends among private well users were variable. **Conclusions:** Despite regulatory progress, environmental and biomonitoring data reveal persistent disparities in arsenic exposure from source to body. Vulnerable populations, particularly those in rural, socioeconomically disadvantaged, and high-SVI areas, continue to bear disproportionate exposure burdens. These findings support the need for enhanced environmental surveillance, targeted exposure reduction efforts, and equitable policy interventions to address the structural determinants of arsenic-related health risk.

PS 4271 Determinants of Placental OATP2A1 and OATP2B1 Transporter Protein Concentrations within the UPSIDE Birth Cohort

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Background and Purpose: Organic anion transporting polypeptides (SLCO gene, OATP) mediate the uptake of a wide range of anionic substrates into human placentas. OATP2A1, a transporter with high affinity to prostaglandins, is primarily found on the maternal-facing side of the blood-placental barrier. On the other hand, OATP2B1 is primarily found on the fetal side and has a broader substrate profile of xenobiotics and endogenous chemicals. By utilizing the Understanding Pregnancy Signals and Infant Development (UPSIDE) cohort (NY, USA), we sought to evaluate how maternal factors, gestational and infant factors, and prenatal environmental toxicant exposures influence concentrations of OATP2A1 and OATP2B1 proteins in healthy term placentas. **Methods:** Villous placenta tissue (N=255) was homogenized and membrane fractions isolated for quantitative targeted absolute proteomic analysis of OATP2A1 and 2B1 using liquid chromatography mass spectrometry. Extant data were used to evaluate bivariate associations between OATP2A1 and 2B1 concentrations and 1) maternal factors: maternal age, gestational age, pre-pregnancy BMI, gestational weight gain, parity, delivery mode, complications (PE, GDM, smoking during pregnancy) and 2) infant factors: birth weight, race, sex. Mutually adjusted models were built including predictors with p<0.2 in bivariate analyses. Extended models also considered exposure to environmental toxicants including PFAS (2nd trimester serum), cadmium (placenta), and mycoestrogens (placenta). **Results:** OATP2A1 and 2B1 protein concentrations in term placentas were modestly correlated (r=0.34, p<0.05). Median OATP2A1 and 2B1 transporter enrichment from UPSIDE was 0.49 pmol/mg protein (IQR: 0.34 - 0.67) and 0.27 pmol/mg protein (IQR: 0.18 - 0.37), respectively. In bivariate analyses, OATP2A1 levels were associated with pregnancy complications, infant weight, gestational weight gain, serum PFHxS, and placental cadmium levels. In extended models fit for each toxicant, a one log-unit increase in circulating PFHxS was associated with a 6.2% (95% CI: 0.00, 13.9) increase in OATP2A1 levels, while a one log-unit increase in placental cadmium levels was associated with a 4.1% (95% CI: 1.0, 8.3) increase. By comparison, in bivariate analyses, OATP2B1 levels were associated with gestational age, parity, serum PFHxS, and placental mycoestrogen levels. In mutually adjusted models, only parity was associated with a 6.18% (95%CI: 2.02, 9.42) increase in OATP2B1 levels. **Conclusions:** To our knowledge, this is the first study to explore predictors of placental OATPs in a human cohort. OATP2B1 was higher in multiparous preg-

nancies similar to other xenobiotic transporters which may indicate a maternal adaptation during subsequent pregnancies. By comparison, OATP2A1 protein levels were positively correlated with cadmium and PFHxS. We speculate that induction of OATP2A1 following toxicant exposure influences overall uteroplacental prostaglandin handling, an important mechanism that regulates induction of labor and rupture of the fetal membranes. Supported by T32ES007148, R01ES029275, R01HD083369, UG3OD023349, UH3OD023349, P30ES005022, UL1TR003017, S10RR024595, and UC2HD113039.

PS 4272 Integrating Toxicological and Economic Evidence to Optimize Repurposing Strategies for Rare Diseases

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Background and Purpose: Rare diseases pose major challenges for drug development due to small patient populations, phenotypic variability, and limited commercial incentives. These barriers hinder conventional efficacy and safety studies, slowing therapeutic progress despite increasing biological insight. Drug repurposing—identifying new indications for existing compounds—offers a practical route to accelerate therapy development by leveraging known pharmacological and toxicological data. However, most repurposing initiatives emphasize biological plausibility while neglecting comprehensive safety or outcomes evidence. Toxicological data defining exposure limits and organ-specific liabilities are seldom examined alongside health economic and outcomes research (HEOR) data describing treatment burden and healthcare utilization. Integrating these domains early in the repurposing process may improve candidate selection, inform dose optimization, and strengthen regulatory justification. This study developed and applied a structured framework that synthesizes toxicological, mechanistic, and economic evidence to support early decision-making in rare disease repurposing. Pirfenidone, an anti-fibrotic agent approved for idiopathic pulmonary fibrosis (IPF), was evaluated for potential use in systemic sclerosis-associated interstitial lung disease (SSc-ILD), a rare fibrotic disorder with high morbidity and limited therapeutic options. **Methods:** A cross-database evidence synthesis integrated toxicological, mechanistic, and translational data across four major resources: **PharmaPendium - Regulatory Toxicology and Clinical Exposure Data:** Extracted exposure-response relationships, no-observed-adverse-effect levels (NOAELs), and organ-specific findings from preclinical and regulatory submissions, normalized using MeDRA taxonomy. Cross-species comparisons established exposure thresholds and dose-limiting toxicities relevant to chronic oral dosing. **Embase - Clinical and Post-Marketing Safety Evidence:** Aggregated safety data from clinical trials, pharmacovigilance reports, and post-marketing studies. Adverse events were standardized via Emtree to enable system organ class-level comparison and longitudinal analysis of chronic antifibrotic therapy. **Embiology - Mechanistic and Pathway Mapping:** Mapped pirfenidone's molecular targets and pathway-level interactions to assess mechanistic overlap between IPF and SSc-ILD, emphasizing TGF- β and PDGF signaling convergence. **Scopus - Bibliometric and Translational Landscape Analysis:** Characterized research maturity, collaboration networks, and topic clustering in fibrosis and rare disease repurposing, highlighting evidence gaps and translational readiness. Findings were synthesized into a qualitative matrix linking toxicological risk domains, mechanistic plausibility, and economic dimensions, including toxicity-related burden and healthcare resource implications. **Results:** Integrated analysis produced a cross-domain safety-mechanistic-economic profile of pirfenidone for rare disease repurposing. Regulatory data defined exposure-dependent toxicities relevant to chronic antifibrotic use. Clinical and pharmacovigilance evidence confirmed reproducible safety patterns across populations. Mechanistic mapping revealed substantial biological overlap between pirfenidone's targets and SSc-ILD fibrotic pathways. Bibliometric analysis identified an active but fragmented translational landscape, underscoring the need for integrative data frameworks. Together, these analyses demonstrated the feasibility and value of harmonizing toxicological, mechanistic, and economic evidence for early repurposing evaluation. **Conclusions:** This study presents a reproducible framework for integrating regulatory toxicology, clinical safety, mechanistic, and translational evidence to inform rare disease repurposing. Applied to the pirfenidone-SSc-ILD case, the approach enhances transparency, reproducibility, and scientific rigor in candidate evaluation. Future extensions will incorporate quantitative modeling of toxicity-value relationships to support prioritization across rare therapeutic areas.

PS 4273 Prenatal mycoestrogen exposure in relation to maternal cortisol during pregnancy

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Background and Purpose: Zearalenone (ZEN) is a highly prevalent mycotoxin contaminating much of the global food supply. ZEN and its metabolites have been designated as 'mycoestrogens' based on the similarity of their structure to estrogens. Our ongoing work in a U.S. pregnancy cohort suggests that over 90% of participants have detectable ZEN concentrations and that levels are linked to

disruptions in sex steroids as well as in placental corticotropin releasing hormone (CRH), a key hormone in the hypothalamus-pituitary-adrenal (HPA) axis. We have additionally linked early gestational ZEN exposure to altered infant cognition and executive function. Here, we continue that line of research examining maternal ZEN exposure in relation to maternal cortisol, an HPA axis hormone that plays a key role in maternal stress as well as fetal growth and neurodevelopment. **Methods:** In a US pregnancy cohort (UPSIDE; Rochester, NY; n=206), we explored associations between ZEN and its metabolites and maternal cortisol across pregnancy. LC-MS was used to measure ZEN in urine samples collected in each trimester. In each trimester, saliva samples were collected at wake, 45 minutes post-wake, 2.5 hours, 8 hours, and 12 hours post-wake using a take-home collection kit. Salivary cortisol was quantified via immunoassay and diurnal area under the curve (AUC) was calculated as a measure of the total daily cortisol production. In preliminary analyses, we examined Pearson correlations between ZEN and its metabolites (ZEN, alpha-zearalenol (AZOL), and the sum of ZEN and its metabolites (SUM)) and cortisol AUC. **Results:** 95% of participants with cortisol AUC measures had detectable urinary concentrations of ZEN or its metabolites during pregnancy. Cortisol AUC increased across trimesters (median = 49.7, 75.6, and 87.7 nmol/L*hour). ZEN was weakly negatively correlated with maternal cortisol AUC in late pregnancy ($r = -0.13$), while correlations earlier in pregnancy and with other metabolites were null. **Conclusions:** Our results suggest that mycoestrogen exposure is likely not associated with maternal daily cortisol load, but further modeling with adjustment for confounders is underway. Furthermore, cortisol follows a diurnal rhythm, thus additional modeling examining the relationship between mycoestrogen exposure and cortisol dynamics, such as awakening response or diurnal slope, is warranted. Supported by P30ES005022, UG3OD035527, T32E5007148, R01ES029275-02S1, R01HD083369, R21ES032047, UG3/UH3OD0223349, T32ES019854, F31ES034269.

PS 4274 An early pregnancy maternal PFAS mixture is associated with decreased gestational age at birth in a prospective birth cohort

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are a synthetic class of persistent chemicals to which pregnant individuals are routinely exposed. However, associations with gestational age have been inconsistent, possibly due to factors related to PFAS congener, timing of exposure, and differences in underlying biologic mechanisms. **Methods:** Maternal serum PFAS levels, demographic, and gestational age at birth came from the Growing prospective birth cohort (case complete n=217). Seventeen PFAS were measured in maternal serum collected around week 10 gestation with liquid chromatography-tandem mass spectrometry. We analyzed 5 PFAS (PFOS, PFOA, PFHxS, PFNA, and PFDA) with limit-of-detection (LOD) rates >70% that also had approximately normal distributions following log2-transformation. Below LOD measures were imputed to LOD/sqrt(2) with an LOD of 0.1 ng/mL. All models were adjusted for maternal age, education, body mass index, and fetal sex. Multivariate linear regression tested the association between each PFAS and gestational age separately. Quantile g-computation and Bayesian kernel machine regression (BKMR) tested the association between a joint mixture of all five PFAS and gestational age. Statistical significance was assessed at a p-value < 0.05 cutoff. **Results:** Mean gestational age was 39.3 $\pm$ 1.1 weeks, or 275.0 $\pm$ 7.4 days. Mean maternal age was 29.5 $\pm$ 3.5 years old. Each doubling in maternal serum PFOS or PFHxS alone was associated with a -1.4 [-2.6, -0.2] day or -0.7 [-1.4, -0.01] day decrease in gestational age, respectively. A quartile increase in all 5 PFAS was associated with a -1.5 [-2.8, -0.2] day decrease in gestational age. This mixture estimate was primarily driven by PFOS accounting for 32.8%, PFDA at 27.3%, and PFNA at 27.2% of the effect. In the nonlinear BKMR model, compared to each mixture PFAS at its median, setting each PFAS to the 65th percentile was associated with a -1.0 [2.0, -0.01] day decrease in gestational age. This inverse trend was similar from the 60th to 75th (p=0.05). **Conclusions:** In an otherwise uncomplicated cohort of pregnant women, maternal serum PFOS alone and PFHxS alone in the first trimester were inversely associated with gestational age in linear models. Mixture approaches quantile g-computation and BKMR similarly identified an inverse association between gestational age and a mixture of PFOS, PFDA, PFNA, PFHxS, and PFOA. These results are consistent with prior findings that PFAS can adversely affect gestational age. However, this analysis provides evidence that exposure to a complex mixture early in pregnancy of commonly experienced PFAS can jointly affect gestational age, an important sentinel of broader perinatal health even with effect sizes on the scale of days.

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are ubiquitous chemicals linked to adverse health outcomes. While diet has been identified as one of the major sources of PFAS exposure, how dietary differences translate into variation in PFAS body burden remains poorly understood. The Planetary Health Diet (PHD), proposed by the EAT-Lancet Commission, is a new dietary framework aimed to reduce chronic disease incidence and environmental degradation by promoting higher consumption of plant-based foods and lower consumption of animal-based foods. We hypothesized that higher adherence to the Planetary Health Diet and specific food components of the dietary framework was associated with lower plasma PFAS concentrations in the Multiethnic Cohort (MEC), a prospective cohort with over 200,000 participants from California and Hawaii. **Methods:** In a subsample of the MEC (N=2,783), plasma PFAS levels were measured in samples collected between 1994 and 2004 using liquid chromatography-mass spectrometry (LC-MS). Planetary Health Diet Index (PHDI) scores were derived from baseline (1993-96) validated food frequency questionnaires and categorized into quintiles. We conducted multivariate linear regression analyses to assess the association between PHDI quintiles and PFAS concentrations. Analyses were adjusted for age at blood draw, sex, race and ethnicity, study area, time between baseline entry and blood draw, body mass index and alcohol intake. Additional models evaluated associations between specific food groups and PFAS concentrations. **Results:** Participants were 56% female with a mean age of 65 years; 56% were Japanese American. PHDI scores ranged from 33 to 120, with higher values reflecting greater adherence. Higher PHD adherence was associated with lower plasma concentrations of sum perfluoro-sulfonic acids (PFSA) (Q5 versus Q1 β : -3.31, 95% CI: -5.55, -1.07; trend $P=0.005$) and sum perfluorocarboxylic acids (PFCAs) (Q5 versus Q1 β : -0.47, 95% CI: -1.00, 0.06; trend $P=0.066$). This association may be explained by reduced intake of PFAS-contaminated animal products and increased consumption of plant-based foods with higher PHDI scores. By food group, each standard deviation (SD) increase in whole fruit intake was associated with 3.36% lower Σ PFSA concentrations (β : -0.034), whereas each SD increase in eggs intake was associated with 4.40% higher Σ PFSA concentrations (β : 0.043). **Conclusions:** Higher adherence to the PHD is associated with lower PFAS concentrations, particularly PFSA. These findings suggest that plant-based diets aligned with the PHD may help reduce PFAS exposure and mitigate associated health risks.

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Background and Purpose: Previous studies investigating neuroblastoma risk and parental pesticide exposure have had mixed findings, with some evidence linking maternal occupational or residential exposure to increased risk. However, most assessed pesticides broadly rather than evaluating specific compounds. To our knowledge, no studies have examined ambient residential exposure to individual pesticides and neuroblastoma risk. The purpose of this study is to evaluate associations between childhood neuroblastoma and prenatal exposure to specific pesticides through residential proximity to agricultural applications. **Methods:** We identified neuroblastoma cases from the California Cancer Registry and cancer-free controls from birth certificates. Analyses were restricted to children born 1998-2016 whose mothers lived within 4,000 meters of an agricultural pesticide application during pregnancy (199 cases, 202,796 controls). Probable and possible carcinogens were selected based on U.S. EPA classifications and pesticide use was collected from the California Pesticide Use Reporting system linked to land-use surveys. Exposures were assessed using a 4,000m buffer around geocoded residential addresses at birth. We used a distance decay model and considered pregnancies exposed when the poundage per acre applied exceeded the pesticide-specific median. We analyzed 68 pesticides with at least 5 exposed cases using unconditional logistic regression and hierarchical models co-adjusted for all pesticides. **Results:** In hierarchical models, we observed elevated risks of neuroblastoma with prenatal exposure to flonicamid (aOR: 1.33; 95% CI: 1.02-1.73), benomyl (aOR: 1.53; 95% CI: 0.98-2.38), cypermethrin (aOR: 1.24; 95% CI: 0.96-1.60), and permethrin (aOR: 1.20; 95% CI: 0.96-1.50). **Conclusions:** Our findings suggest that ambient exposure to certain pesticides during pregnancy may increase the risk of neuroblastoma.

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Background and Purpose: Quantifying and communicating the health risks of environmental exposures remain central challenges in toxicology and risk assessment. Air pollutants such as fine particulate matter (PM_{2.5}), volatile organic compounds, and wildfire-related air toxics are well-known contributors to respiratory and cardiovascular disease, yet the data used to assess these risks are often siloed, technical, and inaccessible to the public. To address this gap, we developed the Montana Healthy Places Dashboard, an interactive, public-facing platform that integrates environmental and public health data to support transparent, community-level risk assessment. **Methods:** The dashboard links datasets on air pollution (e.g., PM_{2.5}, wildfire smoke, and air toxics), meteorology, and social determinants with state level health outcomes such as asthma, premature death, and other exposure-related conditions. Data are displayed through interactive maps, trend visualizations, and downloadable summaries that enable users to explore exposure response relationships, temporal patterns, and geographic disparities. **Results:** Importantly, the platform was designed for diverse stakeholders, state health departments, county and local public health agencies, state legislators and policy representatives, environmental and toxicological researchers, educators, and community organizations. Each group can use the Dashboard to support their unique objectives: public health officials can identify local environmental risk hotspots; policymakers can evaluate data to inform evidence-based legislation; and scientists can access curated datasets for exposure modeling and hypothesis generation. By grounding data visualization in toxicological principles such as cumulative exposure, and population susceptibility, the platform enhances both scientific and public understanding of environmental health risks. **Conclusions:** This work demonstrates how integrating toxicology-based data interpretation with open-access design can advance environmental justice, promote informed policy, and empower local communities to engage in evidence-based decision-making. The Montana Healthy Places Dashboard serves as a model for how risk assessment can move beyond the laboratory making the science of toxicity visible, usable, and actionable for all stakeholders.

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Background and Purpose: Native Americans face disproportionate exposure to environmental contamination that affects their physical and mental health, food sovereignty, and culture. The Ramapough Turtle Clan, residing on the Ringwood Mines Superfund Site in Ringwood, New Jersey, has been the intentional target of toxic dumping for over a decade. This qualitative interview study revealed in-depth information to explore and understand concepts, opinions, and experiences related to dealing with and facing environmental pollution on a daily basis. **Methods:** In-depth, semi-structured interviews were conducted with ten members of the Ramapough Lenape Turtle Clan. Participants were asked about their sense of place, perceptions of risk associated with Superfund sites, the impacts of the Ringwood Superfund site on their health, food availability, nutrition, and access to healthcare. Interviews were transcribed and analyzed using Dedoose for a thematic analysis. Codes were developed to identify frequent themes related to food sovereignty, loss of cultural traditions, and physical and mental health. **Results:** Thematic analysis revealed five major themes out of 64 codes in total: (1) Loss of cultural traditions such as fishing, hunting, and gardening due to contamination; (2) Food sovereignty, including restricted access to safe and culturally significant food sources; (3) Health disorders and disparity, highlighting disproportionate self-reported personal health issues and/or those identified among interviewees' family and friends (e.g., cancer and type-2 diabetes); (4) Mental Health aspects associated with historical contamination and cultural loss; and (5) Intersection of contamination and specific diseases, reflecting the community's thoughts on the connection between their health issues and the dumping. Participants generally described how their lives had changed since the contamination, including the burden of uncertainty about whether their ancestral land and homes were still contaminated, the authorities' neglect, and their desire for meaningful and effective remediation efforts. **Conclusions:** The findings from this study highlight the importance of culturally and community-driven approaches in environmental health research, which focus on the impacts of toxic waste dumping on Indigenous communities and acknowledge the interconnection between land, culture, and health. Moreover, such research contributes to protecting public health by addressing structural inequalities and prioritizing environmental justice (in this case) through the voices of the Ramapough Lenape Turtle Clan members. Qualitative data are essential for designing equitable interventions/preventions that sustain both environmental and cultural balance.

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Background and Purpose: Observational epidemiological evidence, including case-control and cohort studies, are increasingly used to support decision making, however these studies have inherent design limitations that introduce uncertainty. Qualitative risk of bias (RoB) approaches assist in identification of potential biases from population selection, confounding, exposure misclassification, and/or outcome misclassification in epidemiological studies. However, RoB judgments are subjective and cannot inform on the magnitude of bias and whether it is sufficiently large to impact study conclusions. Quantitative bias assessments (QBA) include post-hoc methods that can quantify the direction and impact of such systemic biases on results and therefore can be used to inform certainty (or uncertainty) in individual studies as well as a body of evidence. The objective of this study was to conduct QBA and evaluate the impact of exposure misclassification and confounding biases in observational studies assessing acetaminophen use and liver cancer.

Methods: Literature that evaluates associations between acetaminophen use and liver cancer incidence was systematically identified. Qualitative RoB assessments were conducted using the National Toxicology Program (NTP) Office of Health Assessment and Translation (OHAT) RoB tool with topic-specific refinements. Key bias areas were evaluated using QBA techniques that augment reported information through probabilistic simulation of marginal tables that include unmeasured confounding and/or exposure misclassification information while maintaining fidelity to the marginal data counts. Bayes' rule was used to determine probabilities of unobserved counts based on assumptions, such as the expected magnitude of confounding or sensitivity and specificity of exposure classification. Misclassification of exposure and unobserved interactions between alcohol and acetaminophen use and their impacts on observed liver cancer incidence were evaluated both independently and in a joint model that considers multiple systemic biases. The impact of unmeasured confounding was evaluated using both an additive and an interactive model. **Results:** Identified studies ($n = 4$) included retrospective and prospective cohorts and nested case-control designs that evaluated the risk of liver cancer incidence associated with acetaminophen use across three distinct populations. Each of the identified studies were qualitatively judged to have a high RoB due to uncertainties in exposure characterization and limited adjustment for confounding factors, including protopathic bias (i.e., use of acetaminophen for cancer-related pain), channeling biases (i.e., preferential use of acetaminophen due to underlying health conditions that contraindicate use of NSAIDs), or interactions between concurrent alcohol and acetaminophen use. QBA for exposure misclassification, alone, showed marginal changes in the observed risks. Models evaluating the impact of unmeasured confounding, alone, shifted relative risks toward null findings, with a greater impact in the interactive model (0 to 88.4% of models identifying significant effects across simulations, depending on study) as compared to the additive models (0.2 to 100% of the simulated models, depending on study). Joint evaluation of exposure misclassification and unmeasured confounding shifted reported relative risks further towards the null indicating a synergism; here, only 0-19.2% of simulated models were significant when an interaction model was included. **Conclusions:** The QBA shows that unmeasured confounding attributable to interactions between alcohol use and acetaminophen intake has a substantial impact on observed liver cancer risks. QBA tools enable informed judgment making on the potential impact of residual or unmeasured confounding and/or biases in observational epidemiological evidence. Incorporation of these methods in hazard and risk characterizations supports uncertainty analysis and quantitative assessments of the strength and confidence in underlying epidemiological evidence.

PS 4280 Nicotinamide shows evidence of protection against breast cancer in prospective pregnancy cohort

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Background and Purpose: Previously we found that maternal floor infarction (MFI) of the placenta, a pathology characterized by fibrin deposition on the maternal surface, protected against breast cancer in mothers and daughters in the Child Health and Development Studies (CHDS). We conducted a Metabolome Wide Association Study (MWAS) in trimester 3 (T3) serum comparing MFI pregnancies with controls to discover breast cancer protective pathways and metabolites. **Methods:** The study is based on an 8% random case-cohort sample of CHDS women with available placental information, pregnancy serum and cancer diagnoses through 1997: $N = 566$ in T3 with 43 MFI pathologies and 153 breast cancer cases. Untargeted pathway and metabolite analyses were performed using Rodin (<https://github.com/BM-Boris/rodin.git>). HLIC ESI+ feature intensities were quantile normalized, log2 transformed and tested using one-way ANOVA to discover MFI-enriched pathways ($p < 0.05$). Identified MFI-related metabolites were then tested in proportional hazards models, fitted to a case-cohort study design, to directly determine whether they were associated with risk of subsequent maternal breast cancer. Interactions between metabolites and MFI were tested by adding a product term between MFI and the metabo-

lite. **Results:** MFI-enriched T3 pathways included serine, aspartate and asparagine, phytanic acid oxidation and Vitamin B3 metabolism. Notable, within the Vitamin B3 metabolic pathway, nicotinamide was identified as a significant down-regulated metabolite ($p = 0.02$). When tested in a proportional hazards model, nicotinamide was associated with a 30% protection against future breast cancer (Relative Hazard = 0.7; 95% Confidence Limit = 0.5 to 1.0), that was independent of MFI. **Conclusions:** Nicotinamide in T3 was down-regulated in MFI pregnancies and lower levels were associated with decreased risk of subsequent breast cancer, consistent with MFI protection against breast cancer, but independent of the protective benefit of MFI suggesting multiple pathways to breast cancer development. Nicotinamide is a vitamin B3 precursor of NAD+ (nicotinamide adenine dinucleotide) which is essential for cellular energy regulation, and can directly impact DNA repair, cell senescence and immune processes. NAD+ has been implicated in tumor initiation through dysregulation of DNA repair and cell death and in promoting tumor progression via cellular energy alterations to satisfy the extensive energy demands required for rapidly proliferating cancer cells. Our findings support current efforts targeting NAD+ metabolism for potential therapeutic strategies. Next steps are feature confirmation and targeted examination of breast cancer associations for the intermediary and terminal metabolic features in the metabolism of NAD+ to explore interactions between NAD+, its precursor metabolites and breast cancer risk.

PS 4281 Vibration of effects analysis on cancer mortality for robust discovery of environmental toxicants of aggressive cancers

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Background and Purpose: Chemical exposures have been linked to cellular processes making cancer cells more aggressive. Epidemiological studies have examined the associations between chemical exposures and cancer mortality, which is a population-level surrogate of cancer aggressiveness. But their findings differ due to variations in model specifications, such as covariate selection and linear vs. non-linear assumptions. These differences reflect how multiple analytical choices can lead to divergent conclusions about which chemicals are associated with increased risk for cancer mortality. A vibration of effects (VoE) approach systematically quantifies how such modeling decisions influence the estimated associations, thereby enabling the identification of associations between chemical exposures and cancer mortality that are most robust across model specifications. Such insights can guide prevention measures and improve patient outcomes. **Methods:** Using biomonitoring data from 34,100 US women in the National Health and Nutrition Examination Study (NHANES) 1999-2018, we assessed which of 259 chemical biomarkers are consistently associated with cancer mortality across model adjustments (1,639 deaths). First, we selected the following biological or behavioral covariates based on prior literature: age, race/ethnicity, parity, menopause status, current smoking habits through cotinine biomarker levels, poverty income ratio, educational level, hormone replacement therapy, BMI, and survey cycles. Second, we ran Cox proportional hazard models for all possible combinations of these covariates to assess how varying model adjustments influenced estimated risks. We evaluated both linear and non-linear associations. For non-linearity, we discretized the biomarker levels into nine quantiles (novemtiles). Third, we quantified the stability of each chemical's associations by defining a VoE p-value from the 1st and 99th percentiles of its distribution of hazard ratios across all models. Finally, we prioritized chemicals with a VoE p-value ≤ 0.02 , implying that the chemical is consistently associated with cancer mortality risk across multiple specifications. **Results:** We identified 27 chemical biomarkers with levels consistently associated with risk for cancer mortality. Participants with cotinine levels indicative of heavy tobacco exposure (170-478 ng/mL) had a 2.68-fold greater risk of dying from cancer (VoE p-value = 6.1×10^{-9}) compared with those with minimal exposure (0.011-0.018 ng/mL). Elevated levels of cadmium (0.50-3.00 ug/L, HR = 2.29-3.58, VoE p-values = 2.04×10^{-6} - 3.46×10^{-3}), lead (2.30-5.37 ug/dL, HR = 3.27, VoE p-value = 0.003), and potassium (4.30-4.80 mmol/L, HR = 1.85, VoE p-value = 0.001) were each associated with increased risk of cancer mortality. o-Desmethylnangolensin exhibited low dose effects at 0.4-0.9 ng/mL (HR = 2.23, VoE p-value = 0.01), but higher biomarker levels were near null and not consistent. Similarly, levels of mono-3-hydroxy-n-butyl phthalate at 0.50-0.70 ng/mL (HR = 8.52, VoE p-value = 0.008) showed elevated risk compared to the minimal levels, and while higher levels were linked to higher risk of cancer death, the risk was not consistent. In contrast, higher biomarker levels of Vitamin D and carotenoid-related dietary components (α -carotene, α -cryptoxanthin, β -cryptoxanthin, trans-lycopene, and phytofluene) were associated with lower risk. Gamma-tocopherol exhibited a non-linear relationship: levels between 91.7-252 ug/dL were protective, whereas risk increased beyond this range. **Conclusions:** Leveraging a population-based cohort study, we identified chemicals consistently associated with cancer mortality across varying model specifications. While meeting Bradford Hill's criterion of consistency strengthens confidence in these associations and supports their plausibility for causation, it does not by itself establish causality. Experimental validation is still needed to elucidate the mechanisms by which such chemicals influence aggressive cancers. Accordingly, we are planning experiments to examine the chemical effects on cell migration and invasion, which are key processes underlying metastasis.

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Background and Purpose: The Human Exposome Project represents the next frontier in precision environmental health—a globally coordinated effort to systematically map, measure, and mitigate the environmental determinants of disease. Approximately 70-90% of chronic diseases have environmental rather than genetic roots, highlighting the critical need to comprehensively map and understand the exposome—the totality of chemical, physical, biological, and psychosocial influences that interact with our biology across the lifespan. Complementing the Human Genome Project, the Human Exposome Project aims to capture the exposure side of disease and translate this understanding into precision prevention and health protection. **Methods:** Utilizing advanced sensors, artificial intelligence (AI), precision analytics, and predictive biological tools, the Human Exposome Project will revolutionize public health by enabling precise interventions based on robust, real-time data rather than relying on retrospective epidemiological analyses. This integration of “Exposome Intelligence”—the convergence of exposomics and AI—will enable the discovery of exposure-disease relationships at unprecedented scale and resolution, transforming how we detect, predict, and mitigate environmental health risks. **Results:** In May 2025, more than 400 global leaders convened in Washington, D.C. for the inaugural Exposome Moonshot Forum, formally launching the Global Exposome Forum (GEF) and signing the Washington D.C. Declaration on the Human Exposome. Building on this foundation, the GEF has begun developing an actionable Roadmap for the Human Exposome Project. The Roadmap defines five foundational pillars: (1) Big Data, AI, and Analytics; (2) Measurement Technologies and Standards; (3) Population and Cohort Integration; (4) Policy, Regulation, and Implementation; and (5) Capacity Building and Workforce Development, supported by cross-cutting themes such as, Global governance and collaboration, Ethics, Legal, and Social Aspects (ELSA), Stakeholder engagement and Communication, FAIR data interoperability, and Economics and Sustainability. The near-term (≤ 2 years) phase focuses on establishing an interim global governance structure, harmonized data standards, and pilot projects linking population cohorts across regions. Mid-term priorities (3-8 years) center on scaling infrastructure, deploying validated measurement technologies, federated data platforms, and AI models that can process heterogeneous exposure data at scale. The long-term horizon (9-16 years) envisions exposome data integration into clinical and regulatory decision-making, the creation of a living Global Exposome Atlas, and measurable reductions in exposure-related disease burden. **Conclusions:** This roadmap transforms exposomics from a conceptual framework into a mission infrastructure—anchored in open science, equitable participation, and human-relevant evidence. By aligning standards, governance, and technology across continents, the Human Exposome Project will enable the transition from reactive medicine to precision prevention, redefining public health for the 21st century.

PS 4283 Accelerating the Adoption of Safer Chemistry in Household and Industrial Cleaners: A Data-Driven Solution for Cleaner and Degreaser Ingredient Optimization

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Background and Purpose: Hazards associated with industrial and household cleaners have previously been underscored by parallel longitudinal studies focusing on household cleaning products, which demonstrate that regular chemical exposure associated with household cleaning is linked to significant lung function decline in women equivalent to smoking 20 cigarettes a day for 20 years (Svanes et al., 2018). Similarly, the extensive use of chemical cleaners and degreasers in electronics (and other consumer goods) assembly presents critical occupational health risks, including, but not limited to, those related to respiratory health. ChemFORWARD is a not-for-profit organization (NGO) that maintains a centralized online platform of comprehensive chemical hazard assessments (CHAs) spanning >20 human and environmental health endpoints designed to support users in selecting safer chemical ingredient alternatives for products across diverse industry sectors, including cosmetics, food packaging, plastic additives, and industrial and household cleaners. **Methods:** To mitigate the risks posed by hazardous ingredients, we collaborated with brands, NGOs and certification programs working to scale the availability and accelerate the adoption of safer cleaners that protect workers and the environment. This presentation will demonstrate how the tool enables users to identify chemicals of high hazard and provide a framework for identifying safer, functional alternatives to high hazard chemicals used in cleaners by leveraging known functional data (e.g. solvent, surfactant) as well as similarity calculations with regard to key characteristics: solubility, molecular structure, and physical/chemical properties. **Results:** The ChemWorks collaboration launched a dedicated, free, web-based Cleaners and Degreasers Ingredient Optimization Tool Application (Chemworks.org) designed to support the transition to safer cleaners, including a registry of trade name materials that are accepted by leading frameworks such as GreenScreen Certified™, EPA Safer Choice, and ToxFMD®. Further, we identified certain hazard traits as failing to meet the criteria for safer cleaners, particularly chemicals with respiratory sensitization and inhalation toxicity. In combination, ChemWorks and CHAs provide a roadmap to substituting hazardous with safer functional alternatives, reducing risk

across the value chain from manufacturing to consumer and end-of-life. We will share case studies where chemical selection using these tools supports data-driven chemical management decisions for the development of safer cleaning products. **Conclusions:** By enabling formulators to optimize their product formulations against rigorous criteria, the platform has potential to significantly reduce development cost, accelerate time-to-market, and simplify the pathway to product verification. The adoption of this transparent, data-driven approach removes technical, financial, and informational barriers, thereby institutionalizing safer chemical stewardship across both the industrial and household cleaner supply chain and accelerating the industry-wide transition toward verified safer cleaning solutions.

PS 4284 Exosomal miRNA Profiles Identify Resolving Versus Non-Resolving Pulmonary Inflammation Following Nanoparticle Exposure

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Background and Purpose: Exosomes, small extracellular vesicles carrying proteins, lipids, and regulatory RNAs, are emerging as key mediators of intercellular signaling, immune regulation, and disease progression. Inhalation of engineered nanoparticles (NPs) has become a significant public health concern due to their widespread use in consumer, industrial, and biomedical applications, as well as unintentional occupational exposures. These particles (<100 nm) can penetrate deep into the lungs, where they interact with epithelial and immune cells, generate reactive oxygen species, and activate pro-inflammatory pathways. While acute inflammation is a protective host response, failure to resolve the inflammation drives chronic disease, including asthma, COPD, pulmonary fibrosis, and cancer. Our previous studies demonstrated that inhaled copper oxide (CuO) NPs produce self-limiting inflammation, whereas cadmium sulfide (CdS) NPs sustain persistent immune activation. This study aimed to determine whether exosomal microRNAs (miRNAs) distinguish resolving from persistent NP-induced inflammation and which miRNAs play a role in these two distinct conditions. **Methods:** Female C57BL/6J mice (4 weeks old) were acutely exposed (4 h, ~3.3 mg/m³) to CuO (40 nm) or CdS (60 nm) aerosols in a nose-only inhalation system. Aerosols were generated by a 6-jet Collision nebulizer, dried, charge-neutralized, and characterized by scanning mobility particle size analysis (geometric means: 33.3 nm CuO, 40.8 nm CdS). Lung dosimetry by ICP-MS confirmed clearance of Cu by day 7, whereas Cd persisted beyond day 7. Bronchoalveolar lavage fluid (BALF) was collected on days 1, 3, and 7. Exosomes were isolated from BALF using ExoRNeasy columns, quantified by Horiba ViewSizer and Qubit assays, and profiled for miRNA cargo using the NanoString nCounter Mouse miRNA Panel. Statistical analyses were performed using GraphPad Prism and R. **Results:** BALF analyses showed neutrophil and total cell counts peaked on day 3 for both NPs. By day 7, CuO-induced inflammation largely resolved, while CdS-exposed mice retained elevated neutrophils, proinflammatory cytokines, and M1 macrophage recruitment. Flow cytometry analyses of lungs confirmed neutrophil infiltration, macrophage polarization, and epithelial injury signatures normalized in CuO lungs but persisted in CdS lungs. Exosome concentrations paralleled these kinetics: CuO-induced transient increases that returned to baseline by day 7, while CdS exposure sustained elevated and larger vesicles. miRNA profiling revealed distinct temporal patterns. CuO triggered early upregulation of miRNAs by day 1, mixed regulation by day 3, and normalization by day 7. In contrast, CdS exposure predominantly induced downregulation of miRNAs at the beginning, followed by a trend toward upregulation at later time points. Both CuO and CdS exposures altered miR-200a, miR-148a, and miR-1224, which are key regulators of epithelial repair, macrophage activation, and neutrophil trafficking, respectively. Distinct signatures also emerged: CuO exposure induced resolution-linked miRNAs (miR-21, miR-146a, miR-223), which promote neutrophil clearance, macrophage M1→M2 transition, and epithelial repair. In contrast, CdS exposure suppressed these protective signals and sustained pro-inflammatory miRNAs (miR-155, miR-31, miR-34a), consistent with chronic immune activation and impaired resolution. Pathway enrichment analyses (GO, KEGG, MSIGDB) demonstrated that CuO-associated miRNAs engaged pro-resolution signaling networks, while CdS diminished immune-suppressive and tissue-repair pathways. **Conclusions:** Exosomal miRNA cargo provides a molecular fingerprint of inflammation trajectory, distinguishing resolving (CuO) from persistent (CdS) pulmonary responses. These findings fill a critical knowledge gap by linking immune cell behavior, nanoparticle properties, and exosome signaling to the fate of lung inflammation. Exosomal miRNAs emerge as both biomarkers of exposure effects and potential therapeutic targets. By elucidating how nanoparticles influence inflammatory resolution, this work lays a foundation for exosome-based diagnostics and therapeutics to mitigate environmentally induced chronic lung disease.

PS 4286 Delta-8-Tetrahydrocannabinol Attenuates Colitis in Mice by Modulating Innate Immune Cell Polarization Involving Epigenetic Alterations

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Background and Purpose: Colitis is an inflammatory bowel disease characterized by persistent autoinflammation in the colon. While Delta-9-tetrahydrocannabinol (Δ^9 -THC) is known to exhibit anti-inflammatory effects and reduce hyperinflammation in colitis, this study specifically investigated the impact of Δ^8 -THC, a less psychoactive analog on colitis. The research focused on how Δ^8 -THC influences key innate immune cells—macrophages, neutrophils, and dendritic cells—and the specific miRNAs that may mediate these effects. **Methods:** Colitis was induced in C57BL/6 mice using dextran sodium sulfate (DSS). Mice were treated with either a vehicle or 20 mg/kg Δ^8 -THC administered intraperitoneally every other day for nine days. Euthanasia occurred on day 10, three days after DSS administration ceased and colitis was established. **Results:** Macroscopic data indicated that Δ^8 -THC treatment significantly reduced the colitis phenotype, evidenced by less colon shrinkage, decreased weight loss, and improved colonoscopy scores. At a cellular level, Δ^8 -THC treatment resulted in an overall decrease in myeloid cells and a direct shift in macrophage populations: pro-inflammatory M1 phenotype macrophages decreased, while anti-inflammatory M2 macrophages increased. This phenotypic shift was associated with specific miRNA changes identified through sequencing and IPA analysis. Notably, levels of let-7a-5p, a miRNA known to inhibit both CD86 and TLR4 expression, increased significantly. Targets were verified *in silico* alignment and qPCR from *in vivo* colon samples. **Conclusions:** Together, these findings demonstrate that Δ^8 -THC treatment alters miRNA expression that promote an anti-inflammatory phenotype in the colon, suggesting therapeutic potential for inflammatory bowel diseases. Also, because Δ^8 -THC exhibits less psychoactive properties than Δ^9 -THC, it may be better suited for clinical treatment of inflammatory diseases (Supported by NIH grants P20GM103641, P30GM154631, R01ES030144, and R01AI160896 to PSN and MN).

PS 4287 Indole-3-Carbinol, an AhR ligand, Attenuates Colitis by Suppressing miR-92a and miR-7a to Restore Antimicrobial Peptide Expression and Microbial Homeostasis

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Background and Purpose: Antimicrobial peptides (AMPs) serve as a primary defense against infections and play a crucial role in maintaining gut microbial and immune balance. An imbalance between pro- and anti-inflammatory cells is a hallmark of ulcerative colitis (UC), but the underlying molecular mechanisms remain unclear. MicroRNAs (miRNAs) are key regulators of inflammatory responses in various diseases, including UC. In this study, we investigated the functional roles of disease-promoting miRNAs in regulating AMPs, which in turn influence immune response, microbial dysbiosis and colitis. **Methods:** AhR-deficient mice and wild-type controls were used to evaluate the effects of Indole-3-carbinol (I3C) in dextran sulfate sodium (DSS)- and anti-CD40-induced models of colitis. Disease severity was assessed by monitoring changes in body weight, measuring colon length, and performing histopathological analysis. To characterize gut microbial composition, 16S rRNA gene sequencing of colonic contents from control and colitic mice was conducted using the MiSeq platform. Molecular analyses, including miRNA sequencing, quantitative PCR, and Western blotting, were performed to examine the expression of relevant microRNAs and antimicrobial peptides. **Results:** In this study, we identified a major disease-promoting role for microRNA-92a-1-5p (miR-92a) and microRNA-7a-5p (miR-7a) in both DSS- and anti-CD40-induced models of colitis. We also observed similar results in murine adenocarcinoma MC38 cells treated with DSS. Elevated levels of miR-92a and miR-7a were associated with reduced expression of antimicrobial peptides (AMPs) Reg3a and Reg3b respectively, accompanied by marked microbial dysbiosis. This dysbiosis was characterized by an increase in disease-promoting Gram-negative bacteria and a decrease in beneficial Gram-positive bacteria, contributing to intestinal inflammation. Treatment with Indole-3-carbinol (I3C), a ligand for AhR, restored microbial and immune homeostasis and significantly attenuated colitis severity. Mechanistically, I3C downregulated the expression of miR-92a and miR-7a, relieving their repression of Reg3a and Reg3b, thereby enhancing AMP expression, improving microbial balance, and mitigating intestinal inflammation in both DSS- and anti-CD40-induced colitis models. **Conclusions:** Our results demonstrate that miR-92a and miR-7a drive intestinal inflammation by reducing antimicrobial peptides and sustaining microbial imbalance, implicating these miRNAs as potential therapeutic targets for colitis. (Supported by NIH grants P20GM103641, P30GM154631, R01ES030144, and R01AI160896).

PS 4288 Potential contribution of sphingosine-1-phosphate to crystalline silica-induced NLRP3 inflammasome activation

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Background and Purpose: Silicosis is a debilitating lung disease caused by respiratory exposure to crystalline silica particles (cSiO₂). It is well known that inhaled cSiO₂ are phagocytosed by alveolar macrophages leading to NLRP3 inflammasome activation and subsequent IL-1 β release resulting in chronic lung inflammation and fibrosis. Furthermore, previous research suggests that sphingomyelin metabolism is activated in alveolar macrophages during silicosis disease pathology. The present study investigated the role of sphingomyelin metabolism in cSiO₂-induced lysosome membrane permeability (LMP) and NLRP3 inflammasome activation. **Methods:** Murine macrophages were pretreated with specific inhibitors of acid sphingomyelinase (aSMase) or sphingosine kinase 2 (SphK2) and exposed to cSiO₂ before measuring LMP, NLRP3 inflammasome activation, and S1P production. LMP was determined based on the detection of lysosomal enzyme activity in cytosol extracts. IL-1 β release from macrophages was used as a proxy measurement of NLRP3 inflammasome activation. S1P levels were measured in cell lysates using ELISA. **Results:** S1P production was elevated in macrophages in response to cSiO₂ exposure. In addition, inhibiting S1P production by inhibiting aSMase or SphK2 enzyme activity, in turn, attenuated cSiO₂-induced LMP and IL-1 β release. **Conclusions:** The results suggest that cSiO₂ uptake by macrophages leads to LMP, which activates the NLRP3 inflammasome through a mechanism that may involve increased S1P production.

PS 4289 HSCs-Macrophage Cross-Talk Mediated by IL-11 Exacerbates Liver Fibrosis.

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Background and Purpose: Liver fibrosis is a pivotal stage leading chronic liver diseases toward cirrhosis. It is consensus that the hepatic microenvironment shaped by intricate networks of non-parenchymal cells plays a decisive role during fibrogenesis, among which the communication between hepatic stellate cells (HSCs) and macrophages aroused researcher's attention. Interleukin-11 (IL-11) has been identified as a pro-fibrogenic cytokine, mainly characterized in the context of hepatocyte-HSCs signaling. However, its potential involvement in mediating the interplay between HSCs and macrophages, as well as the underlying molecular mechanisms, remains poorly understood. **Methods:** To explore the function of IL-11 in hepatic fibrosis by overexpressing it in HSCs of CCl₄-administrated mice using AAV6. The underlying mechanisms were probed with RNA sequencing and *in vitro* assays. Additionally, *in vivo* macrophage depletion studies were performed to validate the function of macrophages in IL-11-associated fibrogenesis. Lastly, an anti-IL-11 nanobody was applied to CCl₄-induced fibrotic mice in order to evaluate the therapeutic potential of inhibiting IL-11 signaling. **Results:** HSCs-derived IL-11 aggravated liver fibrosis by driving macrophages into a pro-fibrotic M2 state. *In vitro*, IL-11 acted synergistically with IL-4 to activate PI3K-mTOR pathway, promoting M2 polarization and TGF- β production, while simultaneously to trigger STAT3 pathway to increase the release of chemokine. *In vivo*, overexpressed-IL-11 in HSCs worsened fibrosis, which was diminished by macrophage depletion. Conversely, treating fibrotic mice with an anti-IL-11 nanobody reduced fibrosis and shifted the macrophage profile, highlighting the therapeutic potential of IL-11. **Conclusions:** Our data reveal a self-reinforcing cycle between HSCs and macrophages: HSCs-derived IL-11 reprograms macrophages to become pro-fibrotic, and these macrophages subsequently exacerbate HSCs activation, fueling the progression of liver fibrosis. This pathogenic loop provides strong evidence for targeting IL-11 as an anti-fibrotic strategy.

PS 4290 Extracellular Vesicles Mediate Inflammatory Signaling in Arsenic-Exposed Bronchial Epithelial Cells

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Background and Purpose: Exposure to arsenic (As) from drinking water is a worldwide public health concern. In the US, 5 million people drink water with As concentrations over the EPA standard limit of 10 μ g/L. Due to its persistence and toxicity, As is a priority contaminant at numerous Superfund sites across the U.S. where cleanup and long-term monitoring remain a challenge. In the state of Texas, over a million residents rely on well water. Since private wells are not routinely tested, millions of people are potentially exposed, especially rural residents living in South Texas. As is a known carcinogen, and recent evidence implicates As exposure with respiratory disease following ingestion. Extracellular vesicles (EVs) are cell-derived structures that represent potential biomarkers of exposure or effect to inform environmentally related diseases. Considering the increased risk of arsenic exposure and the heightened prevalence of asthma in South Texas, developing novel biomarkers is essential to identify susceptible populations and clarify the mechanisms by which EVs contribute to respiratory toxicity. **Methods:** To address these objectives, we exposed human bronchial epithelial (16HBE) cells to 0, 5, or 10 μ M of NaAsO₂ for

24 hours (acute exposure) to establish phenotypic endpoints, including cytotoxicity and inflammation at the gene and protein level. Next, to model sub-chronic exposure levels, 16HBE cells were treated with 0, 0.1, or 0.5 μM of NaAsO_2 for 1 week. To further study the mechanism underlying inflammation, conditioned media saved from the 24-hour NaAsO_2 -exposed 16HBE cells was transferred to naïve (unexposed) 16HBE cells for 24 hours. EVs were then isolated from 16HBE cell culture media and characterized using nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), and Western blotting of EVs were performed. Isolated EVs were applied to naïve 16HBE cells for 24 hours to assess their functional effects. **Results:** After 24 hours, lactate dehydrogenase activity was slightly elevated, indicating toxicity. Gene expression of the pro-inflammatory cytokine *IL-1 β* was significantly increased by 5 μM ($p < 0.01$) and 10 μM ($p < 0.01$) after 24 hours of exposure to NaAsO_2 . Protein expression of IL-1B was not statistically increased compared to control, but with an increased trend at the 10 μM dose. At 1 week, *IL-1 β* was also slightly elevated as NaAsO_2 exposure increased. *IL-1 β* was noticeably increased by the conditioned media from the low dose and high dose, by 3 and 2-fold, respectively. For the EV exposure, *IL-1 β* was statistically increased at the high dose. **Conclusions:** Collectively, these findings implicate soluble inflammatory mediators and EV signaling in arsenic-induced epithelial toxicity. EVs isolated from exposed bronchial epithelial cells may serve as novel biomarkers of respiratory inflammation. Future work will validate these mechanisms in primary pediatric lung cell models to enhance translational relevance.

PS 4291 Rodent Liver Tumors Produced by Activators of the Constitutive Androstane Receptor (CAR) and the Peroxisome Proliferator-Activated Receptor Alpha (PPAR α) are Not Relevant to Human Cancer Risk

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Background and Purpose: The hepatocarcinogenic potential of CAR and PPAR α activators in rodents has been debated for over five decades, particularly regarding relevance to human cancer risk. Although metabolic responses are qualitatively similar across species, hepatocellular proliferation appears specific to rodents. This study aims to clarify species-specific differences in CAR- and PPAR α -mediated liver carcinogenesis, incorporating recent molecular findings. **Methods:** This review examines literature to assess the human relevance of liver carcinogenesis induced by CAR and PPAR α activators in rodents. A comprehensive search was conducted using PubMed, ScienceDirect, and relevant references cited in selected publications. Comparative analyses included *in vitro* proliferation assays in hepatocytes from mice, rats, and humans, and *in vivo* studies using human and rat hepatocytes in chimeric mice. Studies involving nonresponsive species, including Syrian hamsters and guinea pigs, were also examined. Epidemiological data were reviewed to assess cancer risk in humans exposed to CAR or PPAR α activators. Particular attention was given to molecular mechanisms, such as Yes-associated protein (YAP) interactions with CAR or PPAR α , to elucidate species-specific responses. The YAP is an effector protein in the Hippo signalling, which controls cell and organ size. **Results:** Findings confirmed that hepatocellular proliferation occurs in rodents but not in humans, as shown by both *in vitro* and *in vivo* studies. *In vitro* assays revealed marked proliferation in rodent hepatocytes upon CAR activator exposure, while human hepatocytes showed no such response. *In vivo* studies using chimeric mice with humanized livers confirmed that human hepatocytes remained quiescent under conditions inducing proliferation in rodents whereas rat hepatocytes in chimeric mice showed proliferation. Molecular evidence, especially from CAR-YAP interaction analyses, supports the absence of proliferative response in humans and primates. In mice and rats, CAR activation triggers YAP activation, followed by nuclear translocation, promoting transcription of proliferation-related genes. In contrast, human hepatocytes show limited or no YAP activation following CAR stimulation, suggesting a species-specific disconnect in downstream signaling. For PPAR α activators, which increase hepatocyte proliferation in mice and rats, no significant proliferation is observed in cultured human hepatocytes, chimeric mice with humanized livers, or human PPAR α mice treated with these activators. These species differences are attributed to variations in receptor activation mechanisms. In mice, interaction with PPAR α -YAP appears necessary for hepatocyte proliferation, whereas this is not the case in humans, although further experimental validation is needed. Epidemiological data show no increased risk of liver cancer or other cancers in humans exposed to either CAR or PPAR α activators. Therefore, CAR and PPAR α share several common characteristics, and these findings support the conclusion that CAR- and PPAR α -mediated liver carcinogenesis in rodents is not relevant to humans. **Conclusions:** This study provides updated molecular insights into species-specific liver carcinogenic responses, which align with previously accumulated evidence and reinforces the conclusion that these rodent-specific mechanisms are not applicable to humans. Consequently, our position is that when a CAR- and/or PPAR α -dependent mode of action (MOA) is demonstrated in rodent studies and other MOAs are excluded, no further testing is needed to determine the absence of human hazard. However, if regulatory authorities require direct experimental evidence, since proliferative responses in human hepatocytes serve as a critical indicator for hazard potential, further methodological standardization of procedures to assess replicative DNA synthesis in cultured human hepatocytes is essential. This is consistent with the 2024 recommendation by the Joint Research Centre, the European Commission's

science and knowledge service. Such standardization would enhance scientific robustness and facilitate efficient and transparent regulatory evaluations, enabling timely and appropriate decision-making.

PS 4292 1,4-Dioxane Induces Epithelial-Mesenchymal Transition and Carcinogenesis in an Nrf2-Dependent Manner

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Background and Purpose: Limited animal evidence suggests that Nrf2 mediates carcinogenesis induced by high doses of 1,4-dioxane (1,4-D), but its role in environmentally relevant concentrations remains unclear. The mechanisms driving the genotoxicity and carcinogenicity of 1,4-D are also challenging to elucidate, underscoring the need for further studies to assess its carcinogenic potential in humans. **Methods:** In this study, BEAS-2B cells, with or without CRISPR/Cas9-editing Nrf2 knockout, were treated with 1.25-20 ppm 1,4-D for two months. Malignant transformation was evaluated through colony formation, migration and invasion assays, while tumorigenicity was confirmed by subcutaneous implantation in immunodeficient mice. RNA sequencing, proteomics, and functional analyses were conducted to identify mechanisms underlying 1,4-D-induced carcinogenesis, with a specific focus on Nrf2's tumor-promoting effects. **Results:** Our results demonstrate that continuous exposure to environmentally relevant concentrations of 1.25-20 ppm 1,4-D over 2 months induces malignant transformation in an Nrf2-dependent manner. Transformed cells exhibit enhanced anchorage-independent growth in soft agar, increased migration and invasion, and tumorigenic potential in a xenograft mouse model. Integrated RNA sequencing and proteomics analyses reveal that 1,4-D robustly activates Nrf2 signaling, driving extracellular vesicle (EV) biogenesis and cargo loading with syndecan 4 (SDC4) and other proteins, including COL12A1, CAPG and NNMT, which are associated with epithelial-mesenchymal transition (EMT) and cancer metastasis. Nrf2 knockout reduces SDC4 expression and its incorporation into EVs, leading to decreased EV uptake by recipient cells. Unlike EVs from 1,4-D-transformed WT cells, which enhance the proliferation, migration and invasion of recipient cells, EVs from 1,4-D-transformed Nrf2 KO cells exhibit a diminished capacity to promote these EMT properties. Furthermore, we demonstrate that the Nrf2 target gene SDC4, induced by 1,4-D and enriched in EVs, plays a critical role in EV uptake by recipient cells, thereby facilitating EMT propagation. **Conclusions:** Our study identifies 1,4-D as a potential human carcinogen, with its effects primarily driven by Nrf2-dependent EV biogenesis, and highlights SDC4-enriched EVs as key mediators of EMT and carcinogenesis.

PS 4293 Cadmium exposure promotes colorectal cancer metastasis through the dysregulation of iron metabolism

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Background and Purpose: Cadmium (Cd) is a toxic environmental pollutant primarily released through agricultural and industrial activities. Human exposure occurs mainly through the ingestion of contaminated food and water or the inhalation of Cd-containing dust and smoke. Colorectal cancer (CRC) is the third leading cause of cancer-related deaths in the United States with metastasis to the liver and lungs being the primary cause of mortality. Emerging evidence suggests environmental factors such as Cd may contribute to CRC progression and metastasis, potentially by disrupting iron metabolism. Our lab has previously demonstrated the importance of iron in CRC metastasis through proteins such as transferrin receptor (TFRC), ferritin heavy chain (FTH1), and nuclear receptor coactivator (NCOA4) which are important for iron uptake, storage, and recycling. It is hypothesized that exposure to Cd promotes CRC metastasis by dysregulating iron metabolism, particularly through NCOA4. **Methods:** Inductively coupled plasma mass spectrometry (ICP-MS) was used to quantify cadmium concentrations in normal, tumor, and metastatic colorectal tissue samples. Human colorectal cancer (HCT116) cells were subsequently exposed to increasing concentrations of CdCl_2 to assess the cellular effects of cadmium exposure. Cell viability and proliferation were evaluated using MTT assays. Protein expression levels of FTH1 and NCOA4 were analyzed by Western blot following Cd treatment. **Results:** ICP-MS tissue analysis revealed an increase in Cd concentrations from normal and tumor colorectal tissue to metastases, with significantly elevated levels in metastatic sites. These findings suggest Cd involvement in the metastatic process. Further, HCT116 cells were treated with increasing concentrations of CdCl_2 . MTT assays indicated no negative impact on cell proliferation at environmentally relevant doses. Finally, Western blot analysis revealed upregulation of FTH1 and downregulation of NCOA4 following Cd treatment. **Conclusions:** These results support the hypothesis that Cd promotes CRC metastasis by promoting iron overload and activating iron-dependent signaling pathways associated with proliferation and metastasis. This work provides insight into the potential cancer risks of environmental toxicants and emphasizes the need for further investigation into iron metabolism as a therapeutic target for Cd-associated colorectal cancer.

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Background and Purpose: The liver is a common target for injury due to xenobiotic exposure. Xenobiotics such as Endocrine-Disrupting Chemicals (EDCs) impact liver function by mimicking naturally secreted hormones and have been associated with liver-related complications, including intrahepatic cholestasis, sinusoidal dilations, and hepatic adenomas. Estrogen Receptor Alpha (ERα), the primary receptor that mediates estrogenic effects in both male and female livers, is a target of EDCs. Disruption in EDC-induced ERα signaling is associated with obesity, metabolic dysfunction, and hepatocellular carcinoma (HCC). However, the mechanisms behind EDC impact on ERα signaling in the liver are not well understood. The objective of this study was to investigate the mechanisms through which ERα activation mediates EDC-induced liver injury. **Methods:** Male and female Sprague-Dawley WT and ERα KO rats received a 10 mg/kg dose of 17β-estradiol (17β, an endogenous estrogen) or diethylstilbestrol (DES, a known EDC) via oral gavage once daily for five days. Liver and serum samples were collected on the sixth day. Liver to body weight (LW:BW) ratio was measured, and Ki67 immunohistochemistry was performed to analyze proliferation. In addition, RNA-seq was performed to identify transcriptomic changes. Ingenuity Pathway Analysis (IPA) was used to identify impacts on upstream transcription regulators and GSEA analyses were used to identify pathways uniquely enriched in male and female WT rats treated with DES or 17β. **Results:** Both 17β and DES treatments increased hepatic proliferation in WT male and female rats, but not in KO rats. However, only DES-treated WT male and female rats showed hepatomegaly, as evidenced by a significant increase in LW:BW ratio. To determine the transcriptional changes underlying these distinct outcomes, KO RNA-seq data were used to remove effects of ERα deletion and to retain genes uniquely regulated by 17β or DES in WT rats. In males, this approach identified 97 unique, WT-dominant DEGs in DES-treated rats compared to 8 in WT 17β-treated rats. IPA revealed that DES treatment increased activation of several proto-oncogenes, including c-Myc, a transcription factor that is known to be involved in liver carcinogenesis. IPA could not be performed for WT rats treated with 17β due to the small number of unique genes. While GSEA identified similar pathway enrichment for both treatments, there were a few key differences. Most notably up-regulation of p53 and DNA repair pathways in 17β-treated rats. In females, RNA-seq analysis identified 85 unique, WT-dominant DEGs in DES-treated rats compared to 88 in WT 17β-treated rats. Unlike with males, IPA was performed for both DES and 17β-treated rats. In WT DES-treated female rats, IPA identified upregulation of several transcription factors associated with cell growth and oncogenic signaling, including SP1, SOX4, and HNF1β. Female rats treated with 17β were surprisingly similar to DES-treated males and showed increased expression of proto-oncogenes, including c-Myc. GSEA also identified many similar pathways for both treatments in WT females, but again, there were key differences. Most notably, upregulation of p53, DNA repair, and apoptosis pathways in 17β-treated rats. **Conclusions:** These data show that DES, but not 17β, induces significant hepatomegaly, which may be linked to treatment-specific upstream regulators. GSEA analyses indicate that WT 17β-treated rats of both sexes exhibit increased proliferative signaling without hepatomegaly, possibly due to concurrent activation of apoptotic and tumor suppressor pathways, which was not observed in WT DES-treated rats. Together, these results suggest that DES and 17β activate distinct molecular mechanisms in the liver.

PS 4295 **Adipocyte-Derived Kynurenine Enhances 3D Growth of Tamoxifen-Resistant Breast Cancer: A Potential Endogenous Toxicometabolic Mechanism**

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Background and Purpose: Every 13 minutes, a woman dies from breast cancer (BC) in the United States. Among women with estrogen receptor-positive (ER+) BC treated with tamoxifen; epidemiological studies show that women with obesity (BMI ≥ 30) experience significantly worse survival outcomes compared to underweight or lean women (BMI < 25). **However, the exact mechanism by which obesity promotes the growth of aggressive, treatment resistant breast cancer remains unclear.** Using a mass spectrometry analysis, the Bernard lab has demonstrated adipocytes (fat cells) are able to metabolize the amino acid tryptophan into kynurenine, an endogenous ligand of the aryl hydrocarbon receptor. Like epidemiological studies that demonstrate circulating kynurenine levels are elevated in women with obesity, our published *in vivo* research indicates mice fed a high fat diet also have elevated serum kynurenine levels. **Therefore, we hypothesize breast cancer cells can take up kynurenine from the microenvironment to induce growth of aggressive, tamoxifen resistant BC cells.** **Methods:** We treated sensitive and tamoxifen resistant ER+ BC cells with increasing concentrations of kynurenine and performed flow cytometry, soft agar colony formation assays, RNA-sequencing, and qRT-PCR. **Results:** Using a novel flow cytometric monitoring system and the auto fluorescent properties of kynurenine, we demonstrated both sensitive (MCF7) and tamoxifen resistant (TAMR7) BC cells are able to take up kynurenine from the microenvironment overtime. Because both MCF7s and

TAMR7s express very low levels of the tryptophan metabolizing enzyme indoleamine 2,3-dioxygenase, these findings suggest the increase in kynurenine uptake from the microenvironment likely results from increased uptake, rather than increased synthesis of tryptophan into kynurenine directly by the BC cells. Moreover, we have developed a novel *in vitro* system to model three-dimensional growth of tamoxifen resistant cells known as a soft agar colony formation assay. We treated two sensitive (MCF7 and ZR-75-1) and two tamoxifen resistant (TAMR7 and ZR-75-1-T) cell lines with increasing concentrations of kynurenine. Over a 10-day period, we demonstrated kynurenine increases colony size in a concentration dependent manner in all cell lines. To understand potential mechanism of this kynurenine-induced 3D cell growth, RNA-sequencing was completed on MCF7 cells treated with a low and a high concentration of kynurenine. Potential pathways of interest revealed include activation of the aryl hydrocarbon receptor, as exhibited by the increased expression of CYP1A1. **Conclusions:** Given that 90-95% of BC cases are associated with modifiable lifestyle factors such as exposure to environmental chemicals, diet, and obesity, this study underscores the potential of kynurenine as a biomarker for identifying patients at elevated risk of developing aggressive, treatment resistant, obesity-driven breast cancer, enabling earlier and more personalized interventions.

PS 4296 **Berberine Modulates Cell Survival in a Peroxisome Proliferator-Activated Receptor (PPAR)-dependent Manner in a Human Malignant Melanoma Cell Line**

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Background and Purpose: Low-survival cancers, including malignant melanoma, require innovative new therapeutic interventions. Peroxisome proliferator-activated receptors (PPAR) is a class of nuclear hormone receptors that can inhibit carcinogenicity and potentially be used therapeutically as a target for melanoma. In particular, pharmacological targeting of PPARβ/δ and PPARγ have been shown to reduce tumorigenesis in several human melanoma models. Berberine, is a naturally occurring isoquinoline alkaloid found in the roots and stems of barberry and turmeric, that may be useful for treating cancers. Interestingly, berberine is reported to be a PPAR agonist. Thus, these studies examined the hypothesis that berberine modulates PPAR-dependent anti-cancer effects in human malignant melanoma. **Methods:** The effects of berberine on melanoma tumorigenesis in a melanoma cell line model (UACC903) with stable modulated PPAR over-expression of PPARβ/δ and PPARγ was examined. A dose response (10.0 - 50.0 μM) of berberine was examined in all UACC cell lines to examine cell proliferation, clonal expansion, soft agar transformation, caspase activation, and cellular metabolic activity. **Results:** Cell proliferation was reduced by berberine in a dose-dependent manner in all UACC903 cell lines compared to controls. Cells over-expressing PPARβ/δ or PPARγ were more sensitive to the effect than control cell lines. Clonal expansion was inhibited by berberine in all cell lines; only cells over-expressing PPARβ/δ exhibited increased sensitivity. Berberine also reduced soft agar colony formation in a dose-dependent manner compared to controls. Over-expression of either PPARβ/δ or PPARγ enhanced this response. Berberine was more potent in inducing caspase activity in cells over-expressing PPARβ/δ or PPARγ compared to controls. Similarly, berberine modulated metabolic activity at lower doses in cells over-expressing PPARβ/δ or PPARγ compared to controls. **Conclusions:** These studies demonstrate 1) berberine has anti-melanoma activities and 2) PPARs contribute to the anti-cancer properties associated with berberine. Mechanistic studies are needed to delineate the molecular signaling pathways of how berberine interacts with PPAR for future development of co-therapeutic approaches for melanoma.

PS 4297 **Evaluation of the Hepatotoxic Effects of Various PFAS in a Short-Term Rat Study**

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Background and Purpose: Perfluoroalkyl and polyfluoroalkyl substances (PFAS) have been used in a wide array of products due to their ability to repel oil and water, reduce friction, and withstand temperature fluctuations. However, some PFAS, including perfluorooctanoic acid (PFOA), have been reported to induce toxicity and tumor formation in the liver of rodents. Therefore, the present study aimed to evaluate the hepatotoxicity of various PFAS using a rat short-term study. **Methods:** Six-week-old male SD rats were administered 6 PFAS including PFOA, undecafluoro-2-methyl-3-oxahexanoic acid (GenX), 3-perfluorohexyl-1,2-epoxypropane (PFHEP), 2,2-difluoroethyl trifluoromethanesulfonate (DFEMS), hexafluoroglutaryl chloride (HxFGC) and perfluorohexanoic acid (PFHxA), for 4 weeks. After treatment, rats were euthanized, and livers were collected for histological examination and RNA extraction. Hepatic gene expression profile was obtained using microarray, and compared with controls to identify altered gene expression. These alterations were subsequently evaluated using a non-genotoxic hepatocarcinogens (NGHCs) prediction model. **Results:** Histopathologically, hepatocellular hypertrophy in Zone 3 was observed in the livers of rats treated with PFOA, GenX, and PFHEP. Periportal necrosis was observed in those treated with DFEMS. In contrast, no histological changes

were noted in the livers of rats treated with HxFGC or PFHxA compared to the control group. Using NGHCs prediction model, PFOA and GenX were identified as NGHCs, whereas the others were classified as negative. **Conclusions:** These findings indicate that PFASs exhibit structural diversity and distinct toxicological properties, suggesting that their individual toxicity should be carefully evaluated.

PS 4298 Investigating the Effects of Chronic Arsenic Exposure in Human Keratinocytes Overexpressing hsa-miR-362

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Background and Purpose: Arsenic is a Group I human carcinogen, a known clastogen that does not directly interact with DNA, but dysregulates DNA repair, chromosomal stability, mRNA alternative splicing, and miRNA (miR) expression. miRs are small non-coding RNAs that can suppress mRNA translation. hsa-miR-362 (miR-362) expression was elevated in arsenic-induced human skin tumors, suggesting a role in arsenic-induced carcinogenesis. **Hypothesis:** miR-362 overexpression will suppress proteins involved in biological pathways known to be dysregulated by arsenic. **Methods:** Ker-CT cells were transduced with lentiviral particles overexpressing either miR-362 or a scrambled miR control (SC-miR). miR-362-5p expression was measured with TaqMan RT-qPCR with hsa-miR-191-5p as control. Four miR-362 or SC-miR clones with similar expression were selected, cultured with +/- 100 nM NaAsO₂, and passaged for several weeks. Predicted miR-362-5p targets were ranked by their combined score from three miRNA - mRNA binding databases: DIANA-MicroT, TargetScan, and miRDP. Expression of target proteins with roles in DNA repair, chromosomal stability, alternative splicing, and their mRNAs were assessed by immunoblot and RT-qPCR. Their downstream protein interactions were determined with STRING. Statistical analyses were completed using unpaired t-tests as well as 2 and 3-way ANOVAs. **Results:** Clonal Ker-CT cell lines transduced with miR-362 and SC-miR lentiviruses were produced. miR-362-5p overexpression was confirmed and ranged between 250- and 2000-fold over basal expression. The 54 miR-362-5p target hits that were found in all three databases were categorized by biological pathway and ranked by combined score. The top results in DNA repair (RMI1), alternative splicing (LUC7L3), and chromosomal stability (RMI1, LUC7L3, and CENPK) were confirmed to be translationally suppressed in miR-362 overexpressing cells. STRING interactions revealed that CENPK primarily interacts with other centromere proteins, RMI1 interacts with members of the Bloom and Fanconi Anemia complexes, and LUC7L3 interacts with several splice factors. Chronic culture assays demonstrated that both miR-362 overexpression and arsenic exposure inhibited cell growth compared to unexposed SC-miR. **Conclusions:** Overexpression of miR-362 was shown to suppress the expression of proteins involved in DNA repair, maintenance of chromosomal stability, and alternative splicing. The resulting dysregulation of these pathways could contribute to arsenic-induced transformation and skin carcinogenesis, but further studies are needed. **Grant support:** R01ES027778, P30ES030283, T35ES014559

PS 4299 Curcumin Analogues Trigger HMOX-1-Mediated Ferroptosis to Halt Endometrial Cancer Growth

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Background and Purpose: Advanced endometrial cancer remains challenging to treat due to limited therapeutic options and drug resistance. Ferroptosis, an iron-dependent form of cell death, offers a potential strategy for overcoming resistance. Curcumin analogues with improved bioavailability, such as HO-3867 and AKT-100, exhibit potent anti-cancer activity, but their mechanisms remain underexplored. **Methods:** KLE and Hec50cc endometrial cancer cells were treated with HO-3867 or AKT-100. RNA sequencing, qPCR, and immunoblotting assessed ferroptosis-related gene expression, focusing on HMOX-1. Intracellular iron and ROS were measured via FerroOrange and DCFH-DA staining. Cytotoxicity and colony formation were evaluated using CyQUANT assays, with pharmacological inhibitors (ZnPP, Liproxstatin-1, Z-VAD-FMK) and HMOX-1 siRNA to dissect the roles of ferroptosis and apoptosis. **Results:** Both analogues upregulated multiple ferroptosis genes, prominently HMOX-1. AKT-100 increased intracellular iron and ROS levels. Inhibition of HMOX-1, ferroptosis, or apoptosis partially rescued cell viability, while HMOX-1 knockdown enhanced clonogenic growth, confirming its key role in AKT-100-mediated cytotoxicity. **Conclusions:** HO-3867 and AKT-100 induce HMOX-1-mediated ferroptosis and apoptosis, effectively suppressing endometrial cancer cell growth. These findings support the therapeutic potential of curcumin analogues and provide a foundation for *in vivo* studies targeting HMOX-1 in endometrial cancer.

PS 4300 Ursolic Acid Modulates Cell Survival in a Peroxisome Proliferator-Activated Receptor (PPAR)-dependent Manner in a Human Malignant Melanoma Cell Line

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Background and Purpose: Low-survival cancers, including malignant melanoma, require innovative new therapeutic interventions. Peroxisome proliferator-activated receptors (PPAR) is a class of nuclear hormone receptors that can inhibit carcinogenicity and potentially be used therapeutically as a target for melanoma. In particular, pharmacological targeting of PPAR β/δ and PPAR γ have been shown to reduce tumorigenesis in several human melanoma models. Ursolic acid, is a naturally occurring triterpene found in apples, cranberries, and peppermint, that may be useful for treating cancers. Interestingly, ursolic acid is reported to be a PPAR agonist. Thus, these studies examined the hypothesis that ursolic acid modulates PPAR-dependent anti-cancer effects in human malignant melanoma. **Methods:** The effects of ursolic acid on melanoma tumorigenesis in a melanoma cell line model (UACC903) with stable modulated PPAR over-expression of PPAR β/δ and PPAR γ was examined. A dose response (10.0 - 40.0 μ M) of ursolic acid was examined in all UACC cell lines to examine cell proliferation, clonal expansion, soft agar transformation, caspase activation, and cellular metabolic activity. **Results:** Cell proliferation was reduced by ursolic acid in a dose-dependent manner in all UACC903 cell lines compared to controls. Cells over-expressing PPAR β/δ or PPAR γ were more sensitive to the effect than control cell lines. Clonal expansion was inhibited by ursolic acid in all cell lines; only cells over-expressing PPAR β/δ exhibited increased sensitivity. Ursolic acid also reduced soft agar colony formation in a dose-dependent manner compared to controls. Over-expression of either PPAR β/δ or PPAR γ enhanced this response. Ursolic acid was more potent in inducing caspase activity in cells over-expressing PPAR β/δ or PPAR γ compared to controls. Similarly, ursolic acid modulated metabolic activity at lower doses in cells over-expressing PPAR β/δ or PPAR γ compared to controls. **Conclusions:** These studies demonstrate 1) ursolic acid has anti-melanoma activities and 2) PPARs contribute to the anti-cancer properties associated with ursolic acid. Mechanistic studies are needed to delineate the molecular signaling pathways of how ursolic acid interacts with PPAR for future development of co-therapeutic approaches for melanoma.

PS 4301 Investigating Cadmium's Adipogenic Effects on Breast Cancer Metastasis in the Bone Niche

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Background and Purpose: Cadmium (Cd), a heavy metal widely used in battery production, electroplating, paints, and metal alloys, is an environmental toxicant with both carcinogenic and obesogenic potential. Human exposure to Cd continues to rise due to occupational hazards in metal industries, inhalation of cigarette smoke, and contamination of water sources through industrial and agricultural pollution. Cd acts as an obesogen and is classified as a Group 1 human carcinogen. Cd exposure has been associated with increased incidence of breast cancer, but its ability to drive tumor progression by enhancing metastasis has not been fully explored. Because Cd has a long biological half-life and accumulates in the bone, which is a frequent site of breast cancer metastasis, it may directly contribute to metastatic progression to the bone niche. We hypothesize that Cd accumulation within bone, combined with its obesogenic effects, serves as a key driver to induced breast cancer metastasis to the bone. **Methods:** An *in vitro* model was established using OP9 mouse bone marrow stromal cells (BMSCs), which are capable of differentiating into adipocytes. Cells were exposed to CdCl₂ at varying concentrations, and cytotoxicity was assessed via MTT assays to determine nontoxic doses. To evaluate Cd-driven adipogenesis, OP9 cells were treated with 0.5, 0.75, and 1 μ M CdCl₂ and 25% serum replacement media. RT-qPCR quantified changes in adipokine and cytokine gene expression, and Oil Red O staining was used to visualize and quantify adipocyte formation. Two *in vivo* models were used to evaluate the effects of Cd on bone marrow adipogenesis and its potential to drive breast cancer metastasis to the bone: a non-tumor mouse model and an orthotopic mouse model. In each model, female BALB/c mice were exposed to either 5ppm Cd or a no Cd control in their drinking water for 8 weeks. In the orthotopic model, mice were orthotopically injected with 4T1 triple-negative breast cancer cells into the mammary fat pad after 4 full weeks of Cd exposure. Fresh Cd-treated water was prepared three times per week, body weights were recorded weekly, and tumor volumes were measured three times per week post-injection. At the terminal endpoint, body, spleen, adipose, and tumor weights were recorded. Femurs were stained for the adipocyte marker Plin1 via immunohistochemical staining, and Cd accumulation in tibias was quantified using ICP-MS. Bone marrow colony assays and H&E staining of lung and liver tissues evaluated metastatic progression. Additional analyses included microCT imaging of femurs, cytokine profiling in plasma, and flow cytometric quantification of myeloid-derived suppressor cells in bone marrow. **Results:** MTT assays revealed that CdCl₂ concentrations above 1 μ M caused significant toxicity in OP9 cells after 48 hours; thus, concentrations $\leq$ 1 μ M were used for subsequent experiments. CdCl₂ exposure is expected to significantly increase adipokine gene expression in OP9 cells and enhance BMSC differentiation into adipocytes, reflected by elevated adipogenic markers and increased Oil Red O staining. *In vivo*,

mice exposed to 5ppm Cd displayed higher adipocyte numbers within bone marrow and had a higher accumulation of Cd within the bone of Cd-exposed non-tumor-bearing mice. Cd exposure did not significantly alter white adipose tissue weight, spleen weight, or overall body weight in non-tumor-bearing mice. In the tumor-bearing mice, Cd exposure is expected to accelerate tumor growth and enhance metastatic markers relative to controls. **Conclusions:** Preliminary data suggest Cd exposure promotes adipogenesis in BMSCs and within the bone marrow of Cd-exposed mice, creating a bone microenvironment favorable for tumor cell colonization and growth. These effects may parallel those of other heavy metals, indicating that environmental metals can remodel the bone niche to promote metastasis. Ongoing studies will further define Cd's role in the metastatic process and microenvironment modulation. Understanding Cd's contribution to breast cancer progression and metastasis could identify it as a modifiable environmental risk factor, with implications for cancer prevention and public health policy.

PS 4302 KEAP1-NRF2 modulation via posttranslational modifications with the nitro-PAH 3-nitrobenzanthrone

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Background and Purpose: Nitroated polycyclic aromatic hydrocarbons (NO₂-PAHs) are a major component of diesel engine exhaust (DEE) - classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC). NO₂-PAHs (including 1-nitropyrene (1-NP) and 3-nitrobenzanthrone (3-NBA)) are formed during the incomplete combustion of PAHs and are adsorbed at the surface of PM_{2.5}. Despite their mutagenic properties, there is insufficient evidence to increase their IARC classification above Group 2A or 2B carcinogens. To become mutagenic, these compounds require metabolic activation through a 6-electron reduction of the nitro-group via a nitroso-, hydroxylamino-, and amine intermediates, which are catalyzed by cytosolic nitroreductases, such as aldo-keto reductases (AKR)1C1-3 and NQO1. The expression of these enzymes is induced by the NRF2-KEAP1 stress response pathway. KEAP1 is part of the cullin 3 (CUL3)-based E2 ubiquitin ligase complex and is responsible for controlling NRF2 stability and activation. The NRF2-KEAP1 pathway is regulated by a "thiol-driven master switch": in the presence of electrophiles and ROS, KEAP1 cysteine residues are modified, which prevents the ubiquitination of NRF2, leading to NRF2 translocation into the nucleus where it induces the expression of genes regulated by the antioxidant response element (ARE). We hypothesized that the reactive nitroso intermediate of 3-NBA can modify KEAP1 cysteine residues, leading to NRF2 activation and consequent induction of enzymes (AKRs and NQO1) responsible for 3-NBA activation increasing its toxicity via a positive feedback loop. **Methods:** The ability of 3-nitrosobenzanthrone (3-NBA) to form covalent bonds with cysteine residues was first assessed using N-acetyl-L-cysteine and glutathione. Recombinant KEAP1 protein was then incubated with 3-NBA. The protein was immunopurified, free cysteine residues were alkylated, and the protein complexes were digested with trypsin. Tryptic peptides were analyzed by nano-LC-MS/MS. Human bronchial epithelial cells HBEC3-KT cells (with wild-type KEAP1) were then exposed to 3-NBA, and the consequent modulation of KEAP1-NRF2 pathway was investigated by measuring post-translational modification (PTM) of KEAP1 by targeted proteomics by nano-LC-MS/MS, nuclear translocation of NRF2 by western-blot analysis, and induction of AKR1C transcripts by RT-qPCR. **Results:** Mass spectrometry analysis of recombinant KEAP1 demonstrated that 3-NBA forms sulfonamide and sulfonamide adducts with multiple key cysteine residues in both the CUL3- and NRF2-binding domains (e.g., Cys151, Cys273, Cys288, Cys319, Cys368, Cys489). Consistent with NRF2 activation, HBEC3-KT cells treated with 3-NBA showed evidence of PTM of KEAP1, NRF2 nuclear translocation, and induction of AKR1C1 gene and protein expression. **Conclusions:** This work demonstrates, for the first time, that a chemical carcinogen can covalently modify KEAP1. This modification activates NRF2, leading to the subsequent activation of ARE genes, such as AKRs, which supports the hypothesis that nitroarenes induce their own toxicity via a positive feedback loop involving KEAP1. These findings provide a novel mechanistic understanding of how NO₂-PAHs may exert their genotoxicity and highlight the NRF2-KEAP1 pathway as a critical molecular target influencing individual susceptibility to the carcinogenic effects of diesel exhaust exposure.

PS 4303 Myricetin and Capsaicin synergistically reduce the proliferation of human hepatoma cells by inhibiting Ki-67, PCNA, and HIF1A expression

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Background and Purpose: Phytochemicals are naturally occurring compounds that are commonly consumed in foods. Numerous studies have demonstrated that certain phytochemicals possess antitumoral properties, including the inhibition of cell proliferation, angiogenesis, and invasion of cancer cells. Myricetin (Myr), a

flavonoid that is found in berries, nuts, and herbs, shows antioxidant properties and causes cell cycle arrest. Capsaicin (Cap), a pungent-smelling alkaloid found in chili pepper, can decrease cell proliferation by triggering cell apoptosis. **Methods:** Human Hep3B and Huh-7 hepatoma cells, cultured in 96-well plates, were treated with Myr, Cap, Myr + Cap, or DMSO and then processed for MTT analysis to assess cell viability and cell proliferation. In addition, Hep3B and Huh-7 cells were cultured in T-25 flasks and treated with Myr, Cap, Myr + Cap, or DMSO for 48 hours (n = 4/treatment). The harvested cells were processed for RT-qPCR analysis of genes involved in regulating cell proliferation. **Results:** MTT assay showed that both Myr and Cap decreased the cell proliferation and viability in both Hep3B and Huh-7 cells. Co-treatment of Myr and Cap synergistically decreased the cell proliferation and viability in both Hep3B and Huh-7 cells. Additionally, both Myr and Cap decreased the mRNA expression of Ki-67, PCNA, and HIF1A in both Hep3B and Huh-7 cells. Co-treatment of Myr and Cap synergistically decreased the mRNA expression of Ki-67, PCNA, and HIF1A in both Hep3B and Huh-7 cells. **Conclusions:** The combination therapy of Myr and Cap may enhance their antitumoral properties.

PS 4304 Genetic Toxicology Studies on trans-1,2-difluoroethene (R-1132-E)

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Background and Purpose: A novel refrigerant gas, identified as R-1132-(E), trans-1,2-difluoroethene, has been extensively evaluated for its genotoxicity potential. A series of *in vitro* and *in vivo* assays have been used for this purpose. A significant mutagenic activity was observed in the bacterial mutagenicity test (Ames studies/OECD471), particularly in the absence of mammalian metabolic enzymes. In the presence of mammalian enzymes the activity in bacteria was markedly attenuated, especially at the exposure level lower than 50%. **Methods:** The potential for genotoxic activity was further investigated in a series of mammalian cell assays *in vitro* (Chromosome aberration test /OECD473, Micronucleus test/OECD487) and also in *in vivo* studies (Micronucleus test in the bone marrow of the rat/OECD474, The activity observed in bacteria and in the Comet study was investigated using the Big Blue® transgenic assay. The Big Blue® assay is a transgenic rodent (TGR) mutation assay, as described in Organization for Economic Co-Operation and Development (OECD) Test Guideline (TG) 488 (OECD 2022). **Results:** Comet assay in 4 tissues of the rat/OECD489). In all of these assays, R-1132-(E) was shown to be not genotoxic, i.e., not clastogenic, not aneugenic, and not mutagenic. R-1132-(E) was observed to induce an apparent increase in DNA damage in lung, kidney, bladder, and liver tissues, but only at dose levels that greatly exceeded the limit dose for the assay. In addition, the DNA damage was only seen in association with an increase in 'hedgheg cells', which were not observed in the tissues of the positive control group. The increase in DNA damage was concluded to be secondary to a separate mode of action (likely excessive cytotoxicity) and not due to direct DNA damage. Since the dose levels of TKN1 used in the comet study were excessively high (up to 8% of the atmosphere breathed by the animals), the possibility that anoxia may have contributed to the response is also considered possible. The purpose of the TGR gene mutation assay is to identify substances that cause mutations due to DNA damage in the tissue being analyzed. The three tissues (lung, kidney, and liver), which showed some evidence of apparent DNA damage in the Comet assay, and bone marrow were analyzed for mutations, and there was no increase in the mutation rate in any of the tissues. It was concluded that the apparent DNA damage in the Comet assay was not caused by a direct interaction with DNA and did not result in mutations. **Conclusions:** The set of genotoxicity data demonstrated that R-1132(E) is not mutagenic.

PS 4305 The Metabolo-Epigenetic Role of AS3MT in Arsenic Exposure

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Background and Purpose: Trivalent inorganic arsenic (iAs^{III}) is a complex epigenetic toxicant and the mechanism underlying its signature of genome-wide DNA hypomethylation remains poorly understood. We previously found that exposure to iAs^{III} within mESCs increased both total and nuclear levels of AS3MT (an arsenic methyltransferase). While AS3MT has been identified as a cytosolic protein, evidence suggests it may also localize to the nucleus and play functional roles in DNA cytosine methylation and histone modification. This project investigates the genomic distribution and mechanistic role of AS3MT within mESCs and human cancer cell lines by testing the hypothesis that nuclear AS3MT is a key mediator of iAs^{III} epigenetic and carcinogenic activity. **Methods:** We exposed human liver, skin, and lung cells to NaAsO₂ at the LOAEL (1 μM) for 48-h with media replenishment at 24-h to assess epigenetic alterations. Additionally, in a separate experiment, we exposed the same cell types to iAs^{III} and then allowed cells to recover for seven days post-exposure, after which samples were counted and collected for RNA sequencing. Trypan blue staining was used to measure cell proliferation across cell types. RNA-seq analysis revealed differentially expressed gene signatures. **Results:** iAs^{III} exposure resulted in significantly decreased cell proliferation without affecting cell viability after 48-h exposure across all cell types. iAs^{III} exposure resulted in distinct genetic signatures of DNA replication and repair machinery in the recovered samples across all cell types. **Conclusions:** This research demonstrates that although AS3MT was

not differentially expressed across all three cell types, DNA replication and repair machinery may be a critical pathway for exploration and future direction in elucidating the mechanistic role of iAs¹¹. Understanding the functional roles of critically impacted replication machinery may help elucidate how environmental toxicant exposures lead to epigenome signatures that perturb transcriptional processes, gene function, and carcinogenicity.

PS 4306 Reconsidering False Positive Rates for a Portfolio of Carcinogenicity Studies in Rats and Mice

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Background and Purpose: Hundreds of chemicals have been evaluated for their potential carcinogenicity via two-year rodent studies conducted by the Division of Translational Toxicology within The National Institute of Environmental Health Sciences. Each study includes two rodent species, two sexes, three or more dose groups, and over 40 tumor types. These tumor data are binary, with tumors being either present or absent. Trend and pairwise tests are used to test for the effect of a given chemical's dose on tumor formation. 485 dose-related tests are conducted per study, leading to potentially inflated false positive rates (FPR), or the likelihood that a statistical test would count a true negative as positive. While statistical significance is not the only consideration for declaring the carcinogenicity of a chemical, it is an important contributing factor. Previous work has examined FPRs in two-year rodent studies, but tumor background rates have shifted over time, and there are now scenarios where more sophisticated statistical models that correct for differential survival are utilized. **Methods:** We built statistical simulations to assess a study's FPR using the Poly-3 test, an extension of the Cochran-Armitage test that includes adjustments for differential survivability among the dose groups. These simulations used the most recent male and female historical control data from 9 chronic mouse studies with 929 unique control animals and 11 chronic rat studies with 1179 unique control animals to assist in computing the FPR of the Poly-3 test for a study. Studies were selected from the 2024 NTP Historical Control Database, with starting dates between 2011 and 2016. Ten thousand simulated datasets were generated per sex and species. Trend and pairwise tests were performed for each tumor type within each simulation dataset, with an alpha level of 0.05 for determining significance. The false positive rate for each species, sex, and tumor type was calculated as the number of significant tests divided by the total number of tests (i.e., 10,000). The expected number of false positive trend and pairwise tests within a study (combining sexes and tumor types) was calculated as the average number of false positives across the simulations. The background tumor rates present in the historical control data were also calculated. Note that there were more tumor types present in rats than mice. **Results:** Across the 10,000 simulations in rats, an average of 1.93 false positive trend tests per study were observed, with 0.85 false positive pairwise tests, ranging from 0 to 12 false positives out of the 260 tumor type and sex combinations tested. Across the 10,000 simulations in mice, an average of 1.57 false positive trend tests per study were observed, with 0.76 false positive pairwise tests, ranging from 0 to 8 false positives out of the 225 tumor type and sex combinations tested. As the background tumor rates increased up to 60%, the FPR increased but remained below the 0.05 level, indicating that the low incidence of tumors controls the FPR. **Conclusions:** This work seeks to emphasize the real-world impact of statistical modeling and enhance confidence in science. In any given carcinogenicity study using the Poly3 test, we would not expect to see more than 2 false positive trend tests or 1 false positive pairwise test. The number of significant tests in some studies has been an order of magnitude greater than these counts, reliably suggesting a true effect compared to the false positive baseline expectations calculated here. This research was supported in part by the NIH, National Institute of Environmental Health Sciences and the Intramural Research Program of the NIH including 75N96022F00055. The contributions of the NIH authors are considered Works of the United States Government. The findings and conclusions presented in this poster are those of the authors and do not necessarily reflect the views of the NIH of the U.S. Department of Health and Human Services.

PS 4307 Getting the big picture of data-rich chemicals: Survey sampling increased efficiency in mapping mechanistic data for polycyclic aromatic hydrocarbons

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Background and Purpose: Mechanistic information is critical for human health hazard evaluation. However, systematic organization of diverse mechanistic information can be resource-prohibitive for data-rich chemicals. Our objective was to organize mechanistic information on polycyclic aromatic hydrocarbons (PAHs) by key characteristics of carcinogens (KCCs) into systematic evidence maps (SEMs) to support cancer hazard evaluations, and to develop methods to increase efficiency

while maintaining accuracy. **Methods:** A literature search for cancer mechanistic data for 45 PAHs with cancer data from animal models was conducted in PubMed, Scopus, and Web of Science using the National Toxicology Program's Report on Carcinogens search syntax for mechanism, mainly KCCs. To effectively identify PAHs and categorize KCCs mentioned in the abstract, we first compared text mining with manual identification of PAHs and KCCs categorization. We divided KCC8 receptor-mediated effects into 8a aryl hydrocarbon receptor (AhR)-related and 8b non-AhR-related. To estimate numbers of references for each PAH-KCC pair without screening every single reference found, we used a survey sampling and statistical analysis approach to predict KCC distribution for each PAH. Furthermore, to validate the performance of this approach, we compared the predicted number of references per KCC per PAH with numbers from manual KCC categorization for five PAHs. To develop SEMs of mechanistic information for PAHs that have animal cancer data from human-relevant exposure routes, we identified 14 priority PAHs and applied the best-performing methods from our tests. **Results:** Nearly 44,000 references were returned from searches conducted through October 2025 for 45 PAHs. PAH identification within the references was more accurate by text mining than by manual review, while KCC categorization was more accurate by manual review. We proceeded with text mining PAHs and manual categorization of KCCs for the rest of the project. The survey sampling method validation demonstrated that predictions of the distribution of references across KCCs per PAH (i.e., number of references per PAH) closely aligned with the results of manual categorization, indicating survey sampling can accurately predict the KCC distribution. We then applied survey sampling methods to predict KCC categorization for data-rich PAHs (i.e., those with 500 or more references). For the SEMs of 14 priority PAHs, over 16,000 references were identified by text mining as containing information relevant to one or more the PAHs and we manually screened nearly 7,000 references for KCC categorization. Three PAHs (benzo[a]pyrene, fluoranthene, and naphthalene) had over 500 references each; thus, the number of references with specific KCC data per PAH was predicted based on survey sampling of the manual categorization results. When all 14 priority PAHs are considered, KCCs 1 (electrophilicity, including DNA adducts), 2 (genotoxicity), 8a (AhR-related), and 5 (oxidative stress) had more references per KCC than any other KCCs. The predictions and the manual screening results are shown in detail in an online, interactive SEM (<https://tinyurl.com/cdatrv33>), where users can explore the data (e.g., sort by chemical structure features). **Conclusions:** Evaluating the performance of new methods helps build confidence in approaches that differ from standard procedures. We recommend chemical identification by text mining coupled with manual categorization of mechanistic information, and we recommend survey sampling prediction for large datasets for appreciable time savings. Our development, evaluation, and validation of approaches for literature categorization by PAHs and KCCs demonstrates how text mining and survey sampling in toxicological topic areas with specific characteristics, such as cancer mechanistic data, can expedite the organization of voluminous mechanistic information into an SEM.

PS 4308 Conserved Alternative Splicing Events Underlie Spontaneous and Chemically Induced Hepatocellular Carcinomas in B6C3F1/N mice

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Background and Purpose: Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related mortality worldwide. In addition to viral etiologies, lifestyle and environmental exposures contribute to hepatocarcinogenesis. In National Toxicology Program (NTP) studies, spontaneous and chemically induced HCCs arise frequently in B6C3F1/N mice and display several morphological and genetic similarities to some human HCCs. Prior genomic analyses indicate that these mouse HCCs share broadly similar mutation profiles. However, the influence of chronic chemical exposure on alternative splicing (AS), a key driver of proteomic diversity, tumor-specific isoform production, and neoantigen formation, in the context of hepatocarcinogenesis remains unclear. Aberrant AS can disrupt apoptosis, cell-cycle control, metabolic homeostasis and stress-response pathways. We hypothesized that AS events may reflect distinct carcinogenic modes of action and reveal molecular features shared across, or unique to, spontaneous and chemically induced HCCs. **Methods:** RNA-seq was performed on HCCs from B6C3F1/N mice arising spontaneously or following one of ten chronic chemical exposures (four genotoxic and six nongenotoxic agents), with age-matched untreated livers serving as controls. Reads were aligned to the mm10 genome using STAR 2.6.0c. Differential AS events were identified using rMATS turbo ($p < 0.05$, $FDR < 0.05$, $|\Delta\text{IncLevel}| > 0.1$) across five event types: skipped exon (SE), mutually exclusive exons (MXE), alternative 5'/3' splice sites (A5SS/A3SS), and retained intron (RI). Visualization and downstream splicing events were performed using rmats2sashimiplot v2.0.2. Enriched RNA-binding protein (RBP) motifs flanking significant AS events were detected using rMATS2 v2.2.0. Differential gene expression was integrated with AS profiles to identify genes undergoing coordinated dysregulation at both levels. Functional implications were assessed using Gene Ontology Biological Processes and Reactome pathway annotations. **Results:** Across treatment groups, tumors exhibited 604-3535 significant AS events (total 13,779 junction + exon counts), demonstrating extensive splicing dysregulation in both spontaneous and chemically induced HCCs. SE and MXE events predominated across all HCCs representing 28-54% and 27-63%, respectively. HCCs from BDCA and DBP exposures showed high MXE proportions, suggesting chemical-specific splice perturbation. Spontaneous HCCs had 39 unique AS-affected genes. Despite this

heterogeneity, a conserved core of 21 SE, 13 MXE, 6 RI and two A5SS/A3SS events (*Arhgap26*, *Dleu2*) events was shared across all HCCs, indicating convergence at the splicing level despite differences in carcinogen class and mode of action. Integration of AS with expression data showed that 11-33% of AS genes were also differentially expressed. Seven genes (*Cep55*, *Dst*, *Ndr2*, *Sgms1*, *Sort1*, *Tnfrsf13*, and *Tpm1*) were consistently dysregulated at both AS and expression levels in every tumor group, highlighting coordinated transcriptional and post-transcriptional remodeling. RBP motif analysis revealed distinct regulatory patterns: SRP20 and RBM proteins in SE events; HuR (ELAVL1) enrichment in MXE events; and HuR, RBM, SRSF, HNRNPCL, and PTBP1 motifs enriched in RI events. Functional analyses showed enrichment of pathways involved in mRNA and nucleic acid metabolism, protein/macromolecule regulation, cellular stress and damage response, and signaling. **Conclusions:** Spontaneous and chemically induced mouse HCCs share a recurrent program of alternative splicing dysregulation, constituting a core molecular hallmark of hepatocarcinogenesis in B6C3F1/N mice. The presence of highly conserved SE and MXE events across all tumors, coupled with consistent dual dysregulation of key genes such as the tumor-suppressor *Ndr2*, suggests that AS influences pathways governing proliferation, cellular stress tolerance, and oncogenic signaling. The AS events in 39 unique genes in spontaneous HCCs may reflect endogenous processes and warrant further validation. Overall, these data suggest that AS may be an important regulatory layer in liver tumor development and support future functional studies to define the mechanistic consequences of specific AS events and their potential value as biomarkers of chemical hepatocarcinogenesis.

PS 4309 Putative lncRNA-miRNA-mRNA Interactions Distinguish Spontaneous and Chemically Induced Mouse Hepatocellular Carcinomas

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Background and Purpose: Hepatocellular carcinomas (HCCs) in B6C3F1/N mice are a frequent endpoint in the National Toxicology Program (NTP) cancer bioassays. Recently, we have shown that HCCs arising due to aging (spontaneously) or due to chronic chemical exposure showed comparable genomic alterations. However, the role of non-coding RNAs (ncRNAs) and competitive endogenous RNA (ceRNA) relationships in shaping etiology-specific tumor biology remains incompletely understood. To address this gap, we have performed RNA-seq and miRNA-seq on B6C3F1/N mouse HCCs arising spontaneously due to age or due to chronic repeated dose exposures to potential human carcinogens. **Methods:** RNA-seq and miRNA-seq were performed on HCCs from B6C3F1/N mice arising spontaneously or due to chronic exposures to one of ten chemicals with diverse modes of action (four genotoxic and six non-genotoxic chemicals), and on age-matched control livers. Differentially expressed lncRNAs, miRNAs, and mRNAs between the untreated control livers and the HCC groups were identified using the DESeq2 R package ($|\log_2FC| > 1$, FDR < 0.05). The miRNA-mRNA functional analyses and pathway enrichment was performed using Qiagen Ingenuity Pathway Analysis (IPA) platform. **Results:** Across chemical-induced HCCs from male mice, 356-1042 lncRNAs, 129-205 miRNAs, and 1622-4060 mRNAs were differentially expressed. In comparison, 731 and 939 lncRNAs, 168 and 205 miRNAs, and 2888 and 3177 mRNAs were differentially expressed respectively in female and male spontaneous HCCs. Two miRNAs (*mmu-miR-879-5p* and *-703*) were upregulated and six miRNAs (*mmu-miR-30c-1-3p*, *-467c-5p*, *-3962*, *-467d-5p*, *-467e-5p*, *-592-5p*) were downregulated across multiple chemical-induced HCCs (n5), mainly BCA, BDCA, GBE, OZ, PCTFT and PM, suggesting chemical-specific miRNA deregulation. Thirty-nine target mRNAs of these DE miRNAs (mainly *mmu-miR-30c-1-3p* and *-212-3p*) were specific to chemically induced HCCs. Despite chemical-specific patterns, 49 miRNAs were upregulated and six miRNAs (*mmu-miR-12195-3p*, *-137-3p*, *-193a-3p*, *-215-5p*, *-455-3p* and *-455-5p*) were downregulated across all HCC groups regardless of exposure. Twenty-two target mRNAs of the DE miRNAs in both spontaneous and chemically induced HCCs were identified. Literature-based analyses identified putative lncRNA-miRNA-mRNA ceRNA relationships, including *NEAT1-mmu-miR-193a-3p-CCND1/MYC/N/TGFB2*, *XIST-mmu-miR-133a-3p-ELF3/PAX7/SOX4*, and *XIST-mmu-miR-137-3p-EPGN*, which were observed in chemically induced but not in spontaneous HCCs. Pathway enrichment suggested that genes within these networks may intersect with AMPK, ERK/MAPK, AKT, Activin/Inhibin, and WNT/ β -catenin signaling pathways, all of which influence hepatocyte growth and metabolic regulation. In contrast, a putative *PVT1-mmu-miR-455-5p-SOX11* axis was detected across all HCCs regardless of the etiology and may signal through the WNT/ β -catenin pathway. **Conclusions:** These findings provide a comprehensive characterization of lncRNA, miRNA, and mRNA dysregulation in spontaneous and chemically induced mouse HCCs, highlighting candidate ceRNA interactions and signaling pathways for further exploration especially for the different exposures examined in this study. The identified ncRNA networks and conserved signaling pathways offer new mechanistic leads and potential biomarkers of chemically induced hepatocarcinogenesis with translational relevance to human disease, and a framework for future validation studies.

PS 4310 Methylation and Transcriptomic Alterations Drive Tumor Promotion Pathways in Chemically Induced Mouse Hepatocellular Carcinomas

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Background and Purpose: Hepatocellular carcinoma (HCC), the third leading cause of cancer mortality worldwide, arises from chronic etiologies including HBV/HCV infection, alcohol misuse, aflatoxin B1 exposure, and lifestyle-associated metabolic dysfunction. The B6C3F1/N mouse, widely used in the National Toxicology Program (NTP) carcinogenicity studies, develops HCCs spontaneously with aging and at higher incidences following chronic chemical exposures. Mouse HCCs share important morphologic and genetic features with subsets of human HCC. Prior work demonstrated that spontaneous and chemically induced mouse HCCs display broadly similar genomic alterations. Here, we hypothesized that chemically induced tumors harbor additional transcriptional and epigenetic dysregulation that distinguishes them from spontaneous HCCs. **Methods:** We analyzed HCCs arising spontaneously or following exposure to one of ten chemicals (4 genotoxic and 6 non-genotoxic) with diverse modes of action. DNA methylation profiling was performed using the Infinium MouseMethylation-12 v1.0 BeadChip, enabling high-resolution assessment of CpG methylation across promoter regions, and gene bodies. RNA sequencing of total RNA was conducted using Ribo-Zero depletion to capture both coding and noncoding transcripts. Differential gene expression analysis was carried out using standard pipelines with thresholds of $p < 0.05$ and $|\log_2FC| > 1.5$ to identify robust transcriptional changes. Methylation alterations were quantified in relation to expressed genes, allowing integration of epigenetic and transcriptomic signals. Functional pathway enrichment analyses were performed using gene sets derived from both DEG and methylation profiles. **Results:** Chemical-induced HCCs exhibited 1820-4585 DEGs, whereas spontaneous male HCCs showed 3789 DEGs. Across all tumors, 39-53% of DEGs also demonstrated altered methylation status, predominantly hypomethylation. Common pathways shared across spontaneous and chemically induced HCCs included FAK-mediated signaling via L1CAM interactions, S100 family signaling, RAF-independent MAPK1/3 signaling, and cell cycle regulatory pathways, indicating conserved oncogenic signaling in murine hepatocarcinogenesis. HCCs from chemical exposures displayed additional transcriptional and epigenetic alterations involving HIF1 α signaling, MSP-RON signaling, and cytokine-mediated pathways linked to IL-4, IL-13, IL-17A, and IL-10. These immune-modulatory changes promoted downstream activation of Wnt/ β -catenin and NF- κ B signaling, suggesting remodeling of the tumor immune microenvironment. Methylation profiling revealed epigenetic alterations in genes associated with alterations in receptor-type tyrosine-protein phosphatases, matrix metalloproteases, and RAF-independent MAPK1/3 signaling across HCCs regardless of the etiology. **Conclusions:** Spontaneous and chemically induced HCCs share activation of conserved oncogenic signaling pathways; however, chemical exposures induce additional transcriptional and epigenetic dysregulation involving hypoxia response pathways, cytokine signaling, and immune remodeling. HCCs from chemical exposures show additional transcriptional and epigenetic alterations that reshape the immune landscape to promote carcinogenesis. These integrated molecular profiles highlight convergent and exposure-specific mechanisms of hepatocarcinogenesis in the B6C3F1/N mouse and may support identification of biomarkers useful for evaluating chemical-induced liver cancer risk.

PS 4311 Mode of Action Analysis of the Tumor Triad Induced by DEHP

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Background and Purpose: The US Environmental Protection Agency's (EPA) Office of Pollution Prevention and Toxics is conducting a risk evaluation for di-ethylhexyl phthalate (DEHP), including a cancer hazard assessment. DEHP is a known activator of peroxisome proliferator-activated receptor alpha (PPAR α). PPAR α activators, including DEHP, are known to induce hepatocellular adenomas and/or carcinomas in rats and mice, as well as pancreatic acinar cell tumors (PACTs) and testicular Leydig cell tumors in rats (often referred to as the "tumor triad" in rats). A postulated mode of action (MOA) for liver tumors involving PPAR α activation has been experimentally supported and discussed by previous existing assessments of DEHP. However, a refined DEHP cancer analysis which also experimentally supports an MOA for PACTs and Leydig cell tumors has not been conducted. Thus, in conducting its DEHP risk evaluation and determining the proper cancer hazard assessment and dose-response approach (i.e., linear low-dose extrapolation vs. threshold approach), EPA performed a cancer MOA analysis of the tumor triad consistent with the 2005 EPA Guidelines for Carcinogenic Risk Assessment. **Methods:** In conducting its MOA analysis of the DEHP tumor triad, EPA considered available epidemiological, animal toxicology, and mechanistic evidence, including published reviews by Corton et al. (2018, 2014) and Klaunig et al. (2003). Further, to address uncertainty in precise mechanisms of DEHP-induced PACTs and Leydig cell tumors with less experimental and direct MOA evidence, EPA considered epidemiological and animal experimental evidence on prototypical PPAR α activators (e.g., WY 14,643) and hypolipidemic drugs (e.g., clofibrate, fenofibrate, gemfibrozil) that mechanistically lower serum lipid levels through activation of PPAR α . Data for these PPAR α activators was used to support inferences for DEHP-induced liver, pancreatic, and testicular tumors that

share postulated mechanistic initiating events. **Results:** Available evidence indicated that DEHP is not directly mutagenic or genotoxic. Experimental studies of mice and rats, as well as mechanistic data, support the conclusion that DEHP-related hepatocellular adenomas and carcinomas in rodents arise through a PPAR α MOA. As a novel approach, EPA included evidence from hypolipidemic drugs and other prototypical PPAR α activators to support inferences. Data for these hypolipidemic drugs and prototypical PPAR α activators demonstrate that these chemicals induce the tumor triad in rats, but not in humans when prescribed at doses several orders of magnitude higher than levels of exposure to DEHP for the general U.S. population based on NHANES biomonitoring data. Based on inferences from hypolipidemic drugs, EPA concluded that PACTs, Leydig cell tumors, and liver tumors are related to PPAR α activation following chronic DEHP exposure. **Conclusions:** Overall, review of available evidence indicated, in addition to hepatocellular tumors, PACTs and Leydig cell tumors in rats are related to PPAR α activation following chronic exposure to DEHP and hypolipidemic drugs. Although some uncertainties remain, there is strong evidence to support the postulated, non-genotoxic, PPAR α MOA inclusive of the tumor triad, indicating a non-linear threshold approach is appropriate in evaluating DEHP cancer risk. Under the EPA Guidelines for Carcinogen Risk Assessment, EPA determined that DEHP is "Not Likely to be Carcinogenic to Humans" at doses below levels that do not result in PPAR α activation. In consideration of this analysis with the overall DEHP human hazard assessment, EPA determined conducting quantitative dose-response assessment of tumor outcomes were not appropriate considering the identified non-cancer point of departure based on effects on the developing male reproductive system consistent with phthalate syndrome is expected to adequately account for all chronic toxicity, including carcinogenicity. The views expressed in this abstract are those of the authors and do not necessarily represent the views or policies of US EPA.

PS 4311a A Multi-Lab Single-Cell and interpretable AI Investigation of Pesticide NGTxC Mechanisms in 3D Lung Models

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Background and Purpose: Non-genotoxic carcinogens (NGTxC) are challenging to identify because they do not induce direct DNA damage but act through progressive alterations in gene regulation, epithelial identity, and tissue homeostasis. The ImmunoSearch-led NewgenTOXiv consortium (3 academic laboratories, ImmunoSearch Labs, and AI partner NukkAI) aims to define early mechanistic signatures capable of distinguishing NGTxC from structurally related non-carcinogens across multiple chemical families. Here, we present the first results obtained on a set of pesticides and highlight the broader applicability of the approach to additional NGTxC compound classes. **Methods:** Human 3D airway tissues (Epithelix MucilAir™) were exposed to three NGTxC pesticides (Aldrin, Dieldrin, p,p'-DDT) and two non-carcinogenic analogs (Endrin, Methoxychlor) using two exposure paradigms: (1) acute, consisting of a single non-cytotoxic topical or systemic dose followed by single-cell transcriptomic analysis at 6 hours; and (2) chronic, involving repeated low-dose exposures for eight weeks with longitudinal sampling. Transcriptomic datasets were analyzed using regulatory network reconstruction and NukkAI's interpretable AI pipeline. Their approach combined an autoencoder to learn low-dimensional latent representations of gene expression, jointly trained with a classifier to separate NGTxC from non-carcinogens. An interpretable classifier was subsequently applied to the latent space to identify the most discriminative regions and extract pathway-level drivers for mechanistic interpretation. **Results:** Across independent analytical strategies, NGTxC pesticides consistently induced a convergent set of pathway-level perturbations absent in structurally related non-carcinogens. Acute exposures triggered disruption of epithelial differentiation, marked loss of ciliary organization, activation of stress-response and pro-inflammatory pathways, and selective engagement of pro-survival and repair programs associated with epithelial plasticity. Chronic exposures amplified these early signatures, revealing progressive activation of regeneration-linked proliferative circuits consistent with early non-genotoxic carcinogenic processes. Importantly, the same analytical pipeline applied to additional NGTxC compound families revealed comparable early mechanistic patterns, demonstrating that the approach is not pesticide-specific but captures cross-chemical NGTxC biology. **Conclusions:** Combining 3D human lung models, single-cell genomics, and interpretable AI provides a robust multi-laboratory strategy for mechanistic discrimination of NGTxC carcinogens. This study offers the first single-cell-resolved, AI-supported map of pesticide-induced NGTxC activity in human airway tissue and demonstrates that the same analytical framework successfully identifies comparable early NGTxC signatures in other chemical classes. These findings support the development of mechanism-based, cross-chemical screening strategies for NGTxC hazard identification in regulatory toxicology.

PS 4312 DNA Damage Evaluation of Benzo[b]Fluoranthene (B[b]F) Using Rat Liver Comet Assay

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Background and Purpose: Benzo[b]fluoranthene (B[b]F) is a polycyclic aromatic hydrocarbon (PAH) produced from the incomplete combustion of organic materials, such as fossil fuels and tobacco. While B[b]F has been shown to be genotoxic in various *in vitro* assays (e.g., Ames test), its *in vivo* genotoxicity and carcinogenicity properties are less well characterized compared to other PAHs such as benzo[a]pyrene. **Methods:** In this study, the genotoxic potential of B[b]F was investigated using rat liver Comet assay. Male Sprague-Dawley rats (N=5) were orally administered B[b]F at 0, 50, 100, and 200 mg/kg/day for 3 consecutive days. Ethyl Methanesulfonate at dose level of 150 mg/kg/day was used as positive control. Animals were euthanized 3-4 hours after the 3rd dose and left lateral liver lobe was collected to conduct Comet assay the same day with fresh liver tissues. **Results:** B[b]F did not induce any adverse clinical signs or decreases in body weight or food consumption over 3 days of dosing. Percent tail intensity (%TI) was used as measure of DNA damage by scoring 150 cells (75/slide) per animal. Group and individual median %TI in vehicle control animals was <6%. No increase in the median %TI was observed in the low (4.75%) and mid (4.54%) dose groups. Median %TI (66.95%) in the high dose group was statistically significant compared to the concurrent control. Positive control %TI (64.89%) was statistically significant and within the positive control historical control range. **Conclusions:** The results indicate that the B[b]F has the potential to cause DNA damage at higher but clinically non-toxic dose levels.

PS 4313 Evaluating the Applicability of the ICH M7 Less Than Lifetime Concept to N-Nitrosomorpholine Using Error-Corrected next-generation sequencing

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Background and Purpose: The ICH M7 guideline introduced the "less than lifetime" (LTL) concept for assessing mutagenic impurities, proposing that cumulative exposure over a shorter duration may present a comparable potential cancer risk to lower-dose lifetime exposure. This framework permits higher daily intake limits for mutagenic impurities under short-term exposure scenarios while maintaining equivalent risk levels. Nitrosamines, classified under the "Cohort of Concern" in ICH M7 due to the potent carcinogenicity of certain compounds in animal studies, present a regulatory challenge when applying LTL adjustments. **Methods:** This study investigates the applicability of the LTL approach to nitrosamine using rodents exposed to N-Nitrosomorpholine (NMOR) at various dose levels for 3, 10, or 28 days. Mutagenicity was assessed using two different error-corrected next-generation sequencing (ecNGS) platforms: Duplex Sequencing (DS), a targeted method, and SMM-seq, a non-targeted approach. These technologies enable ultra-accurate detection of endogenous mutations across species. **Results:** The results demonstrated a linear relationship between genotoxicity and total exposure, consistent with Haber's law, suggesting that total exposure may serve as a valid metric for NMOR-induced mutagenicity. Both ecNGS platforms produced comparable mutation profiles, reinforcing the robustness of the analytical methods. **Conclusions:** These findings offer critical insights into the risk assessment of nitrosamines under LTL exposure scenarios and may inform future regulatory considerations regarding the application of the LTL concept to this class of compounds and adoption of ecNGS methods in the genetic toxicology field.

PS 4314 Investigation of the Effects of Dimethyl Sulfoxide on the *in vivo* Micronucleus Test in Rats

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Background and Purpose: In order to assess the safety of a drug candidate compound under development, a high systemic exposure level corresponding to the maximum tolerated dose in animals is generally required in the non-clinical toxicity studies. However, we often encounter difficulties obtaining higher systemic exposures despite investigating various dosing formulations. Therefore, identifying a vehicle applicable to toxicity studies that has high solubility and is well-tolerated and which could help to obtain high systemic exposures would be of considerable benefit for safety assessment. Dimethyl sulfoxide (DMSO) is widely used as a solvent in *in vitro* toxicity studies, but data on its role as a vehicle in *in vivo* micronucleus tests in rats is limited. The purpose of this study is to investigate whether oral or intravenous administration of dimethyl sulfoxide could be applicable for a rat micronucleus test. **Methods:** DMSO was administered orally at the dose levels of 5 and 10 mL/kg or intravenously at 1 mL/kg to non-fasted male Sprague-Dawley rats (8 weeks of age, 5-9 animals/group) for 2 days. The animals in the negative control group were given saline in the same manner. The animals in the positive control group (5 animals) received a single intraperitoneal injection of 50 mg/kg cyclophosphamide. During the dosing period, clinical observations and measurements of the body weights and

food consumption were conducted. Bone marrow smears were prepared approximately 18 to 24 hours after the last dose. The frequency of micronucleated polychromatic erythrocytes (MNPCEs) in the bone marrow smear was examined under the microscope and the proportions of reticulocyte (RETs) to erythrocytes in the bone marrow smears, a marker of bone marrow toxicity, were also determined. **Results:** The following changes were observed in the group intravenously administered DMSO: decreased locomotor activity immediately after dosing on Day 2 in 1/9 animal, reddish urine in 8/9 animals on Day 1 and 9/9 animals on Day 2. No DMSO treatment-related findings in the clinical observations were observed in the group administered DMSO orally. In both administration route groups, there were no DMSO treatment-related findings in the body weights and food consumption. DMSO did not increase the frequency of MNPCEs and there were no treatment-related changes in the proportion of RETs in both administration route groups when compared with the negative control group. In the positive control group, the frequency of MNPCEs was statistically significantly higher than that in the negative control group. **Conclusions:** DMSO is considered to be a useful vehicle for rat bone marrow micronucleus tests at doses up to 10 mL/kg for oral administration and up to 1 mL/kg for intravenous administration.

PS 4315 Optimization of S9 metabolism protocols for *in vitro* genotoxicity testing

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Background and Purpose: S9 liver fractions (S9) from rat, hamsters or humans are used in *in vitro* assays to mimic the metabolism in the liver. This metabolism can increase or decrease the DNA reactivity of a substance, resulting in different responses in absence and presence of S9. For the bacterial or mammalian cell genotoxicity assays with OECD Test Guidelines, typically cells are exposed for 24-30h in absence of S9 and 3-4h in presence of S9 mix. Cytotoxicity of S9 often limits exposure time and S9 concentration that are tolerated in the *in vitro* assays. New Approach Methodologies (NAMs) that investigate multiple direct and indirect genotoxic modes-of-action, would benefit from tailored S9 metabolism protocols. In addition, some classes of chemical, e.g. N-nitrosamines, required dedicated *in vitro* metabolism protocols to investigate their genotoxicity. Recently an enhanced S9 protocol for the Ames test (EAT) was developed. In this study, we explored various modifications to the standard *in vitro* metabolism protocol based on S9 liver fraction from phenobarbital/ 5,6-benzoflavone-exposed rats and hamster. **Methods:** To investigate optimizations of S9 metabolism protocol, the mammalian cell-based GFP reporter assay ToxTracker was used. This NAM includes six different fluorescent reporter genes for direct DNA damage as well as indirect genotoxicity related to oxidative stress, protein unfolding and cytotoxicity. Differential activation of the reporter genes was assessed using flow cytometry. Different concentrations of S9 extract, different co-factor mixes, exposure times and S9 from different species were explored. To test the optimized S9 metabolism protocols, a selection of 15 reference chemicals, including NDMA, Aflatoxin B1, Cyclophosphamide and Benzo[a]pyrene, was applied to compare different S9 exposure protocols. **Results:** We first determined the maximum concentration of rat S9 mix that would be tolerated by the ToxTracker cells for 4h and 24h exposure. Importantly, the maximum S9 concentration did not induce cytotoxicity nor activated any of the (geno)toxicity reporters in ToxTracker. Decreasing the S9 mix concentration from 1% to 0.4% allowed for 24h continuous exposure. Next, we compared the metabolic effectiveness of the short and long exposure protocol for 15 pro-genotoxic compounds. No significant difference between the two exposure protocols was observed for direct DNA reactive genotoxins. However, indirect genotoxicity related to oxidative stress after S9 metabolism for benzo[a]pyrene was only observed in the 24h 0.4% S9 protocol. For the *in vitro* genotoxicity assessment of N-nitrosamines, S9 mixes from rat and hamster liver extracts were compared. Hamster S9 was more efficient in metabolism of NDMA. To improve metabolic activation, the hamster S9 concentration was increased to 10% and exposure time reduced to 30 minutes, followed by 4 h exposure at 3% S9. The addition of calcium chloride to the hamster S9 mix further improved the metabolic activity. This enhances metabolism protocol was successfully validated with 7 genotoxic N-nitrosamines, including NDMA, NDEA, NDELA and NF. **Conclusions:** By reducing the S9 concentration and extending exposure to 24h, both direct and indirect genotoxic effects of compounds could be detected in ToxTracker. Metabolism of 15 pro-genotoxins was comparable using a 3h and 24h exposure protocol. For genotoxicity assessment of N-nitrosamines, a dedicated hamster S9-based metabolism protocol was developed.

PS 4316 Repair Assisted Damage Detection (RADD), a versatile tool for measuring DNA damage across species and damage types

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Background and Purpose: Numerous DNA damaging events occur daily from the environments we live in, the lifestyles we lead, and through our normal metabolic processes. Damaging events that directly modify DNA bases have been linked to mutagenesis and carcinogenesis. There is a substantial need to characterize DNA damage events and their repair to understand how they contribute to disease etiol-

ogy. Current methods to characterize and quantify the formation of DNA adducts have significant drawbacks because they often require a substantial amount of biological sample, highly specialized expertise and equipment, and depending on technique, may be limited to the detection and quantification of only a handful of DNA adducts at a time. There is a pressing need for high-throughput, easy-to-use, fluorescence-based methodologies for the measurement of a broad spectrum of DNA lesions across biological samples. We have validated the Repair Assisted Damage Detection (RADD) assay to address this need. **Methods:** RADD utilizes a DNA damage processing repair enzyme cocktail to detect and modify sites of DNA damage for a subsequent gap filling reaction that labels the DNA damage sites. This ability to detect and label a broad spectrum of DNA lesions within cells offers a novel and easy-to-use tool for assessing levels of DNA damage in cells. **Results:** Here, we present several applications of the RADD methodology to map DNA damage at promoter sites, identify DNA lesions from emerging environmental chemicals, examine DNA repair defects, and understand environmental stressors and their impact on genomic stability across different species, i.e., mice, rats, zebra finches, and *Drosophila*. Using RADD, we have identified unique DNA lesion signatures for the e-cigarette component, dihydroxyacetone, and monitored the induction of DNA lesions from acute, sub-acute, and chronic exposures. We also identified fluctuations in DNA damage across a population of zebra finches that experiences different stressors. Finally, we identified novel DNA damage induction and DNA repair changes induced in mice that were fed a high carbohydrate and fat diet. **Conclusions:** These applications demonstrate that RADD offers researchers a unique tool for investigating DNA adducts and DNA repair across biological specimens that may provide new insight into DNA repair defects and DNA damage induction, particularly for non-genotoxic chemicals or complex exposures.

PS 4317 Regulatory-Compliant Genetic Toxicology Assessment for Tides

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Background and Purpose: Peptide and oligonucleotide therapeutics, commonly grouped as TIDES, are two of the fastest-growing and most promising modalities within the pharmaceutical industry. Oligonucleotides (ONs), comprising single or double strands of RNA or DNA, act via mechanisms such as antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), microRNAs (miRNAs), small activating RNAs (saRNAs), and aptamers. Peptides mean any polymer that are composed of 40 or fewer amino acids. With the unique properties, Tides have emerged as a prominent force in innovative drug development, to better understand the genotoxicity of Tides, we conducted a retrospective analysis of genotox data for both FDA-approved Tides and the Tides previously evaluated in our laboratory. **Methods:** No single assay is able to detect all types of DNA damage, a test battery has been adopted to evaluate the genotoxic liability of novel pharmaceuticals. ICH S2(R1) guidance outlines appropriate *in vitro* and *in vivo* tests. The guidance provides two options for the standard genetic toxicology battery that includes an assessment of mutagenicity in a bacterial reverse mutation assay and genotoxicity evaluation in mammalian cells using *in vitro* and/or *in vivo* tests. Genotoxicity data of Tides that were either approved for marketing by the FDA between 2016 and 2025, or evaluated at WuXi AppTec (Suzhou) Co., Ltd. between 2018 and 2025, were collected for this retrospective analysis. **Results:** Percentage of different classes of novel therapeutics approved by the FDA in the span of 2016-2025 of which Tides attribute for 11.2%. A total of 20 Oligonucleotide drug products that includes 1 aptamer, 11 antisense oligonucleotides (ASOs), 7 small interfering ribonucleic acids (siRNAs), and 1 mixture of single-stranded and double-stranded polydeoxyribonucleotides have been identified. All oligonucleotide drug products were evaluated for genotoxicity and the results are all negative. A total of 33 peptides that includes 9 Peptide-Drug Conjugates (PDCs); 19 oligopeptides (2-9 amino acids); 1 Polypeptide (PP), 32 of them are synthetic. 24/33 (73%) were evaluated for Ames: 1 positive, 2 false positive (due to feeding effect), others are negative. 23/33 (70%) were evaluated *in vitro* mammalian cell test, 16 run for chromosomal aberration assay (CAA), others are for MLA/micronucleus assay (MNT), and they are all negative. 22/33 (67%) were evaluated for *in vivo* micronucleus (12 in rats and 10 in mice), and they are all negative. From 2018 to 2025, there are 19 peptides and 35 oligonucleotides tested in our laboratory. For the 19 peptides, 12/19 (63%) were evaluated for Ames; 15/19 (79%) were evaluated in CAA, and 11/19 (58%) were evaluated for *in vivo* micronucleus, they are all negative. For the 35 oligonucleotides, 35/35 (100%) were evaluated for Ames and CAA, 31/35 (89%) were evaluated for *in vivo* micronucleus, 1 equivocal (WoE was used for evaluating the marginal increase), 2 false positives (one positive is contributed by the excipient, and the other is impacted by the binding between drug product and fluorescent dye), the others are negative. **Conclusions:** Consistent with reports in the literature, the Oligonucleotide Safety Working Group's genotoxicity testing subcommittee believes that therapeutic oligonucleotides are unlikely to be genotoxic and peptides are large with poor membrane permeability and are not expected to reach the cytoplasm or nucleus, thus they would not interact directly with DNA or the mitotic apparatus, and their degradation releases endogenous amino acids which should pose no genotoxic risk. In summary, most Tides have undergone genetic toxicology testing, and the results have generally been negative.

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Background and Purpose: Glyphosate-based herbicide formulations (GBFs) are used world-wide to control unwanted vegetation. Genotoxicity testing is a key part of their safety evaluation, addressing potential for risks of heritable mutations and genotoxic mechanisms of carcinogenesis. Comprehensive science-based regulatory reviews have consistently concluded that approved uses of Bayer GBFs do not present a human health concern. These evaluations include consideration of numerous regulatory and published studies on genotoxicity of diverse GBFs. Many regulatory genotoxicity studies of Bayer GBFs have not previously been published; their experimental details and results are summarized here. **Methods:** Regulatory studies included *in vivo* mammalian (mouse) bone marrow micronucleus, *in vitro* human lymphocyte cytokinesis block micronucleus, and bacterial reverse mutation studies, which were conducted under good laboratory practices (GLPs) and according to international test guidelines that were applicable at the time of the studies. Where there were exceptions to GLPs or test guidelines, they did not affect the integrity or interpretation of the study data or the overall conclusions on genotoxicity. The tested Bayer GBFs included concentrated products, relevant dilutions, and ready-to-use products containing various glyphosate salts (isopropylamine, potassium, and ammonium) of differing concentrations, and various proprietary surfactant blends ranging from 0% to 20%. Twelve *in vivo* micronucleus studies (1998-2016) generally followed OECD Test Guideline 474. Mice received two doses separated by approximately 24 hours, with bone marrow harvested 24 hours after the second dose. Six of the studies used the intraperitoneal route of administration while the other six used the oral (gavage) route of administration. Three dose levels were tested, and generally five males and five females were analyzed for each treatment group. Depending on the study, 1000 to 4000 polychromatic erythrocytes (PCEs) per animal were scored using coded slides. Eighteen *in vitro* micronucleus studies (2016-2023) generally followed OECD Test Guideline 487, using a cytokinesis-block design, and were either dosed the limit dose of 2 mg/mL or the highest dose producing acceptable cytotoxicity. Treatment conditions included 4-hour exposures with and without metabolic activation and an extended exposure without metabolic activation. Two thousand binucleated cells (1000 each from duplicate cultures) per treatment were scored for micronuclei using coded slides. Twenty-seven bacterial reverse mutation studies (1998-2024) generally followed OECD Test Guideline 471, assessing five bacterial strains using both plate incorporation and/or pre-incubation methods at up to nine dose levels, in triplicate, with and without metabolic activation. Plates were incubated (48-72 hours) and scored for the presence of revertant colonies. **Results:** All twelve *in vivo* mammalian micronucleus studies were negative for genotoxicity. All eighteen *in vitro* micronucleus assays in human lymphocytes and all twenty-seven bacterial reverse mutation assays were also negative. **Conclusions:** Across fifty-seven previously unpublished studies - combined with earlier published data - Bayer GBFs consistently showed no evidence of genotoxicity. Results were uniformly negative across *in vivo* mammalian micronucleus, *in vitro* micronucleus, and bacterial reverse mutation studies, covering multiple formulations, concentrations, glyphosate salt forms, and proprietary surfactant blends. These findings substantially expand the genotoxicity database for Bayer GBFs and strengthen the overall weight of evidence that Bayer GBFs are not genotoxic.

PS 4319 **Not all literature-based structural alerts lead to mutagenicity**

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Background and Purpose: DNA-reactive substances are capable of directly damaging DNA even at low concentrations, which can result in mutations and potentially lead to cancer. According to reports, there is a strong correlation between structural alerts indicative of DNA reactivity and mutagenic potential, and structure-based assessments are useful for predicting bacterial mutagenicity outcomes. There are a variety of approaches to conduct this evaluation including a review of the literature-based structural alerts (LBSAs), and/or computational toxicology assessment. The mutagenic potential can be typically identified using bacterial reverse mutation assay. In order to gain a better understanding of the correlation between structural alerts and mutagenic potential, and to facilitate expert review using LBSAs, we conducted a retrospective analysis of bacterial reverse mutation data with structures generated in our laboratory. **Methods:** The bacterial reverse mutation (Ames) assay was performed in compliance with ICH S2(R1) and OECD TG471, some studies are conducted using miniaturized versions of standard assays, such as the mini-Ames assay. The Ames studies conducted at WuXi AppTec (Suzhou) Co., Ltd. from 2018 to 2025 were collected for the retrospective analysis. Only data with available structural information are included, and these mainly pertain to impurities. The structures are classified based on LBSAs, such as those published by Ashby, Tennant and Müller. **Results:** Of the 392 compounds tested in the Ames, 279 (71.2%) were concluded to be negative and 113 (28.8%) were mutagenic. Among these 392 compounds, there are 242 compounds containing LBSAs, and the remaining 150 compounds lack LBSAs. Out of 150 compounds without LBSAs, 14 (9.3%) were mutagenic. For 242 compounds containing LBSAs (totally 15 categories), only 99 (40.9%) were

actually mutagenic in the Ames test, indicating that relying solely on LBSAs without taking mitigating factors into account can result in excessive sensitivity. The highest frequencies of LBSAs found were in three categories that together comprise over 70% of all alerts: aromatic amines and derivatives, alkyl halides and aromatic nitro compounds. The percentage (58.3%) was higher for aromatic nitro compounds, another two classes (aromatic amines and alkyl halides) that were between 40% and 45% Ames positive. The mutagenicity of LBSAs is impacted by physicochemical properties (such as molecular weight), chemical reactivity and metabolic activation and these expertise can be useful in expert reviews of *in silico* predictions. Each alerting structure has its own mutagenic mechanism and can also lead to positive results in specific strains. Two troubleshooting cases are presented here by utilizing these knowledge. Case 1: compound A is a small molecule for metabolic diseases. Compound A with lower purity is positive in TA98/TA100 with S9, but negative in the absence of S9 mix and other strains. Since no LBSAs are identified in compound A, considering the specific positive strains, it is suspected that the positive result is caused by impurities (such as aromatic amines). Aromatic amines are deemed to be specifically acting through the nitrenium ion mechanism of action and generally be positive in TA98/TA100 in the presence of S9 mix. The follow-up studies confirmed the suspicion. Case 2: Invalid Ames due to extreme high revertants even in negative control. Based on the specific positive strains and the alerting structure knowledge, ethylene oxide (EO), a common reagent for sterilization, was suspected (sensitive strains are TA100 and TA1535). The vendor confirmed that EO was used to sterilize the petri dishes. After we switched to petri dishes sterilized by gamma irradiation, everything returned to normal. **Conclusions:** In summary, 392 compounds were tested over an 8-year period in our laboratory, of which 113 (28.8%) were found to be mutagenic in the Ames test. The probability of a positive result for compounds containing LBSAs was 40.9%, compared to only 9.3% for those without LBSAs. LBSAs can be a useful tool in expert reviews of *in silico* predictions and in troubleshooting. However, relying solely on LBSAs without taking mitigating factors into account may lead to a significantly higher rate of false positives.

PS 4320 **Control Charting and Variance Partitioning of Historical Negative Controls: Rat Liver Micronuclei as a Real-World Example**

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Background and Purpose: Historical control data (HCD) from negative/vehicle animals can provide valuable context for interpreting genotoxicity assay results, yet recent guidance from the International Workshops on Genotoxicity Testing (IWGT) emphasizes that their use should depend on the data's overall quality and stability. We applied these principles to liver micronucleus (%MNHEP) data from 26 independent rat studies (Crl:CD; 3 male and 3 female vehicle control rats per study; age at time of tissue harvest 5 or 7 weeks). **Methods:** %MNHEP were scored via flow cytometry, and log₁₀-transformed values were evaluated with JMP Pro (v18) software using Individual (I-) charts, Nelson Rules, and Variance Components Analysis (VCA). **Results:** Most %MNHEP values fell within ± 2 -sigma limits, as defined by the I-chart methodology, indicating overall process control. Even so, there was a low-value time interval encompassing studies 16-24 suggesting a temporary yet sustained downward shift. Although the underlying cause of this shift has not been identified, its persistence warrants analytical attention. Including this low-response time interval widened the HCD distribution and increased inter-study variability: the Stability Index (SI) for the full data set was 1.24, improving to 1.14 after excluding studies 16-24. VCA showed total variability estimates as Study = 43.2 %, Sex = 0.6 %, Age at Time of Harvest = 0 %, Vehicle = 0 %, and Inter-animal = 56.3 % when all data were included; after excluding studies 16-24 these values were Study = 24.4 %, Sex = 1.1 %, Age at Time of Harvest = 0 %, Vehicle = 0 %, and Inter-animal = 74.5 %. To use these HCD for "Criterion C" purposes (i.e., comparison of index study results to HCD), we have been experimenting with a compatibility check/requirement. For example: if the concurrent negative-control group mean falls below the 20th percentile of the majority-era HCD distribution of group means, Criterion C is not applied and interpretation relies on concurrent vehicle controls. When instituting this requirement, 2 of 17 majority-era studies and each of 9 low-response studies were deemed ineligible for Criterion C-type comparisons. **Conclusions:** The use of HCD as an aid for interpreting index study results should be highly transparent, and also provide some flexibility given the underlying structure of the data and real-world issues that may be encountered. The approach described herein illustrates how process-control tools can support nuanced, evidence-driven use of HCD in genotoxicity assessment.

PS 4321 **Generalizability of an S9 Metabolic Activation System Optimized for Nitrosamines: *In vitro* MicroFlow and MultiFlow Assay Results**

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Background and Purpose: Certain N-nitrosamines are DNA-reactive, and this genotoxic activity often requires CYP-mediated metabolic. Although exogenous rodent S9 systems contain relevant CYP isoforms to varying degrees, the genotoxicity of some important N-nitrosamines can be challenging to detect in mammalian cell assays. To address this, an optimized exogenous S9 metabolic activation protocol was developed that combines rat and hamster S9 fractions with CaCl₂-supplemented cofac-

tors according to the report by O'Neill and colleagues [Environmental Mutagenesis 4(1982)7-18]. We report here on the evaluation of set of nitrosamines that have now been extended to include several non-nitrosamines in order to more broadly evaluate the generalizability of the method. **Methods:** Human lymphoblastoid TK6 cells (functional p53) were treated with each of eight nitrosamines (NDMA, NDEA, NNK, NNN, NPIP, NPYR, NMOR, NDELA), three metabolically activated genotoxiciants (cyclophosphamide [CP], benzo[a]pyrene [BaP], and 2-amino-1-methyl-6-phenylimidazo[5-b]pyrene (PhIP)), and two cytotoxicants (Tunicamycin and Curcumin). All chemical treatments occurred for 4-hours in the presence of the optimized S9 mix (1.5% Rat plus 1.5% Hamster liver S9 fractions, a NADPH generating cofactor mix that includes NADP, glucose-6-phosphate, KCl, MgCl₂, and excess CaCl as described by O'Neill et al.). At the 4-hour time point, cells were washed and cells were allowed to recover for 20-hours. At the 4- and 24-hour timepoints, cells were processed with MultiFlow DNA Damage Assay reagents to provide γ -H2AX and p53 activation measurements. At the 24-hour timepoint, cells were processed with *in vitro* MicroFlow (micronucleus, or MN) measurements. All these biomarkers were acquired via flow cytometry. Quantitative benchmark dose (BMD via PROAST v70.0) and area-under-curve (AUC) metrics were complemented by ToxPi visualization and principal component analysis (PCA) to evaluate mechanistic clustering and potency rankings. **Results:** Aggregate biomarker responses revealed that nitrosamines exhibited a wide range of potencies (NNK ~ NNN > NDMA > NDEA > NPIP ~ NPYR > NMOR >> NDELA). Interestingly, temporal γ -H2AX readings revealed what appears to be remarkable repair kinetics of NNN and NNK, whereby robust γ -H2AX signals at the 4hr time point where absent following the washout and 20 hour recovery period. CP, BaP, and PhIP produced strong S9-dependent responses, validating the generalizability of the nitrosamine-optimized metabolic activation system. Tunicamycin and Curcumin were cytotoxic, and curcumin induced what appeared to be an MN response. Even so, MultiFlow's biomarkers were unresponsive to curcumin, which tends to suggest an irrelevant MN response. ToxPi and PCA analyses using BMD and AUC values resulted in clustering of compounds by mechanistic class and potency. **Conclusions:** The optimized exogenous S9 metabolic-activation protocol, featuring a 4-hour exposure and 20-hour recovery design, improves assessment of nitrosamines, while remaining applicable to other classes of pro-genotoxiciants. When combined with MultiFlow and MicroFlow assays, this system represents an economical hazard ID platform that is likely complementary to Ames assays, given the important advantages stemming from its mammalian cell-based nature.

PS 4322 Hemoglobin N-(2-Hydroxyethyl)valine as a Noninvasive Surrogate of Ethylene Oxide Exposure Correlating with DNA Adduct Formation

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Background and Purpose: Ethylene oxide (EtO) is a reactive epoxide and a known human carcinogen that alkylates DNA to form mutagenic adducts, including N7-(2-hydroxyethyl)guanine (N7-HEG) and O6-(2-hydroxyethyl)-2'-deoxyguanosine (O6-HEdG). Quantifying genotoxicity induced by EtO remains challenging and invasive because it requires tissue sampling. In contrast, hemoglobin N-(2-hydroxyethyl)valine (HEV) is a blood-based biomarker that can reflect cumulative EtO exposure. This study aimed to determine whether HEV can serve as a surrogate of internal EtO exposure that quantitatively correlates with DNA adduct formation across major tissues. **Methods:** B6C3F1/J mice were exposed to EtO via whole-body inhalation at concentrations ranging from 0.05 to 200 ppm (6 h/day for 28 days). Blood and tissues, including liver, lung, mammary gland, and bone marrow, were collected after exposure. HEV was released from hemoglobin by acid hydrolysis and quantified using a highly sensitive LC-MS/MS method. DNA adducts (N7-HEG and O6-HEdG) were simultaneously quantified in tissues using validated LC-MS/MS assays. Relationships between HEV and DNA adduct levels were evaluated using Pearson correlation analysis. **Results:** HEV levels in hemoglobin increased in a dose-dependent manner with EtO exposure. Parallel dose-dependent increases were observed for both N7-HEG and O6-HEdG across all four tissues. Strong linear correlations ($R^2 = 0.72-0.95$) were found between blood HEV and DNA adduct levels, showing that HEV formation in hemoglobin mirrors tissue DNA alkylation. **Conclusions:** This study establishes hemoglobin N-(2-hydroxyethyl)valine as a noninvasive and mechanistically relevant surrogate biomarker of ethylene oxide exposure. The strong quantitative correlation between HEV and tissue DNA adducts supports its use in translational biomonitoring and in improving risk assessment of EtO-induced genotoxicity.

PS 4323 Optimization of an Exogenous Metabolic Activation System for the Detection of Nitrosamines with Human Cell-Based Genotoxicity Assays

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Background and Purpose: Nitrosamines such as NDMA, NDEA, NNK, NMOR, NPYR, NNN, and NDELA are established mutagens and carcinogens that require metabolic activation to elicit genotoxic effects. However, standard *in vitro* assays and the exogenous metabolic activation systems applied, i.e. rat S9 fractions, often show

limited sensitivity or inconsistent detection of such compounds. Recent advances such as those utilized in the Enhanced Ames Testing (EAT) protocol show that hamster-derived S9 can at times provide more robust genotoxicity responses. Additionally, O'Neill et al 1982 [Environmental Mutagenesis 4(1982)7-18] reported on enhancement of mutagenicity of nitrosamines following the addition of excess calcium chloride with consequential calcium phosphate precipitation. The current study aimed to optimize the S9 activation system for detecting genotoxic nitrosamines by evaluating species source, concentration, cofactor composition, and exposure duration. **Methods:** TK6 human lymphoblastoid cells were exposed to at least a 10-point concentration range of each nitrosamine under several metabolic activation conditions. A standard continuous low-dose exposure with 0.25% S9 was compared to short-term "NA-optimized" conditions incorporating 3% rat S9, 3% hamster S9, or a 1.5% rat + 1.5% hamster combination. Each system included NADPH-generating cofactors (NADP, glucose-6-phosphate, KCl, MgCl₂). CaCl₂ was introduced into the co-factor mix according to O'Neill et al. 1982. At 4 and 24 hours, cells were evaluated for γ -H2AX, phospho-Histone H3, and p53 using a multiplexed genotoxicity assay (i.e., MultiFlow). Additionally, cells from the 24 hour timepoint were also evaluated for micronucleus induction via the MicroFlow method. The resulting flow cytometry data were evaluated for point of departure using PROAST benchmark dose (BMD) software (v70.0; critical effect size 0.5; model averaging). **Results:** The several 3% S9 CaCl₂-enriched systems produced stronger and more consistent concentration-response relationships compared with the standard 0.25% continuous exposure. Distinct species-related activation patterns were observed. The 3% rat S9 system provided the most robust activation for bulky nitrosamines such as NNK and NNN, producing low BMD values and steep γ -H2AX induction curves. In contrast, the 3% hamster S9 system preferentially activated smaller methylating nitrosamines (NDMA and NDEA), yielding clearer responses and tighter confidence intervals. The combined 1.5% rat + 1.5% hamster S9 condition offered the broadest metabolic coverage, capturing the full spectrum of nitrosamine reactivity within a single exposure system. This dual-species mix improved reproducibility for NMOR and NPYR and minimized false negatives observed when using either species alone.

Conclusions: Systematic optimization of S9 source, concentration, and cofactor composition markedly improved assay sensitivity and reproducibility across several (geno)toxicity biomarkers for the reference nitrosamines studied. The optimized S9 activation system represents an improved hazard identification for nitrosamines, and future work will be directed at evaluating the degree to which resulting *in vitro* biomarker responses correspond to *in vivo* genotoxicity and carcinogenicity.

PS 4324 Elucidation of Mitochondrial Nucleic Acid Signals Under Genotoxic Stress

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Background and Purpose: Mitochondria are vital organelles in higher eukaryotic cells, playing a crucial role in energy production, cell signaling, and biosynthesis. The mitochondrial DNA (mtDNA) genome, which encodes key components of the oxidative phosphorylation system, is susceptible to damage by endogenous and exogenous chemicals. When compromised, both mtDNA and mitochondrial double-stranded RNA (mt-dsRNA) can be released into the cytosol, where they act as damage-associated molecular patterns (DAMPs), triggering innate immune and inflammatory responses. While their role as signaling molecules is recognized, the relative contributions of mtDNA and mt-dsRNA under different genotoxic chemicals are not well understood. We hypothesize mtDNA and mt-dsRNA molecules contain signaling codes to inform different cellular defense mechanisms. This study aims to quantify the contributions of mtDNA and mt-dsRNA in mitochondrial homeostasis and innate immune response to various genotoxic agents. **Methods:** The initial phase of the project focuses on a mitochondria-targeting chlorambucil derivative developed in my laboratory that selectively alkylates mtDNA without damaging nuclear DNA. We used human bronchial epithelial (BEAS-2B) cells as a model system, which is relevant to inhalation exposures. Quantitative PCR was used to quantify the abundance of mtDNA and mt-dsRNA in the cytoplasm and assess the mtDNA integrity, as well as to determine the effects on the mitochondrial network and activation of an immune response downstream of the sensors cGAS and RIG1. Immunofluorescence using the J2 anti-dsRNA antibody was used to verify the release of mt-dsRNA species. Changes in mitochondrial homeostasis were measured as a function of respiration efficiency using the mitochondrial stress tests by the Seahorse apparatus. **Results:** Our findings revealed that exposure led to a significant increase in mitochondrial copy number and a concomitant decrease in mtDNA integrity. Release of both mtDNA and mt-dsRNA into the cytosol was confirmed, accompanied by changes in mitochondrial and cellular dynamics. We observed the upregulation of both fission and fusion genes, indicative of a shift in mitochondrial dynamics. Furthermore, a significant increase in the transcription of cytosolic sensors and immune response components was observed. Seahorse assays revealed that respiration efficiency was negatively affected after a 24-h exposure. **Conclusions:** Taken together, this research establishes a robust and quantitative platform to assess the distinct contributions of mtDNA and mt-dsRNA in mitochondrial respiration, dynamics, and innate immunity under genotoxic stress. Future work will extend this platform to decode the mitochondrial nucleic acid signals from additional genotoxic chemicals and cell models, particularly at doses relevant to human exposure.

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Background and Purpose: Enteric methane emissions from cattle are a major contributor to global greenhouse gas emissions. Use of anti-methanogenic feed additives, including the seaweed *Asparagopsis*, can substantially decrease methane emissions and thus reduce the climate impact of dairy and beef production. Brominata™, a patented strain *A. taxiformis*, is highly effective at reducing methane emissions and has recently been approved as a feed additive in Mexico and some states across the U.S. As part of the feed additive approval process in Europe, the European Food Safety Authority (EFSA) requires two *in vitro* genotoxicity studies and an *in vivo* subchronic oral toxicity test. Depending on the results of these studies, EFSA may recommend follow up studies. Therefore, the objective of this study was to evaluate Brominata™ in the standard set of Organisation for Economic Co-operation and Development (OECD) guideline toxicity tests, including any follow up studies, required by EFSA to assess the safety of a feed additive for the ultimate human consumer. **Methods:** Brominata™ was evaluated in two *in vitro* genotoxicity studies, including the bacterial reverse mutation assay (Ames test) in *Salmonella typhimurium* and *Escherichia coli* (OECD 471) and the mammalian cell micronucleus assay in human lymphocytes (OECD 487). For each *in vitro* assay, two fully independent studies were conducted using different batches of material. A follow up *in vivo* mammalian alkaline comet assay was conducted in Wistar rats (OECD 489). Brominata™ was evaluated in a 28-day dose range finding (DRF) study and a full 90-day oral repeated dose toxicity study (including a 28-day recover period) in Wistar rats (OECD 408). All studies were conducted in compliance with OECD test guidelines and principles of Good Laboratory Practice (GLP) (except the 28-day DRF study). **Results:** In one of the Ames tests, Brominata™ did not cause gene mutations by base pair changes or frameshifts in the genome of any of the five tester strains with or without metabolic activation and was considered to be non-mutagenic. In the second test, Brominata™ was negative in most of the tester strains (TA 98, TA1535, and TA1537) with and without metabolic activation. In TA100, Brominata™ caused an increase in revertant colony numbers without metabolic activation but not with metabolic activation (suggesting the substance would not be mutagenic *in vivo*). Increased revertant colonies were also observed in *E. coli* uvrA (pKM101) in both the presence and absence of metabolic activation; however, the threshold concentration was higher with metabolic activation (suggesting metabolism is detoxifying and limited to no effect would be observed *in vivo*). Based on the results of the Ames tests, a follow up *in vivo* mammalian alkaline comet assay was conducted in Wistar rats (OECD Test No. 489), consistent with EFSA guidance. Brominata™ did not induce biologically relevant DNA-strand breaks in the liver after oral administration of up to 1,200 mg/kg/day on two consecutive days to Wistar rats. Thus, Brominata™ was considered to be non-DNA damaging *in vivo*. In both of the *in vitro* micronucleus assays, Brominata™ did not induce structural and/or numerical chromosomal damage in human lymphocytes in the presence or absence of metabolic activation. Brominata™ was thus considered to be non-mutagenic with respect to clastogenicity and/or aneugenicity. In the DRF study, no adverse test-item related effects were observed at up to 600 mg/kg/day (highest dose tested). In the 90-day study, there were no Brominata™-related effects on clinical signs, functional observation battery, body weight development, food consumption, clinical pathological parameters, organ weight, or gross or microscopic findings related to a systemic effect. The no observed adverse effect level (NOAEL) was 400 mg/kg/day (highest dose tested). **Conclusions:** Based on the results of the genotoxicity and oral repeated dose toxicity studies, Brominata™ was found to be not systemically toxic and non-DNA damaging *in vivo* in rats. Overall, these results support the safety profile of this feed additive. The next step is to conduct a human health risk assessment, using the NOAEL from this study as the safe dose, to evaluate the safety of this feed additive for the ultimate human consumer.

PS 4326 DNA Adductomics to Support the Assessment of Alcohol's Genotoxicity

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Background and Purpose: Alcohol consumption is a known risk factor for head and neck cancer as one in three cases are associated with drinking. Early detection leads to an increase of over 28% in relative survival rates. However, identification of the early stages of alcohol-related carcinogenesis is difficult as the mechanisms of this process are not well understood. Alcohol may induce a number of different processes leading to cancer initiation, including the induction of DNA damage through its major genotoxic metabolite, acetaldehyde (AA). Individuals who consume alcohol and garner a mutation (rs671) in their ALDH2 gene have a higher risk for developing head and neck cancer. Increased levels of the major AA-derived DNA adduct N²-ethyl-dG have also been found in oral cells of individuals after drinking alcohol. Using a DNA adductomics approach, a mass spectrometric methodology that chemically characterizes DNA damage, we are able to better understand the entire scope of damage that occurs from exposure to AA in the oral cavity. This work aims at better elucidating the genotoxic effects of AA through the genera-

tion of a library of DNA adducts, and provide an example of how DNA adductomics can be used to precisely characterize genotoxicity of chemical exposures. **Methods:** Normal oral keratinocyte (NOK) cells were exposed to AA (0, 4, 8, and 12 mM) for 4 hours, after which DNA was isolated. Enzymatic hydrolysis was performed to isolate modified nucleosides. After samples were processed, DNA adduct analysis was performed using liquid chromatography coupled to high resolution accurate mass (LC-HRAM) mass spectrometry techniques. For targeted analysis of known DNA adducts, a data independent method was used. For screening for unknown DNA adducts, a data dependent approach was utilized that harnesses the instrument's ability to scan for known neutral losses of nucleobases or deoxyribose and further fragment these specific ions. Data for unknown adducts was then uploaded to Compound Discoverer™ to aid in data analysis. **Results:** DNA from exposed NOK cells was evaluated for N²-Et-dG and Cro-dG levels. These adducts showed significant increases after exposure. A dose dependent response was seen for both adducts with levels rising 300 times and 533 times higher in 12 mM vs. 0 mM. In order to identify unknown DNA adducts and form a library that can be used for future studies, an untargeted screening method was performed. Beginning with the 12 mM exposure level, DNA was hydrolyzed and analyzed using a data dependent approach. Data was then uploaded into Compound Discoverer and a list of 36 masses was produced. This list was achieved through the filtering of neutral loss of deoxyribose or one of the nucleobases, obtaining correct fragmentation patterns, and having higher levels in the exposure group compared to controls. This list of 36 masses was then targeted in triplicate analysis to obtain further validation. This yielded 8 masses that were higher in the 12 mM exposure group compared to control. After these findings, a similar process was done comparing the 8 mM exposure group to the control group. A pooled sample of the 8 mM replicates was hydrolyzed and analyzed compared to controls. This data followed the same filtering and validation process as the 12 mM exposure stated above. The filtering yielded a mass list of 45 putative masses that were targeted in triplicate. The triplicate analysis resulted in 7 masses having higher levels in the exposure group compared to control. Comparing the two sets of results together, 4 masses were in common between the groups, leading to an overall library of 11 masses. **Conclusions:** Confirmation of exposure was done by measuring N²-Et-dG and Cro-dG. Screening for unknown DNA adducts led to the putative identification of 11 masses that were confirmed to be associated with AA exposure. This list of adducts can be formed as a library of adducts that can be extended to future studies in animal models or human samples. This library can better explain one of the key events within the carcinogenic process and this experiment shows the value of using DNA adductomics to fill gaps within an AOP of a chemical exposure to cancer formation. Having a library resulting from this *in vitro* analysis shows that DNA adductomics is a viable new approach to address genotoxicity.

PS 4327 Inclusion of structural alerts for the detection of substances from the Cohort of Concern in a combined mutagenicity prediction model

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Background and Purpose: Toxicological risk assessment aims to identify safe exposure levels for humans, but some compounds lack sufficient data. Highly carcinogenic substances classified under the Cohort of Concern (CoC), require special regulatory attention according to ICH (International Conference on Harmonization) guidelines [1]. These guidelines focus on assessing DNA-reactive (mutagenic) compounds to evaluate potential genotoxic carcinogens by promoting computational methods (rule-based expert systems (SAR) and statistical models (QSAR)). Thus, this research is focused on the characterization and generation of structural alerts for the identification of the carcinogenic potential and the implementation of a consensus prediction combining these alerts (SAR) with QSAR models for carcinogenicity assessment. **Methods:** A systematic literature review was conducted to retrieve data on the genotoxic and carcinogenic potential of the substances from various scientific databases (Google Scholar, PubMed, Scopus, and Web of Science) by using an in-house developed and an external software. The obtained data were then analysed to identify to find structural patterns characteristic of genotoxic carcinogens versus non-genotoxic carcinogens. To this end, the presence of different functional groups in the carcinogenicity dataset was analyzed for both positive and negative molecules by using the "fragments" module of the Python package rdkit [2]. Finally, the validated substructures were codified as SMARTS (Specific Alert and Response System) and incorporated in python scripts, to automatically screen databases for the identified alerts. **Results:** Our analysis identified 87 relevant molecular substructures, from which a total of 26 functional groups showing relative presence greater in carcinogenic substances than in non-carcinogenic ones were identified. The SMARTS for the 26 functional groups were compiled and compared to compounds for which validated with the retrieved experimental data for diverse toxicological assays evaluating the genotoxic and carcinogenic potential (Ames test, chromosomal aberration assay, COMET assay, micronucleus generation assay, HPRT assay and *in vivo* carcinogenicity assay), showing that the key functional groups present in genotoxic compounds were nNH0, nAniline, nArN and nNitro. To verify the application of these alerts to sets of molecules with a high carcinogenic risk, the presence of the SMARTS for the 26 identified functional groups was analyzed in the IARC database [3] and experimental mycotoxin database that is part of the MICOTOXILICO project (<https://chemopredictionsuite.com/MicotoXilico>) [4]. **Conclusions:** Specific rules or alerts have been

established to detect compounds structurally similar to those included in the CoC and which, therefore, should be considered for their potential carcinogenic and genotoxic effects. The generated SMARTs from the identification of functional groups or substructures responsible for genotoxic carcinogenic potential have been implemented as rule-based expert systems alongside the computational models already included in the ProtolCH tool (https://protopred.protoqsar.com/ProtolCH_info). Thus, the ProtolCH tool is able to detect compounds included in the CoC, as well as other compounds with carcinogenic potential not included in the CoC, thanks to the implementation of a consensus prediction that combines QSAR statistical models and rule-based expert models (SAR). Acknowledgments: This research was funded by MICIU/AEI /10.13039/501100011033/FEDER, UE through the project PID2024-160527OB-I00 and the Generalitat Valenciana CIAEST/2022/035. References: [1] ICH guideline M7(R1): European Medicines Agency (2015), ICH guideline M7(R1) on assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk. [2] Landrum, G. A. RDKit: Open-source cheminformatics; 2015. Zenodo. DOI: 10.5281/zenodo.591637. [3] <https://monographs.iarc.who.int/list-of-classifications> [4] Tolosa, J. et al. 2023. MicotoXilico: An interactive database to predict mutagenicity, genotoxicity, and carcinogenicity of mycotoxins. *Toxins*, 15(6), 355.

PS 4328 Implementation of an hOGG1-Enhanced *In Vivo* Comet Assay to Identify Particle-Induced DNA Damage in Rat Lung Tissue Following Instillation

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Background and Purpose: The *in vivo* alkaline comet assay according to OECD 489 can detect both DNA-strand breaks and oxidatively damaged DNA upon enzyme modification and is currently an integral part of regulatory genotoxicity testing strategies. While the liver is the primary organ examined for chemicals and pharmaceuticals, the assay is also valuable for genotoxicity testing of particles in the lung after instillation. This study aimed to establish a robust protocol for particle testing in lung tissue. Key challenges included identification of an efficient and reliable cell isolation method that minimizes basal DNA damage, finding appropriate technical positive controls for better adhering to the 3R principles, and implementing an effective enzyme incubation step for detection of oxidatively damaged DNA, relevant for particle-exposed lungs. **Methods:** Initially, we addressed cell isolation from lung tissue by comparing various buffers and mechanical devices (e.g., douncer, scissors, pistils). Optimal tissue dissociation was finally achieved using tissue punches and a grinding step with disposable pistils in tissue buffer-filled reaction cups. Vehicle controls showed low baseline tail intensities (TI) of less than 4% increasing to 9-12% in animals treated with ethyl methanesulfonate (EMS, 300 mg/kg, 3h). But for 3R reasons, also technical *in vitro* positive controls were established. EMS-treated L-929 cells (4.85 mM, 1h), cryopreserved in large batches, served to show appropriate electrophoresis. Both freshly treated and thawed cells (stored up to 11 months) showed consistent TI values of 12-16%. For sensitive detection of oxidatively-damaged DNA, an incubation step $\pm$ human 8-oxoguanine DNA glycosylase 1 (hOGG1) enzyme was chosen. Potassium bromate (KBrO₃)-incubated L5178Y/TK⁺ cells served as technical positive control to ensure hOGG1-functionality. **Results:** A respective incubation protocol with optimized KBrO₃ (5 mM KBrO₃) and hOGG1 concentration (100 μ l of a 2.5 μ g/ml solution of hOGG1) and incubation times (1 h and 15 min, respectively) was finally developed to allow for clear distinction between buffer- and hOGG1-treated KBrO₃ samples. **Conclusions:** In conclusion, the optimized *in vivo* comet assay protocol enables reliable detection of both DNA strand breaks and oxidative DNA damage in rat lung tissue following particle exposure. Future work will focus on enhancing oxidative damage detection by combining hOGG1 with endonuclease IV, which removes AP sites that can inhibit hOGG1 activity.

PS 4329 Mitochondrial Glutathionylated DNA Adducts: Accumulation, Regulation, and Biological Implications

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Background and Purpose: Mitochondrial DNA (mtDNA) is crucial for cellular energy production, metabolism, and signaling. Its dysfunction is implicated in various diseases, including mitochondrial disorders, neurodegeneration, and diabetes. mtDNA is susceptible to damage by endogenous and environmental factors; however, unlike nuclear DNA (nDNA), mtDNA lesions do not necessarily lead to an increased mutation load in mtDNA. Instead, mtDNA lesions have been implicated in innate immunity and inflammation. Here, we report a novel type of mtDNA damage: glutathionylated DNA (GSH-DNA) adducts. These adducts are formed from abasic (AP) sites, key intermediates in base excision repair, or from alkylation DNA damage. The study aims to establish the importance of GSH-DNA adducts by quantifying their level, clarify their regulation and biological effects. **Methods:** Our study utilized mass spectrometry to directly compare the formation and persistence of GSH-DNA adducts in both nDNA and mtDNA across two distinct human cell lines under various genotoxic stress conditions. To identify the key enzymatic regulators, we quantified adduct levels in DNA repair-deficient cells and characterized the repair efficiency of

candidate enzymes using *in vitro* enzyme kinetic assays. To elucidate the biological effects of adduct accumulation, we performed quantitative proteomics to assess protein level changes and measured cellular glutathione (GSH) levels. Lastly, we determined how GSH-DNA adduct accumulation influences DNA conformational stability and DNA-protein interactions through a combination of molecular dynamics (MD) simulations and *in vitro* binding assays. **Results:** Our data demonstrate that GSH-DNA adducts accumulate in mtDNA of two different human cell lines, with levels one or two orders of magnitude higher than in nDNA. The formation of GSH-DNA adducts is influenced by TFAM and polyamines, and their levels are regulated by repair enzymes AP endonuclease 1 (APE1) and tyrosyl-DNA phosphodiesterase 1 (TDP1). The accumulation of GSH-DNA adducts is associated with the downregulation of several ribosomal and complex I subunit proteins and the upregulation of proteins related to redox balance and mitochondrial dynamics. Their accumulation did not perturb cellular or mitochondrial GSH levels. Molecular dynamics (MD) simulations revealed that the GSH-DNA lesion stabilizes the TFAM-DNA binding, suggesting shielding effects from mtDNA transactions. **Conclusions:** This study provides critical insights into the formation, regulation, and biological effects of GSH-DNA adducts in mtDNA. Our findings underscore the importance of understanding how these lesions may contribute to innate immunity and inflammation.

PS 4330 Benzo[b]fluoranthene induces mutation accumulation and human cancer-relevant mutational signatures in mouse lung alongside steady state levels of chromosome damage in blood.

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Background and Purpose: Benzo[b]fluoranthene (BbF) is one of the 16 polycyclic aromatic hydrocarbons (PAHs) prioritized by the US Environmental Protection Agency due to high levels of human exposure, and the known mutagenic and carcinogenic effects of substances in this chemical class. This study examined the effects of increasing exposure durations on the mutagenic effects of BbF. **Methods:** MutaMouse males were exposed to 5 doses of BbF or a vehicle control via repeated oral gavage for 28, 60, 90, 120, or 180 days, with the dose range modified to account for duration of exposure (top doses were 25, 50, or 100 mg/kg/day). To comprehensively assess genotoxicity, mutagenesis was evaluated in lung tissue (n = 4) at 28-, 90- and 180-days via duplex sequencing (DS), a targeted, error-corrected sequencing method; chromosomal damage (n = 8) was evaluated using the peripheral blood micronucleus assay (MN) at all 5 time points. Mutational signature analysis was performed using the SigProfiler suite to fit single-base substitution (SBS) signatures obtained from the Catalogue of Somatic Mutations in Cancer (COSMIC) database. BMD modeling of mutation and MN data was conducted using PROAST 71.1 in the R programming environment. **Results:** Dose-dependent increases in mutation frequency (MF) and C:G > A:T mutations were observed after 28-, 90-, and 180-days of exposure. BbF-induced mutations were lower in genic targets, consistent with transcription-coupled repair of BbF-DNA adducts. As early as 28 days, BbF exposure resulted in enrichment of the cancer-associated mutational signature SBS4, which is linked to tobacco smoking. SBS4 accounted for 72.4% of the reconstructed spectrum at 28 days, which increased to 87.5% at 180 days. Induction of chromosomal damage by BbF was observed at all five time points. Mutations accumulated over time in lung tissue, whereas chromosomal damage in peripheral blood erythrocytes reached a steady state by 28 days. Benchmark dose (BMD) confidence intervals (CIs) for MF became more precise with extended exposure, while BMD CIs for micronucleus frequency remained relatively stable. **Conclusions:** These data demonstrate that BbF is a potent mutagen capable of inducing persistent mutations in lung with cancer-relevant signatures, supporting its potential role in human lung carcinogenesis.

PS 4331 Correlation of Dietary Antioxidant Levels to Incidence and Severity of Oteseconazole-induced Nuclear Opacities in Peri-Postnatal Development (PPND) Studies

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Background and Purpose: Oteseconazole (VIVJOA®) is an antifungal agent approved for recurrent vulvovaginal candidiasis (RVVC); however, its use is restricted to women not of child-bearing potential. This restriction is due to rat PPND studies where a higher incidence of spontaneous background ocular abnormalities (nuclear opacities & bleeding events) were noted in pups born and nursed from a small percentage of oteseconazole-treated dams during gestation and lactation. Assessing direct agent causality is challenging when the incidence of spontaneous background opacities in young rats can range up to 50% (Ban et al., 2008) and ophthalmic examinations are not conducted in rat PPND studies for currently marketed drugs. Since antioxidants are linked to cataract prevention, the mechanism of nuclear cataract formation and its dependence on rat chow dietary antioxidant status and various antioxidants were examined *in vivo* and *in vitro*, respect-

tively. **Methods:** For drug registration, pregnant Sprague-Dawley Crl:CD(SD), Hsd(SD), or Crl:WI(Han) dams in 4 PPND studies were dosed with oteseconazole (7.5 mg/kg/d) from gestation day 6 (GD6) through lactation day 20 (LD20), inclusively, with ophthalmic examinations by indirect ophthalmoscope and slit lamp biomicroscope and/or gross assessments along with clinical pathology sampling for aberrations in hematology, coagulation and serum chemistry parameters performed during study. Subsequent studies used cross-fostering designs at two contract research facilities to determine a susceptibility period during lens development. Recent PPND studies used diets of decreasing antioxidant content (PMI 5002>Teklad 2016C>custom) as determined by the Ferric Reducing Antioxidant Power (FRAP) assay. *In vitro* studies used both mouse lens explants and mouse and human lens epithelial cells. Oxidative stress was quantified by 4-hydroxynonenal (4-HNE) staining in lenses from treated rat pups and in mouse and human lens cells. **Results:** Rat pups from control and treated dams showed variable incidence of predominantly nuclear opacities across strains (range 0-15% for Sprague-Dawley Crl:CD(SD), 0-40% for Crl:WI(Han), and 13-48% for Hsd(SD)). Subsequent PPND and cross-fostering studies demonstrated variability in spontaneous background and treatment-associated ocular abnormalities between contract labs later suspected to be diet related (5002 and 2016C diet). Isolated serum γ -glutamyltranspeptidase (GGT) elevations across studies and diet type in treated rat pups and dams indicated liver oxidative stress. Two PPND studies showed no treatment nuclear opacities when dietary FRAP values were >3000 μM Fe^{3+} reduction potential (5002 and 2016C diets), but were observed with lower FRAP values (2016C, custom) with dietary FRAP variability based on lot surveys. 4-HNE staining was found in the anterior suture lines that touched the outside fetal nuclear area in lenses from treated pups that increased in intensity and severity with declining dietary FRAP values, suggesting opacity formation during lactation due to higher exposure. Mouse lens explants confirmed increased 4-HNE and disrupted glutathione homeostasis. Liproxstatin-1 and menaquinone-4 delayed opacification in whole lens culture, suggesting a small role for ferroptosis. Combinations of antioxidants (lutein/zeaxanthin, eicosapentanoic acid/docosahexanoic acid, β -carotene/ α -carotene/retinoic acid/ascorbic acid and N-acetylcysteine/ascorbic acid) were protective of mouse and human lens epithelial cell death, where the minimal toxic concentration of oteseconazole was 5 and 10 μM after 96 hr in mouse and humans lens cultures, respectively. **Conclusions:** While rat strains and contract research facilities influenced ophthalmic findings, nuclear cataract formation was linked to innate differences and intrinsic variability in dietary antioxidant capacity. Oxidative stress is a primary mechanism of nuclear cataract formation and inadequate dietary nutrition to maternal dams leads to nuclear cataract formation in rat pups. *In vitro* studies indicate that a wide variety of antioxidants commonly found in the human diet are protective. Given the broad nutritional status of human, it is inferred that these rat findings have limited relevance to humans.

PS 4332 Dietary Vitamin K-Dependent Coagulopathy and Ocular Bleeding Events in Rat Pups Treated with Oteseconazole During Peri-Postnatal Development (PPND) Studies

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Background and Purpose: Oteseconazole (VIVJOA®) is an antifungal agent approved for recurrent vulvovaginal candidiasis (RVVC) however its use is restricted to women not of child-bearing potential. This restriction is based on rat PPND studies describing a higher incidence rate of ocular opacities and bleeding events in treated pups compared to untreated controls. Ocular opacity incidence rates range from 0-50% in young rats (Ban *et al.*, 2008), but no comparable data exists for pups. Investigations were initiated to better define the translational relevance of PPND findings and gain mechanistic understanding of an apparent treatment-related coagulopathy. **Methods:** Initial PPND studies used pregnant Sprague-Dawley Crl:CD(SD), Hsd(SD), or Crl:WI(Han) dams dosed with oteseconazole (7.5 mg/kg/d) from gestation day 6 (GD6) through lactation day 20 (LD20) that included full ophthalmic and gross necropsy examinations performed on PND21/35. Clinical pathologic testing included blood cell counts, coagulation screening tests (aPTT/PT), and serum chemistry panels. Because subsequent PPND and cross-fostering studies demonstrated variability in spontaneous background and treatment-associated ocular abnormalities between contract research sites, potentially related to feed composition (PMI 5002 vs Teklad 2016C), assays to detect vitamin K deficiency were performed. These assays included measurement of plasma vitamin K and procoagulant activities of vitamin K-dependent factors (II, VII, IX, X). In addition, a subset of rats was administered intramuscular vitamin K daily (10 mg/kg phyloquinone). Subsequent PPND studies evaluated diets with varying vitamin K content (2016C>5002>custom). Analytical sensitivity issues precluded determination of vitamin K-epoxide formation, therefore the inhibitory potential of oteseconazole was measured in rat and human microsomal vitamin K-epoxide reductase (VKORC1) prepared from transfected yeast cells, using warfarin as a known inhibitor. Banked human plasma samples from previous clinical trials were obtained with IRB approvals. **Results:** PPND clinicopathologic findings in pups included subcutaneous and ocular hemorrhage, red cell mortality, pronounced prolongation of aPTT/PT, and decreased fibrinogen and red cell counts (C_{max} >40 μM). The presence and severity of signs appeared variable between sites that fed either 5002 or 2016C diets as their standard rodent chow. Studies revealed reductions in maternal/pup plasma and liver vitamin K and low activities of factors

II, VII, IX, X. Marked prolongation of aPTT/PT after 28 days of oteseconazole dosing (15 mg/kg/d) in young adult rats fed 5002 diet were reversed by daily phyloquinone administration through day 35. Coagulopathy with ocular bleeding were attenuated/absent in pups from maternal rats fed the 2016C diet but present for diets with lower vitamin K (5002 and custom). Oteseconazole inhibition of rVKORC1 and hVKORC1 showed IC_{50} values of 74 and 53 μM compared to 0.14 and 0.56 μM for warfarin, respectively. Systemic exposure margins of 0.8x the IC_{50} for rVKORC1 on PND10 lead to coagulopathy and ocular bleeding in rat pups on the 5002 and custom diet while systemic/liver exposure margins of 0.42/9.8x the IC_{50} for rVKORC1 inhibition on GD18 led to aPTT increases in pregnant rat dams on the custom diet. In humans, aPTT/PT were unchanged in Phase 1 single and multiple ascending dose trials, plasma vitamin K was not altered and protein induced by vitamin K absence or antagonist-II (PIVKA-II) was unchanged in clinical trial subjects after 12-week of treatment in Phase 3, randomized, double-blind, placebo-controlled studies where C_{max} =5.3 μM . **Conclusions:** While rat strain and husbandry differed across sites, wide variability in vitamin K content across diet selection (natural phyloquinone or added menaquinone) had direct effects on coagulopathy and exposure differences could account for variable incidence of ocular bleeding events in PPND studies. Oteseconazole was determined to be a weak rVKORC1 and weaker hVKORC1 inhibitor, at least 100x less potent than warfarin. Weak hVKORC1 enzyme inhibition results in a therapeutic exposure margin of at least 10x, suggesting limited relevance to humans.

PS 4333 Multifocal ERG and VEP-Based Evaluation of Retinal and Cortical Function in Cynomolgus Monkeys with Simulated Deficits and Ocular Hypertension

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Background and Purpose: Glaucoma, typically associated with elevated intraocular pressure, is a neurodegenerative disease characterized by the irreversible loss of retinal ganglion cell axons within the optic nerve, ultimately resulting in localized visual field defects. Even in toxicological studies, early identification of these localized functional impairments including visual field defects is essential for safety assessment and translational research. Multifocal electroretinography (mfERG) and multifocal visual evoked potentials (mfVEP) enable sector-wise mapping of retinal and cortical responses, respectively. Stimulus design (On-Off white/black alternation for mfERG; pattern reversal for mfVEP) plays a pivotal role for reliable recordings. This study aimed to establish and validate mfERG/mfVEP protocols in cynomolgus monkeys following a sequence: first, evaluate reproducibility under intact (normal) conditions; second, introduce localized masking to simulate sector-specific deficits and validate abnormality detection; finally, apply the protocols to laser-induced ocular hypertension to assess sensitivity to pathological change. **Methods:** Purpose-bred cynomolgus monkeys (three males, 2-8 years, 2.0-8.0 kg, B virus antibody-negative) were studied: two with laser-induced unilateral ocular hypertension (≥ 30 mmHg) and one normotensive bilaterally. Recordings were acquired under ketamine (i.m.) followed by propofol (i.v.) anesthesia. Retinal signals were measured using contact lens electrodes; cortical signals were recorded with transcutaneous cup electrodes at Fz and Oz. Visual stimuli were presented on a 23.8-inch monitor at 35 cm ($\approx 34^\circ \times 34^\circ$ visual angle). Using a 511-frame m-sequence, three paradigms were tested: (1) On-Off white/black alternation across 61 hexagonal sectors; (2) whole-field pattern reversal across 48 sectors; and (3) quadrant-based pattern reversal across 48 sectors. Each paradigm was recorded twice: intact, then with two localized black circular masks to simulate sector-specific impairment. The evoked signal was quantified as signal-to-noise ratio (SNR, dB) from peak-to-peak amplitudes relative to baseline noise. Reproducibility was assessed by intraclass correlation coefficients (ICC) for P1 or N1 amplitudes between the first and second recordings. Group comparisons used two-sample t-tests. **Results:** Under intact conditions, mfERG reproducibility varied by paradigm: On-Off alternation showed moderate stability for P1 (ICC = 0.589), whereas quadrant-based reversal yielded the highest reproducibility (ICC = 0.943). Reproducibility in mfVEP was consistently high across paradigms (P1 ICC = 0.792-0.927; N1 ICC = 0.731-1.000). Localized masking significantly reduced SNR in targeted sectors for both modalities, confirming sensitivity to simulated deficits: mfERG showed reductions under On-Off alternation (-4.65 dB, $p < 0.001$) and quadrant-based reversal (-1.69 dB, $p = 0.0351$), while mfVEP exhibited the largest drop under quadrant-based reversal (-3.70 dB, $p = 0.0002$). In the ocular hypertension model, mfERG under pattern reversal showed higher SNR compared to normotensive eyes (whole-field: +3.13 dB; quadrant-based: +4.29 dB; both $p < 0.001$), likely reflecting reduced baseline noise rather than improved retinal function. In contrast, mfVEP demonstrated consistent SNR decreases in hypertensive eyes (whole-field: -2.67 dB; quadrant-based: -1.80 dB), supporting reduced cortical responses resulting from visual pathway disturbance. Quadrant-based pattern reversal provided the most stable and sensitive detection of simulated deficits and cortical changes. **Conclusions:** We demonstrated that mfERG and mfVEP provide complementary and reproducible assessments of retinal and cortical visual function in non-human primates. Quadrant-based pattern reversal showed higher stability and sensitivity, making it a preferred paradigm for detecting localized and disease-related functional changes. Responses of mfERG under pattern reversal, on the other hand, exhibited variable SNR changes in hypertensive eyes, suggesting that its role in disease detection requires further investigation. The present analysis was performed using sector-level averages; future studies should incorporate higher-resolution spatial mapping to clarify the extent of localized functional impairment.

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Background and Purpose: Chemical injuries, including nitrogen mustard (NM), cause acute and prolonged corneal damage, emphasizing the need for therapeutics to enhance healing. MG53 is a membrane repair protein with known cytoprotective effects in multiple tissues, while its ocular function remains largely unexplored. This study investigates the therapeutic potential of recombinant human (rh)MG53 in mitigating NM-induced corneal injury. **Methods:** Human corneal epithelial cells (hCEC) were treated $\pm$ NM (100 μ M, 2h) $\pm$ rhMG53 (50 μ g/mL, 12h) (n=4); ELISAs were used to evaluate expression of γ H2AX, 4HNE, and VEGF. Dissected and isolated rabbit corneas were stabilized on agar and randomly assigned to treatment groups. NM (100 μ M, 2h) was applied to the central cornea using filter paper discs; uninjured corneas were used as controls. After rinsing, corneas received 50 μ L of topical thermosensitive hydrogel (thermogel) $\pm$ rhMG53 (0.8mg/mL, 24 h) (n=5). Corneas were collected and processed for real-time PCR to evaluate expression of Cox2 and MMP9. To evaluate the effects of rhMG53 *in vivo*, the right eye of wildtype (WT) mice received a 2% NM injury and was randomly assigned to a treatment group. rhMG53 treated mice received 5 μ L of thermogel loaded with 0.8mg/mL rhMG53 (n=11), while control mice received thermogel alone (n=10). Treatment was applied once a day for 14 days. Fluorescein staining and photographs were taken on days 0, 1, 3, 5, 7, 10, and 14 post-NM injury. Corneal opacity and fluorescein staining were quantified using a published scoring system. At study termination, corneal flat mount staining was performed for the nerve marker β -III tubulin. To evaluate how MG53 influences baseline corneal nerve density, uninjured corneas from WT, MG53 knockout (KO), and MG53 overexpressing (OE) transgenic mice were stained for β -III tubulin (n=3). Expression of β -III tubulin was measured and quantified using confocal microscopy and ImageJ. ANOVA and Mann-Whitney U tests were performed to determine statistical significance. **Results:** hCEC exposed to NM had increased expression of γ -H2AX (p<0.001), 4HNE (p<0.05), and VEGF (p<0.05), compared to NM-injured cells treated with rhMG53. NM-injured corneas treated with thermogel alone had significantly higher Cox2 (p<0.01) and MMP9 (p<0.05) expression compared to NM-injured corneas treated with rhMG53-loaded thermogel. NM-injured mice treated with rhMG53 showed significantly (p<0.05) reduced fluorescein staining, an indicator of re-epithelialization, and significantly (p<0.05) reduced corneal opacity, compared to controls. Injured corneas treated with rhMG53 had a corneal nerve density and branching pattern similar to uninjured corneas. Injured corneas treated with unloaded thermogel had a significant (p<0.05) reduction in nerve density and a disrupted nerve pattern, compared to uninjured eyes. Uninjured eyes from MG53 OE mice showed a significantly higher corneal nerve density than KO (p<0.01) and WT mice (p<0.05); KO mice showed significantly (p<0.01) lower corneal nerve density than WT mice. **Conclusions:** rhMG53 significantly reduces NM-induced acute DNA damage, oxidative stress, and pro-angiogenic signaling in hCEC. *Ex vivo*, rhMG53 significantly reduces NM-induced corneal inflammatory and protease signaling. *In vivo*, topical rhMG53 significantly accelerated re-epithelialization and reduced opacity, supporting its pro-regenerative effects. Notably, our findings identified a neuroregenerative function of MG53. The observed variation in baseline corneal nerve density in MG53 KO and OE mice highlights MG53's potential role in regulating corneal nerve homeostasis. These findings provide molecular insights into rhMG53's regenerative mechanisms, supporting its therapeutic potential for treating corneal injury.

PS 4335 Development of a Quantitative and Objective Scoring System for Drug-induced Ocular Toxicity

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Background and Purpose: Growing awareness of ocular diseases such as glaucoma, diabetic retinopathy and age-related macular degeneration (AMD) has prompted varied approaches to pharmaceutical development worldwide. Advances in drug administration techniques have enabled specialized delivery methods, including intravitreal (IVT), subretinal, and intracorneal injections. While the importance of evaluating ocular toxicity is widely recognized, challenges persist in achieving quantitative and objective assessments of ocular toxicity. The Draize test in particular, although commonly used for evaluating ocular toxicity, is limited to assessing the external structures of the eye. To overcome these limitations, we have developed a modified scoring system designed to enable quantitative and objective evaluation of overall ocular condition. **Methods:** The modified scoring system evaluates eight parameters: Conjunctival Hyperemia (CH), Keratic Precipitates (KP), Aqueous Cell (AC), Aqueous Flare (AF), Iris Involvement (II), Anterior Lens Capsule Deposits (AL), Posterior Lens Capsule Deposits (PL), and Vitreous Haze (VH). Each parameter is scored on a scale from 0 (normal) to 3 or 4 (severe). The criteria for scoring the severity of each finding were established based on substantial references. The evaluation was performed using slit-lamp biomicroscopy and ophthalmoscopy. **Results:** This scoring system was applied to evaluate ocular toxicity in a single IVT injection nonclinical toxicity study. There were no significant changes in parameters, other than KP and AC, in either the control or low-dose groups. These findings suggest that KP and AC were likely induced by the injection procedure, rather than the test article. Other findings-particularly those related to the posterior segment of the eye (PL,

VH)-showed dose-dependent increases in severity, indicating a likely correlation with the test article. **Conclusions:** Using the newly developed scoring system, ocular toxicity was evaluated quantitatively and objectively, allowing a more detailed assessment of both the anterior and posterior ocular structures. This system builds upon the Draize test in providing quantitative and objective evaluations, but overcomes its limitations by enabling comprehensive assessments of the posterior segment of the eye. As specialized ocular administration methods continue to evolve, and as ocular toxicity evaluations can be anticipated to grow in importance, this scoring system has the potential to become an essential tool in ocular toxicity assessment. Although this scoring system was applied to New Zealand White rabbits, it is planned to expand its use in future studies to evaluate ocular toxicity in rodents, dogs and monkeys.

PS 4336 Reproductive Risk Assessment of Intravitreal Biopharmaceuticals in the Last 20 Years: A Comparative Evaluation of Marketed IVT Biologics

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Background and Purpose: Intravitreal (IVT) biologics targeting VEGF and Ang-2 pathways have transformed the management of retinal diseases. However, despite the availability of these approved therapeutics, fewer than half of treated patients experience meaningful visual improvement. This highlights the need for novel drugs that address both endothelial dysfunction and structural vascular changes. At the same time, concerns about the reproductive safety of these agents remain. **Methods:** This review compares nonclinical reproductive toxicity data for four biologic agents approved in the last 20 years, faricimab (Vabysmo®), brolucizumab (Beuvo®), aflibercept (Eylea®), and ranibizumab (Lucentis®), using publicly available regulatory documents and literature across species and study designs. **Results:** Species selection and study design varied significantly across reproductive studies presented in regulatory documents and US package inserts. Nonhuman primates (NHPs) were the pharmacologically relevant species selected for faricimab and brolucizumab dosed IVT, while rabbits were dosed intravenously for aflibercept and ranibizumab, achieving systemic exposures far exceeding clinical intravitreal (IVT) levels. In an NHP embryofetal development study, faricimab induced a non-dose-dependent increase in pregnancy loss in NHPs at IV doses 158 $\times$ human peak concentration (Cmax), with no NOAEL identified. Aflibercept caused fetal malformations and maternal toxicity in rabbits at all tested doses, with no developmental NOAEL identified. In an NHP fertility study, aflibercept disrupted menstrual cycles and sperm morphology at systemic exposures up to 1500 $\times$ human levels. Ranibizumab showed minimal skeletal abnormalities in NHPs at high IVT doses (~13 $\times$ human exposure), with no placental or maternal toxicity. Brolucizumab demonstrated no reproductive toxicity in NHPs at 10 $\times$ human IVT exposure. Anti-VEGF mechanisms are associated with impaired placental angiogenesis, fetal resorption, and fertility disruption. Molecular design influences placental transfer and systemic exposure; agents lacking intact Fc regions (e.g., ranibizumab, faricimab) may offer reduced fetal risk. While anti-drug antibody (ADA) formation was observed across products, its impact on toxicokinetics and reproductive outcomes was generally limited. However, ocular inflammation following intravitreal administration of recombinant biologics is a well-documented phenomenon across preclinical species and is often linked to both local and systemic ADA responses. These immune reactions can compromise animal health and confound study interpretation, particularly in species like rabbits that can exhibit heightened immunogenicity. Systemic ADA formation has been shown to precede ocular ADA detection, suggesting a systemic origin that may contribute to broader physiological changes such as inflammation, weight loss, and organ toxicity. These systemic effects can exacerbate pregnancy loss and developmental abnormalities, complicating the interpretation of DART findings. Regulatory guidance and peer-reviewed literature emphasize that ADA-related effects in animals may not reliably predict human immunogenicity or clinical risk, underscoring the importance of integrating mechanistic understanding, improved bioanalytical tools, and thoughtful species selection into nonclinical study design. **Conclusions:** In conclusion, these findings underscore the importance of species selection, dosing route, and molecular format in evaluating reproductive risks per ICH S5 (R3) and S6 (R1) guidance. While systemic exposure is limited with IVT administration, the shared antiangiogenic mechanism warrants caution, particularly in women of childbearing potential. Interpretation of animal data should consider species-specific sensitivity, ADA-related inflammation, and pharmacokinetic profiles to inform clinical decision-making and labeling.

PS 4337 Comparison of Ocular Features in Bama and Göttingen Minipigs

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Background and Purpose: Minipigs have become increasingly popular in ocular research due to their anatomical similarities to human eyes. In the USA and Europe, the Göttingen minipig is commonly used for ocular research, while the Bama minipig is typically the strain of choice in China. This study aims to provide comparative data on the ocular features between the two strains. **Methods:** Bama mini-

pigs aged 4-9 months (females: n=14 eyes; males: n=12 eyes) were used for ocular parameters collection. The anatomical parameters—axial length, anterior chamber depth, lens thickness, and vitreous chamber depth—were measured with an ultrasound scanner; central corneal thickness was measured with a corneal pachymeter; retinal thickness was measured by Optical Coherence Tomography (OCT); vitreous volume was measured using *ex vivo* eyeballs. The physiological parameter, intraocular pressure (IOP), was measured with a tonometer. Fundus photographs (FP) were also collected. The data obtained from Bama minipigs was analyzed across three age groups (4-5, 6-7, and 8-9 months) and compared with the published data on Göttingen minipigs of comparable ages. Toxicity bridging studies were also conducted to compare the test-article-related responses between the two strains. **Results:** With increasing age, the measured anatomical parameters were as below: axial length was 16.39±0.44, 17.89±0.60, and 19.72±0.04 mm; anterior chamber depth was 1.55±0.23, 2.82±0.29, and 3.27±0.19 mm; lens thickness was 5.79±0.24, 6.40±0.22, and 7.20±0.10 mm; vitreous chamber depth was 8.02±0.32, 8.59±0.38, and 9.25±0.05 mm; central corneal thickness was 632±42, 674±58, and 776±13 µm; retinal thickness ranged from 264±21 to 273±12 µm; intraocular pressure ranged from 15±1 mmHg to 17±3 mmHg. The measured vitreous volume was 2.1±0.2 mL. Compared with the published data, the ocular anatomical parameters, IOP, and FP in Bama minipigs were comparable to those in Göttingen minipigs. In toxicity bridging studies, the endpoints, including ophthalmologic examination, IOP, electroretinography (ERG), OCT, and histopathology examinations, were consistent in both strains. **Conclusions:** Based on the comparable ocular anatomical parameters, IOP, and FP results, and consistent test article-related responses observed between age-matched Bama and Göttingen minipigs, Bama minipigs could also serve as an appropriate species selection for ocular pharmacokinetics, pharmacodynamics, and toxicology research.

PS 4338 Safety Profiling of Ocular Buffers in the Context of Posterior Segment Gene and Cell Therapeutics

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Background and Purpose: Ocular gene and cell therapies have shown transformative potential in treating inherited and acquired retinal disorders, including retinitis pigmentosa, age-related macular degeneration (AMD), and diabetic retinopathy. The success of these therapeutic modalities depends not only on vector or cell design but also on the physicochemical properties and tolerability of the formulation excipients used for intravitreal (IVT) and subretinal (SR) administration. Ocular formulations are often supplemented with stabilizers, salts, and surfactants such as albumin, hyaluronate, and Pluronic to enhance vector or cell viability; however, their intraocular safety has not been comprehensively profiled. The objective of this study was to evaluate the ocular tolerability of commonly used buffer formulations following IVT or SR delivery in preclinical rodent models. **Methods:** A total of 60 Rowett Nude (RNU) rats (N=10/group) received either IVT or SR injections with one of the following formulations: balanced salt solution (BSS), BSS Plus, 0.2% sodium hyaluronate in BSS, 0.1% human serum albumin (HSA) in BSS, and 1% or 5% dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS). Additionally, 20 C57BL/6 mice (N=10/group) were injected IVT with high-salt PBS with or without 0.001% Pluronic F68. Retinal structure and injection quality were assessed immediately post-dose (Day 1) using optical coherence tomography (OCT) and fundus imaging, and again at Day 30 to assess recovery and tolerability. Retinal function was evaluated using full-field or pattern electroretinography (ERG) on Day 30. **Results:** Successful delivery into vitreous and subretinal compartments was confirmed by Day 1 imaging. Animals with lens damage or vascular injury were excluded. By Day 30, all groups exhibited normal retinal architecture, except for mild to moderate vitreous inflammation observed in both IVT and SR groups administered 0.1% HSA in BSS. ERG analysis revealed preserved a- and b-wave amplitudes in all buffer groups except DMSO formulations. A dose-dependent SR toxicity was evident with 1% and 5% DMSO, characterized by reduced ERG amplitudes and outer retinal disruption, whereas IVT delivery of DMSO at comparable concentrations was well tolerated. **Conclusions:** These findings demonstrate that ocular buffers such as BSS, BSS Plus, and sodium hyaluronate are well tolerated following IVT and SR injection, whereas formulations containing albumin or DMSO can induce route-dependent retinal stress or inflammation. The results underscore the importance of excipient selection and imaging confirmation for safety evaluation in the development of posterior segment gene and cell therapeutics.

PS 4339 Ocular Topical RTA 408-Mediated NRF2 Activation Mitigates Chloropicrin-Induced Corneal Injury

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Background and Purpose: Chloropicrin (CP; CCl₃NO₂, trichloronitromethane) is a volatile chemical that causes immediate ocular irritation, intense pain, excessive tearing, and temporary blindness. Currently, there are no approved treatments for CP-induced ocular toxicity. Beyond its historical use in warfare and terrorism, CP-related occupational and accidental exposures have risen in the United States

due to its increasing use as a fumigant and pesticide. The nuclear factor erythroid 2-related factor 2 (NRF2) signaling pathway regulates cellular defense against oxidative stress and inflammation through the induction of antioxidant genes and detoxifying enzymes. Previous studies using NRF2 knockout (KO) mice have shown that loss of NRF2 exacerbates CP-induced oxidative stress and inflammation in the cornea. Based on this, we evaluated the efficacy of RTA 408 (omevaloxone), a synthetic triterpenoid and an FDA-approved NRF2 activator, in mitigating CP-induced corneal injury. **Methods:** Male Balb/C mice (8-10 weeks old; 4-5 mice per group) were exposed to 2.5% CP vapor for 1 minute using a vapor cap. Control mice were exposed to the vehicle only. Mice in treatment groups received either a 30-minute pre- or post-exposure application of topical RTA 408 (0.1 and 1%), while control groups received corn oil. Clinical examinations were performed using slit-lamp microscopy from 3 hours up to 7 days post-exposure. Mice were euthanized at 1- and 7-days post-exposure, and corneal tissues were collected for cDNA preparation. Gene expression analyses were conducted using qPCR for oxidative stress marker [NAD(P)H: quinone oxidoreductase 1 (*Nqo1*)] and proinflammatory cytokines [Interleukin-1 beta (*Il-1β*), C-C motif chemokine ligand 2 (*Ccl2*), C-C motif chemokine receptor 2 (*Ccr2*), Interleukin-6 (*Il-6*)]. Statistical analyses were performed using one-way ANOVA with multiple comparisons, and significance was set at *p* < 0.05. **Results:** Topical RTA 408 treatment increased *Nqo1* expression in the cornea, confirming NRF2 pathway activation. In contrast, CP exposure suppressed *Nqo1* expression at both day 1 and day 7 while significantly upregulating *Ccl2*, *Il-1β*, *Ccr2*, and *Il-6* at day 1 post-exposure. By day 7, mice treated with RTA 408 exhibited a 16-99% reduction in proinflammatory cytokine expression compared to untreated CP-exposed mice (*p* < 0.05). These molecular findings correlated with clinical observations: untreated mice developed severe corneal ulceration, whereas RTA 408-treated mice showed markedly reduced ulceration at 1-day post-exposure. **Conclusions:** Our findings demonstrate that topical RTA 408 effectively activates the NRF2 signaling pathway in the cornea, thereby reducing inflammation and tissue injury following CP exposure. These results identify NRF2 activation as a promising therapeutic strategy for CP-induced ocular injuries. Ongoing studies are exploring the combined efficacy of NRF2 activators and super-saturated oxygen emulsion therapy in further mitigating CP-induced corneal damage.

PS 4340 Generation of a Novel Immortalized Human Corneal Epithelial Cell Line for Reliable In Vitro Ocular Toxicity Studies

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Background and Purpose: The corneal epithelium is a transparent cell layer that covers the cornea and allows light to enter the eye. It serves as a critical barrier against pathogens and environmental insults, protecting deeper ocular tissues. Due to its protective and regenerative functions, the corneal epithelium is widely used in studies of wound healing, drug development, permeation, and toxicity. While animal models are commonly employed in ocular toxicity testing, they often fail to accurately reflect human responses. Human primary corneal epithelial cells offer a more relevant alternative, but their short lifespan and donor-dependent variability limit their suitability in long-term, reproducible studies. To address these limitations, we developed an immortalized human corneal epithelial cell line that retains key functional characteristics of primary cells while enabling extended culture and consistent performance in ocular toxicity assays. **Methods:** We established a clonal immortalized human corneal epithelial cell line by stably expressing human telomerase reverse transcriptase (hTERT) and a mutant cyclin-dependent kinase 4 (CDK4^{R24C}) in normal primary human corneal epithelial cells. We assessed cell morphology, proliferation rate, expression of epithelial markers, migratory capacity, and performance in *in vitro* eye irritation assays. **Results:** The resulting cell line, hTERT HCEC (ATCC® CRL-4067™), was cultured for over 125 population doublings without signs of replicative senescence. It exhibited an abnormal female human karyotype and maintained epithelial-like morphology. Both primary cells and hTERT HCEC expressed cytokeratin 14 (KRT14) and demonstrated migratory ability. In irritation assays, treatment with glycolic acid or benzalkonium chloride led to a dose-dependent reduction in cell viability, confirming the cell line's responsiveness to toxic agents. **Conclusions:** The hTERT HCEC cell line successfully overcomes the limitations of primary human corneal epithelial cells while preserving essential functional attributes. This model provides a robust and reproducible platform for *in vitro* eye irritation testing and broader ocular toxicology applications.

PS 4341 Novel putative mechanism of corneal toxicity associated with netarsudil

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Background and Purpose: Ocular irritation manifesting as instillation pain, hyperemia, and erythema are common adverse events associated with topical administration of the Rho-associated protein kinase (ROCK) inhibitor, netarsudil, and occur in >50% patients. Generally attributed to the vasodilative effects of ROCK inhibitors, the purpose of this study was to evaluate whether netarsudil elicits changes in corneal permeability that may contribute to ocular irritation. **Methods:** Human corneal epithelial cells (HCE-T) were seeded in 12 mm transwell inserts with 0.4 µm pore polycar-

bonate membranes (cellQART®) and cultured in Dulbecco's modified Eagle's medium (supplemented with 5% fetal bovine serum, 0.5% dimethyl sulfoxide, 5 mg/mL insulin, 10 ng/mL human recombinant epidermal growth factor and 100 U/mL penicillin-100 µg/mL streptomycin; 0.3 mL donor, 1 mL receiver chambers) until confluent (1 wk). In a separate set of experiments, HCE-T cells were stratified in an air-liquid interface in transwells (4 wk). Cells in the donor chamber were exposed to 0.02% netarsudil or vehicle (DMSO) for up to 1 h. Permeability of 6-carboxyfluorescein (6-CF; 376 Da, paracellular), Dextran-Texas Red (DTR; 10 kDa, paracellular), Rhodamine B (479 Da, transcellular) and AlexaFluor™488-conjugated immunoglobulin G (IgG; 150 kDa, low permeability) were calculated from fluorescence values measured with Cytation5 plate reader (Agilent) and the apparent permeability coefficient was calculated. **Results:** In monolayer corneal epithelial cells, netarsudil elicited a time-dependent decrease in transcellular transport (RhoB, $n=7$, $p<0.01$) and a concomitant increase in paracellular transport for 6-CF ($n=11$, $p<0.01$) and DTR ($n=6$, $p<0.05$). Permeability of AlexaFluor™488-conjugated IgG was similar between vehicle and netarsudil treated cells ($n=6$, $p=0.31$). In stratified corneal epithelial cells, netarsudil resulted in a decrease in the P_{app} ($n=6$, $p<0.01$). **Conclusions:** Our findings suggest that netarsudil perturbs epithelial barrier function via disruption of tight junctions. Diverging effects observed in monolayer vs. stratified corneal epithelial cells likely result from differences in actin cytoskeletal tension and mechanics, and diffusion kinetics across a multilayered corneal epithelium. Our findings are consistent with the reported adverse events associated with netarsudil and may offer an additional mechanism contributing to corneal toxicity associated with ROCK inhibitors.

PS 4342 Particulate matter PM₄ and PM₁₀ elicit neutrophil pathology *in vitro*

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Background and Purpose: Particulate matter (PM) poses a significant toxicologic concern to the ocular surface, causing acute ocular toxicity and increasing the risk for developing long term sequelae including chronic dry eye disease. Formation of neutrophil extracellular traps is a contributor to ocular surface pathophysiology in >50% of patients with different subtypes of dry eye. The purpose of this study was to investigate effects of small and large fine atmospheric particulate matter on human neutrophils *in vitro*. **Methods:** Standard Reference Material PM₄ (SRM® 2786) and PM₁₀ (SRM® 2787) were obtained from the National Institute of Standards and Technology. Primary human neutrophils were isolated from human whole blood using bead-based immunopurification (Miltenyi). Extracellular DNA was quantified by Sytox™ Green labeling and quantification of neutrophil markers by flow cytometry. Effects of PM on neutrophil adhesion to corneal epithelial cells (HCE-T) was determined using plate-reader based quantification of calcein-labeled neutrophils. Data were analyzed by One-Way or Two-Way ANOVA with Holm-Sidak's multiple comparisons test, as appropriate, and graphed in Prism software. **Results:** Freshly-isolated primary human neutrophils were exposed to PM₄ or PM₁₀ for 2 h, or the positive control, PMA (20 nM), for 20 min. Exposure resulted in increased expression of CD11b and CD16. Furthermore, exposure to PM₄ (75 and 150 µg/mL) and PM₁₀ (150 µg/mL) significantly increased the percentage neutrophils undergoing NETosis as measured by Sytox™ Green fluorescence ($n=5$, $P < 0.01$). PM₄ (10-300 µg/mL) dose-dependently increased neutrophil adhesion to corneal epithelial cells ($n = 3$). The level of adhesion was similar to that elicited by pathophysiologically relevant anti-citrullinated autoantibodies (ACPA, 100 ng/mL). Pooled human immune globulins (ocular surface immune globulins, OSIG) are currently developed for the treatment of dry eye disease and prevented PM-induced adhesion to corneal epithelial cells ($n=3$, $P<0.01$). **Conclusions:** These findings extend prior evidence that PM exacerbates neutrophilic inflammation and provide mechanistic insight into the association between PM exposure and both acute ocular toxicity and chronic ocular surface disease. The ability of OSIG to block PM-induced neutrophil adhesion highlights a potential therapeutic strategy for mitigating pollutant-driven ocular surface inflammation.

PS 4343 Distinct retinal epithelial responses to carbon black nanoparticles versus PM2.5: oxidative stress-driven EMT and barrier disruption

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Background and Purpose: Retinal fibrotic diseases affect more than 11 million people in the United States, leading to irreversible vision loss in these patients. Emerging evidence links particulate matter to fibrotic processes in ocular tissues. Our previous studies in C57BL/6 mice demonstrated that chronic exposure to ultrafine particulate matter alters retinal and corneal thickness and induces TGF- β -mediated α -SMA-expression consistent with fibrotic remodeling. Activation of the TGF- β /SMAD axis and α -SMA overexpression are known to initiate epithelial-mesenchymal transition (EMT) in epithelial cells, a pivotal event in ocular fibrosis. Particulate matter of less than 2.5 microns (PM2.5) generally exhibits an elemental carbon core coated with diverse organic compounds, which together influence their reactivity and biological effects. Currently, there is limited understanding of how the carbon core from PM2.5 interacts with and affects the retinal layer. The present study investigates how expo-

sure of human retinal pigmented epithelial (ARPE-19) cells to PM2.5 or carbon black nanoparticles (CBNPs) activates the TGF- β /SMAD/ α -SMA pathway, leading to EMT and barrier disruption. **Methods:** To demonstrate the differential effect between the carbon core and the complete PM2.5, CBNPs were employed as a simile of the carbon core in this study. PM2.5 was collected during the Summer 2025 in Irvine, California, using a PM2.5 High Volume Air Sampler with a HiVol cascade impactor (Tisch, USA). Monarch 120 CBNPs were purchased from Cabot Corporation (USA) (particle size range 10 - 300 nm). ARPE-19 cells were exposed to increasing concentrations (0 - 10 µg/cm²) of PM2.5 or CBNPs for 24, 48, and 72 hours. After exposure, cell viability was assessed using MTS and LDH assays. Additionally, cells were fixed and stained with an α -SMA antibody (to evaluate protein expression) or a BODIPY antibody (to assess lipidic oxidative damage) at 72 hours. Images of the fixed cells were collected in a High-Resolution Fluorescence Microscope Keyence KV-8000. Lastly, trans-epithelial electrical resistance (TEER) was measured after 24, 48, and 72 hours. **Results:** Cell viability assays showed no cytotoxicity at any concentration or time point tested for either particle. After 72 hours, CNBP exposure produced a concentration-dependent increase in lipid oxidation and α -SMA protein expression, accompanied by an increased TEER at 1.0 µg/cm² but a decrease at 5.0 µg/cm², indicating loss of epithelial integrity at higher doses. In contrast, exposure to PM2.5 exhibited elevated lipid oxidation, and increased TEER values at 1.0 and 5.0 µg/cm², suggesting an adaptive or barrier-strengthening response. These findings indicate that CBNPs, but not PM2.5, induce dose-dependent oxidative stress and α -SMA upregulation consistent with the initiation of epithelial-mesenchymal transition (EMT). The contrasting TEER responses highlight particle-specific effects on retinal epithelial barrier function, likely driven by differences in particle size and chemical composition. **Conclusions:** Together, these findings demonstrate particle-specific molecular responses in retinal pigmented epithelial cells. CBNPs induced oxidative stress, α -SMA overexpression, and loss of barrier integrity, consistent with the initiation of epithelial-mesenchymal transition (EMT) and early fibrotic remodeling. In contrast, PM2.5 exposure caused lipid oxidation but preserved or enhanced barrier function, suggesting a transient or adaptive response. Overall, the results indicate that retina epithelial is more prone to interact with smaller particles, underscoring the potential role of ultrafine particles in promoting ocular fibrosis and vision impairment.

PS 4344 Topical dexamethasone intervention reduces acute and delayed ocular damage in sulfur mustard vapor-exposed rabbit eyes

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Background and Purpose: Sulfur mustard (SM) is a potent alkylating agent, causes acute corneal injury and progressive, irreversible damage termed mustard gas keratopathy (MGK). MGK is characterized by chronic discomfort, corneal degeneration, vision loss, and dose-dependent recurrent lesions (RELs). No therapy currently halts MGK progression. This study investigates early dexamethasone (Dex) treatment to suppress inflammation, limit initial injury, and reduce delayed lesion incidence and chronic corneal degeneration following SM exposure. **Methods:** New Zealand White rabbits ($n = 32$) were SM exposed for 60 s using a novel vapor cap model, inducing recurrent lesions in 50% of eyes within 8 weeks. Post-exposure, 0.1% dexamethasone was administered topically three times daily for 5 days. Weekly assessments via slit-lamp and optical coherence tomography (OCT) monitored disease progression. At study termination, corneal tissues were harvested for histopathological and molecular analyses. **Results:** Slit-lamp and OCT images reveal progressive corneal opacity and edema post-SM exposure, with fluorescein uptake indicate epithelial disruption at day 3 and recovery by day 5. -Dex eyes show sustained thickening, hypercellularity, and stromal edema, while +Dex mitigates these changes ($p = 0.0135$). At day 56, -Dex exhibits bullous keratopathy as recurrent lesions, +Dex suppresses recurrence and preserves corneal integrity. The Kaplan-Meier analysis for corneal thickness confirms delayed lesion onset and significantly ($p = 0.0300$) reduced recurrence with Dex treatment through day 56. **Conclusions:** Dexamethasone reduces acute corneal edema following sulfur mustard (SM) vapor exposure and suggests its potential to attenuate acute ocular inflammatory responses. It also mitigates recurrent lesion emergence and chronic pathologies. Early intervention with dexamethasone reduces both acute and later long-term SM-induced ocular injury, potentially by suppressing inflammatory cascades that drive progressive corneal degeneration. These findings advance ocular toxicology by establishing a pharmacological framework for counter-acting vesicant-induced tissue damage and guiding therapeutic strategies in chemical injury models.

PS 4345 The Effect of Glucagon-like Peptide 1 (GLP-1) Receptor Agonists and Estradiol Treatment on the Diabetic Inner Retinal Blood Barrier

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Background and Purpose: Diabetic Retinopathy (DR) is a major cause of vision loss in millions of U.S. adults. Here, toxins and other harmful compounds enter the retina via lowered integrity of the inner blood-retinal barrier (iBRB), a multi-partite cellular tissue that regulates molecular transport between the bloodstream and neuroretina.

A rapidly increasing number of American adults are prescribed Glucagon-like Peptide 1 Receptor Agonist (GLP-1RA), such as Semaglutide, as treatment for diabetes and/or obesity. However, rapid weight loss and significant improvements in blood glucose caused by Semaglutide have been linked to a higher risk of DR exacerbation in some overweight or obese patients. Our research focuses on estradiol (E2), a known regulator of glycemic control and retinal health. Studies show that E2 helps preserve retinal ganglion cells and maintain iBRB integrity. Previous studies from our lab have also shown that moderate E2 levels are critical for maintaining iBRB integrity and lowering harmful reactive oxygen species production in iBRB cells. It is our hypothesis that estrogen can be used as a co-treatment to improve Semaglutide actions that impact vision. **Methods:** Rat progenitor retinal cells (R28) were used to model Muller glia, which are regulators of iBRB integrity. Cells were conditioned in media containing 1 µg/mL of advanced glycation end products (AGEs) for at least 7 days to create diet-induced hyperglycemic condition. The AGE treated cells were treated with Semaglutide (100 nM), E2 (10 pM), or a combination of Semaglutide and E2 treatments for 7 days. AGE treated and non-AGE treated cells serve as controls in the following readouts. Cells were seeded to collagen and fibronectin coated transwell inserts on day 3. Permeability assay was performed on day 7 by adding Rhodamine B (1 mg/mL) and 4kDa fluorescein isothiocyanate dextran molecules (1 mg/mL) to the apical side of the transwell inserts containing the cells. Media from the basolateral side were collected at 1, 3, 5 and 12 hr time points for fluorescent plate reading. Immunocytochemistry was done for AGE receptor, AGER and the mean fluorescent intensity was quantified to determine the effect of Semaglutide and E2 treatments. **Results:** Rhodamine B diffused easily across cells, irrespective of treatment, likely facilitated by paracellular transport. On the other hand, AGE conditioning caused a statistically significant increase in the passage of 4 kDa Dextran. Semaglutide and E2 treatments did not improve the permeability of the 4 kDa Dextran. AGER expression in AGE conditioned cells which received both Semaglutide and E2 treatments were statistically lower than cells that received only the AGE conditioning without treatment. Additionally, combined treatment of Semaglutide and E2 treatments were significantly lower than either treatment alone. In non-AGE treated cells, AGER expression was higher in the nucleus as per the nucleus to cell mean intensity ratio. AGE treatment statistically reduced AGER expression in the nucleus. Only the combined treatment of Semaglutide and E2 to the AGE treatment restored the increased nuclear localization. Our current studies examine the expression of GLP-1R and GPER30 through immunocytochemistry and perform permeability assay of 70 kDa Dextran molecules to increase our mechanistic understanding of the effect on iBRB cells. **Conclusions:** Our current results show that combined treatment of Semaglutide and E2 may restore the AGE receptor expression that is associated with cell permeability and barrier function. To increase our understanding of the permeability function, we aim to perform Transepithelial Electrical Resistance (TEER) across the cells in the future. This current investigation behind iBRB disruption could lead to improved understanding on adverse effects from GLP-1RA treatment to the retina as well as offer improved treatment plan to mitigate those effects.

PS 4346 Development of an *in chemico* test battery to assess eye irritation of surfactants and surfactant-dominant consumer products

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Background and Purpose: Assessment of eye irritation following accidental exposure is critical to the holistic quantitative risk assessment of surfactant-dominant consumer products. Historically, albino rabbits were used to classify materials based on their extent of ocular damage in the Draize test. However, the use of the Draize method is restricted by major regulatory authorities because of ethical concerns, the subjective nature of the methods data interpretation, and human relevancy due to species differences. Given that new chemistries with no eye irritation data are continually innovated, alternative methods that allow for the efficient assessment of eye irritation must be innovated. Protein denaturation and lipid disruption are key molecular initiating events in the eye irritation adverse outcome pathway. Multiple *in chemico* assays measuring protein denaturation and lipid disruption have been proposed as methods to assess eye irritation. Still, to date no single test has been demonstrated to sufficiently predict the eye irritation potential of surfactant-dominant mixtures. Therefore, our objective is to develop an *in chemico* test battery to determine relative eye irritation potencies based on a test materials ability to disrupt cellular proteins and lipids. Therefore, we are 1) investigating the physical chemical properties of surfactants which drive these effects to understand assay applicability; and 2) building a mechanistic understanding of the molecular changes which promote eye irritation **Methods:** **Hemoglobin Denaturation Assay (%HDR):** %HDR is utilized to determine the ability of a test item to denature hemoglobin as a representative protein hemoglobin. A dilution series of the test material is prepared in water and added to a 96-well plate. An Hb/phosphate buffer solution is then added to the samples. This mixture is incubated for 5 minutes before its optical absorbance is measured by a spectrophotometer. The %HDR is then calculated $(100 - (100 \times (\text{Hb} + \text{test item}/\text{Hb} + \text{H}_2\text{O})))$ using values blanked to H₂O. **Ca²⁺ Sequestration Assay:** Intracellular Ca²⁺ maintains the structural integrity of tight junction proteins that preserves corneal epithelium connections. A dilution series of the test material is prepared in HEPES buffer solution containing CaCl₂ and added to a 96-well plate. A calcium detecting dye is added to the samples, which are then incubated for 5 minutes before the optical absorbance is recorded. The % sequestered calcium is then calculated $(\% \text{ sequestered calcium} = 100 - (100 \times (\text{test item}/\text{H}_2\text{O})))$ using values blanked to the buffer without CaCl₂. **Liposomal Disruption Assay:** A commercially

available liposome permeability fluorescence assay (ProFoldin, Hudson, MA) is used as a surrogate to understand the potential of a test material to disrupt a lipid bilayer. The assay is performed according to manufacturers instructions. **OptiSafe:** OptiSafe is a commercially available *in chemico* test method which measures changes in macromolecules to assess eye irritation potential (Lebrun Labs, Anaheim, CA). It is validated to classify chemicals that are non-classified (OECD TG 496), but has not been validated for mixtures. To investigate mixture potency, surfactants and formulas with well characterized eye irritation potencies were diluted and prepared according to manufacturer's directions. **Results:** The tested surfactants exhibited reliable inter and intra-experimental reproducibility. Moreover, more potent eye irritants as determined by historical methods (e.g., isolated chicken eye (ICE) test) were demonstrated to induce elevated responses across the *in chemico* test battery. Finally, surfactant-dominant formulations were observed to induce scores in each assay that correlated with their established eye irritation potential. **Conclusions:** Development and optimization of eye irritation assays that are reproducible and relevant for surfactant-dominant cosmetic formulations is critical for assessing the human safety of finished products. This work demonstrates that an *in chemico* test battery, which measures protein and lipid macromolecular disruptions central to the development of eye irritation, has promise to be used in a weight of evidence assessment of surfactants and surfactant-dominant consumer product formulations.

PS 4347 NWD-19: A First-in-Class Mitochondrial Therapeutic Demonstrating Safety and Efficacy in Chemical-Induced Ocular Surface Injury

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Background and Purpose: This study was performed to evaluate the cytotoxicity of NWD-19 in human corneal epithelial cells *in vitro*. Additionally, *in vivo* therapeutic efficacy of NWD-19 was determined when administered by topical ophthalmic instillation in mice following benzalkonium chloride (BAK) induced chemical toxicity. **Methods:** For *in vitro* assessment, cells were seeded (10⁵ cells/well) on 96-well plates and grown for 2 days. Cells were then exposed overnight to a dose-range of NWD-19 (0.005-10 µM) in complete culture medium, DMSO vehicle (0.1%), or media only for 16 h. Toxicity was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake and lactate dehydrogenase (LDH) release assays. For *in vivo* effect, BAK toxicity was induced by instilling 10 µL of 0.2% BAK once daily to both eyes in C57BL/6J mice (n=10/group) from Day 0 to Day 21. Mice received either vehicle (1% carboxymethylcellulose) or 0.003% NWD-19 (5 µL/eye) by three times daily (TID) bilateral topical ophthalmic instillation from Day 8 to Day 21. Mitochondrial selectivity and kinetics were studied for dosing regimen determination. **Results:** *In vitro* concentrations up to 5 µM had no biologically or toxicologically relevant effect on LDH release as a surrogate marker of cytotoxicity or MTT absorbance as a surrogate marker for proliferation. In mice eyes, BAK caused severe dryness and corneal lesions. NWD-19 resulted in a significant reduction in fluorescein scoring at Day 14 and Day 21 compared to the vehicle (p<0.05). Corneal fibrosis predominantly noted by Day 7 was significantly cleared following 7 days of NWD-19 administration. Corneal neovascularization was noted in both vehicle and NWD-19 treated eyes at Day 14 and Day 21 but was lower in the NWD-19 treated group at both timepoints. **Conclusions:** In extended time exposure experiments, NWD-19 was well-tolerated by human corneal epithelial cells at concentrations up to 5 µM. C57BL/6J mice, topical instillation of 0.003% NWD-19, equivalent to a dose of 0.45 µg/eye/day was well tolerated and resulted in a significant attenuation of BAK-induced ocular surface and internal tissue pathology. NWD-19 is being developed as a novel first-in-class mitochondrial therapy for the treatment of dry eye disease.

PS 4349 Modeling Exposure to Polycyclic Aromatic Hydrocarbons (PAHs) in Placentae using Antibody-based Array Proteomics and Machine Learning

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Background and Purpose: The placenta plays a critical role in fetal development and is sensitive to both clinical and environmental influences. In this study, we employed high-throughput proteomic and epigenomic profiling to explore how placental molecular features relate to clinical outcomes and environmental exposures. **Methods:** Using reverse phase protein arrays (RPPA) and gas chromatography-mass spectrometry (GC-MS), we analyzed protein expression, histone post-translational modifications, and levels of three polycyclic aromatic hydrocarbons (PAHs) in placental samples from 41 term and 36 preterm deliveries. Analysis approaches included Weighted Gene Correlation Network Analysis (WGCNA) and machine learning (ML). **Results:** Unbiased analytical approaches, including WGCNA and machine learning ML, revealed distinct protein modules associated with gestational age, birthweight, and maternal BMI. Notably, expression of lactate dehydrogenase A (LDHA) emerged as a top predictor of gestational age and birthweight. PAH levels, particularly benzo(a)pyrene (BaP), were significantly correlated with specific protein expression patterns and maternal BMI. WGCNA revealed a blue module correlated with BaP

at $r=0.328$, enriching for apoptosis, PI3K/AKT/MTOR, and p53 signaling. Machine learning regression enabled modeling of BaP with a goodness-of-fit $R^2=0.47$ using the k-Nearest Neighbor method; top informative proteins with negative association with BaP were pAkt, pAMPKb1, Vimentin, and pBCL2; as little as 5 proteins were sufficient for effective machine learning regression of BaP. **Conclusions:** These findings highlight the utility of integrative molecular profiling in identifying biomarkers of placental function and environmental exposures, enabling the study of their interaction on pregnancy outcomes.

PS 4350 ToxIndex: An agentic AI orchestrator for toxicology expert tools

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Background and Purpose: Toxicologists face expanding heterogeneous data sources (regulatory dossiers, mechanistic literature, and cheminformatic repositories) that are difficult to synthesize within time-sensitive decision windows. ToxIndex is a web-based, agentic AI orchestration platform coordinating analytical workflows, enabling scientists to employ automated processes that interrogate public data, and execute analytical pipelines. The platform integrates cheminformatic engines for metabolite prediction, adverse outcome pathway (AOP) extracting agents, probabilistic toxicology modules, and report-generation utilities. We describe the architecture and application for endocrine and general toxicity assessment. **Methods:** ToxIndex employs a scalable graph-directed workflow engine built with Kubernetes, Redis, and CloudSQL that manages multi-step agent and tool invocations. Users initiate analyses through the web application, where large language model (LLM) powered agents sequence tasks such as (1) invoking the SyGMA metabolite predictor and structure normalization pipelines (RDKit, PubChem); (2) querying curated, ontology mapped, property databases, and literature search APIs; (3) parsing PDFs or regulatory studies to extract AOP evidence; and (4) synthesizing probabilistic risk summaries by fitting beta distributions to observed toxicity rates and calculating posterior probabilities of adversity. Intermediate data products are cached in Google Cloud Storage and surfaced through interactive dashboards for iterative review. **Results:** ToxIndex demonstrates the impact of agentic workflows that determines the potential of developmental neurotoxicity of chemicals through a case-study on acetaminophen. SyGMA metabolite enumeration generated 31 candidate products, which mapped to downstream hazard indices through integrated knowledge bases. The automated system retrieved 2766 publications from PubMed, CTD, and PubTator3 databases. This corpus was reduced to a subset of candidate papers after screening for relevance and reliability checks as defined by the Toxicological data Reliability Tool (ToxRTTool) criteria and classified via an LLM-based approach using Gemini-2.5-Pro. For systematic evaluation of mechanistic plausibility, a weight-of-evidence framework was employed on the selected papers to extract 8394 key events related to developmental neurotoxicity, which were then clustered into 2774 key event clusters based on their causal relationships. Across broader workflow validation, ToxIndex reduced expert review cycle time by an order of magnitude compared to human-based manual document review. The evidence-supported key events converged on ten core mechanistic pathways underlying toxicity in the evaluated chemicals, including disruptions such as redox imbalance with associated antioxidant depletion, as well as Ca^{2+} signaling defects in developing neurons. Probabilistic toxicity modules, integrating clinical cohorts and *in vivo* data, produced posterior risk distributions consistent with historical assessments in validation cases. Stakeholders reported substantial gains in transparency through the system's audit trail, including step-level logs, evidence citations, and reproducible Python artifacts. **Conclusions:** ToxIndex demonstrates how orchestrated LLM agents, cheminformatics, and probabilistic modeling converge in a regulatory-compliant web application to accelerate toxicological evaluations. By unifying document ingestion, pathway mapping, metabolite prediction, and evidence synthesis, ToxIndex enables reproducible, explainable decision support for endocrine disruption and broader toxicity domains. Ongoing development expands the tool catalog and refines active learning loops. This approach provides a blueprint for deploying agentic AI systems that augment expert judgment without sacrificing scientific rigor.

PS 4351 ToxInsightor: Exploring Alternatives to Animal Use for Safety Assessment - a Case Study of Antibody-Drug Conjugates (ADCs)

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Background and Purpose: New Approach Methods (NAMs) are increasingly adopted in risk assessment as regulatory agencies prioritize predictive, human-relevant, and ethical strategies. Selecting a "fit-for-purpose" derisking and mitigation approach requires a robust understanding of current practices for marketed drugs and clinical candidates. Manual collection of this information is time-consuming, labor-intensive, and often incomplete. **Methods:** To address this gap, we developed ToxInsightor, an agentic AI-based platform leveraging a deep tox-specific research framework built on LangChain architecture, Model Context Protocol (MCP), and GPT-4o with tailored toxicology prompt engineering. ToxInsightor provides an integrated solution to curate relevant data, perform automated analysis, and generate evidence-based recommendations for nonclinical risk strategies. **Results:** To validate ToxInsightor, we applied it to antibody-drug conjugates (ADCs) focusing on nonclinical risk strategies. The platform autonomously aggregated and analyzed animal models

and NAMs for ADCs, extrapolating findings to human toxicities and recommending "fit-for-purpose" strategies. For example, extrapolation rates for bone marrow toxicity were 0.82, 0.54, and 0.48 for cynomolgus monkeys, rats, and dogs, respectively; hepatotoxicity rates were 0.43, 0.28, and 0.22 for the same species. Recommended NAMs for hepatotoxicity included cell-based *in vitro* assays and *in-silico* models, with detailed context of use. Toxicologists reviewed these outputs and provided positive feedback on their potential utility for facilitating decision-making. **Conclusions:** ToxInsightor demonstrates strong potential as a tox-specific, multi-agent AI assistant to streamline data curation, enhance risk assessment, and support strategic decision-making in nonclinical safety evaluation.

PS 4352 A Comparative Assessment of Large Language Model (LLM) Performance in Answering Target Safety Assessment Questions

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Background and Purpose: Target safety assessment (TSA) is a critical first step in assessing the potential hazards associated with modulating a biological target in an organism. This requires synthesizing information on phenotypes of genetically engineered organisms and human mutations, expression profiles, and known risks. This process traditionally requires extensive manual evaluation of disparate data from public databases and a vast body of scientific literature. This manual process is time-consuming, resource-intensive, and faces limitations in scalability and speed. Large Language Models (LLMs) present a significant opportunity to accelerate evidence synthesis and analysis in toxicology. However, their reliability and accuracy in addressing specialized, high-stakes domains like TSA require rigorous validation before they can be integrated into toxicology workflows. This study evaluates the performance of several internal and external LLMs in answering complex, expert-generated toxicology questions. **Methods:** A dataset of 120 TSA-related questions was crowd-sourced from practicing toxicologists, covering over 20 drug targets. Target safety themes were categorized by their primary focus i.e., on-target toxicities/risks. Phenotypic outcomes were the most common (40%), followed by established toxicities/risks (20%), tissue expression (15%), and biological function (15%). Target structure and specific molecules in development were less frequent (5% each). Four LLMs were evaluated that included two internally developed LLMs and two external LLMs (Gemini 2.5 Flash Preview 05-20 and Gemini 2.5 Pro Preview 06-05) from Google. The models were tested in two settings: (1) with retrieval-augmented generation (RAG), where models were provided automatically-retrieved scientific literature snippets and asked to synthesize an answer, and (2) without RAG, generating free-form answers without retrieval of external knowledge. A comprehensive scoring rubric was developed to evaluate response quality based on length appropriateness, well-formedness/coherence, relevance, completeness, accuracy, overconfidence, underconfidence, and use of citations (when applicable). All responses were scored by both expert human evaluators and the GPT-4.1 model from OpenAI. **Results:** A comparison of model performance revealed that the internal models (both with RAG and without RAG) and Gemini 2.5 Pro provided the highest-quality responses, scoring particularly well in terms of length appropriateness (80-95%) and well-formedness (60-90%) while Gemini 2.5 Flash provided suboptimal responses particularly with RAG for both length appropriateness (45%) and well-formedness (35%). For all models, the accuracy of the responses improved with RAG, whereas the overconfidence was more pronounced without RAG. Across the evaluation, a good agreement was observed between the scores from the human annotators and the GPT-4.1 evaluator, further indicating the potential for using LLMs in scalable evaluation frameworks. **Conclusions:** These results are encouraging and demonstrate the capability of state-of-the-art LLMs to generate relevant and coherent answers to key TSA related questions. The strong performance, particularly with RAG, supports the continued development and integration of LLMs into internal pipelines to assist in evaluating target safety.

PS 4353 Predicting Multi-Protein Corona Adsorption Patterns Using a Deep Conditional VAE-GAN Model

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Background and Purpose: The protein corona (PC) has widely been demonstrated to impact the pharmacokinetics, safety, and efficiency of nanoparticle (NP) systems for clinical applications such as drug or vaccine delivery. Recently, machine learning (ML) algorithms have increasingly been used to analyze PC formation datasets to predict PC formation and identify complex patterns in NP-PC interactions to improve NP design optimization and pharmacological outcomes. While current models have proven to be robust, they largely focus on single-task objectives, such as predicting individual protein adsorption, which limits their ability to capture complex and dynamic NP-PC interactions like protein co-adsorption that occur *in vivo*. To address this gap, this study aimed to develop a variational autoencoder-generative adversarial model (VAE-GAN) to predict multi-protein adsorption profiles from NP and experimental parameters. **Methods:** NP design and experimental parameters alongside corresponding quantitative PC profiles were obtained from the Protein

Corona Database (PC-DB), a publicly accessible repository. A conditional VAE-GAN model was implemented in PyTorch. Abundance data from 100 frequently adsorbed proteins were min-max scaled, and NP condition features were one-hot encoded. The model comprised an encoder-decoder pair with LayerNorm, LeakyReLU activations, and dropout for latent representation learning, and a batch-normalized discriminator to distinguish real from synthetic profiles. Training minimized composite loss integrating weighted reconstruction, Kullback-Leibler divergence, and adversarial terms, optimized with KL-annealing and a one-cycle learning rate schedule. Model performance was evaluated using RMSE and R^2 metrics, while latent space structure and generated protein corona profiles were analyzed through PCA, t-SNE, and histogram visualizations. **Results:** The optimized VAE-GAN model demonstrated high predictive performance, achieving an average test set R^2 of 0.780 and RMSE of 0.767 across the 100 selected proteins. Latent space visualizations revealed distinct clustering patterns within the generated PC profiles that closely match real experimental data, indicating effective encoding of underlying NP-PC interaction features. These findings demonstrate that the conditional VAE-GAN framework effectively captures multivariate dependencies in NP-PC interactions and can generate realistic, high-fidelity PC compositions *in silico*. **Conclusions:** This study demonstrates that a VAE-GAN framework can effectively generate realistic PC composition predictions that reflect the variability and complexity of experimental datasets. Future work will focus on expanding the dataset with experimental NP-PC data, particularly for understudied *in vivo* exposure and organic NP (e.g., lipid) formulations. In addition, further incorporation of explainable artificial intelligence (XAI) techniques will enhance model interpretability and accessibility for NP-PC researchers.

PS 4354 Dual Readout: Predicting Affinity and Actionable Holo-Complex Structures with *In Silico* Safety Screening

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Background and Purpose: The increasing use of secondary pharmacology screening early in drug discovery programs has led to superior drugs with fewer patient adverse events. However, discovering one or more off-target hits in a secondary pharmacology screening panel does not immediately provide guidance on how to derisk such potential liabilities. **Methods:** Using a physics-based computational platform consisting of induced fit molecular docking and free energy perturbation we created an *in silico* safety panel of kinases, GPCRs and hormone receptors. We found that the approximate assignment of protein re-organization free energy was the missing component needed to successfully employ existing computational tools. Furthermore, using the results of the physics-based calculations we can generate sufficient training data to develop ML models particular to individual anti-targets to rapidly predict binding affinity as a function of the predicted protein-ligand complex. **Results:** We quantify the accuracy of the panel by predicting selectivity of selected compounds against a range of off-targets. Specifically, correlating predicted to experimental affinity, we observe R^2 values of 0.53, 0.69, and 0.60 for sets of unrelated small molecules bound to Estrogen Receptor Alpha, SSTR2, and ACM1, respectively. In the task of predicting kinase selectivity for a set of 19 inhibitors, we observe an R^2 of 0.85 correlating predicted versus the experimental number of hits for each inhibitor against a panel of 55 kinases. A hit is defined as a K_D of 10 μ M or stronger. **Conclusions:** The computational screening panel provides a highly valuable dual readout: (1) a predicted binding affinity (K_D) and (2) an atomistic structural model (holo-complex structure) of the ligand binding to the off-target. This structural model is a superior readout to affinity alone, as it offers mechanistic insight into the binding interaction that is often unavailable from a standard experimental assay. This combined information allows drug discovery teams to efficiently iterate safer molecular designs by using the structural models to rationalize chemical modifications that disrupt the off-target interaction without affecting the primary target.

PS 4355 Evaluation of Single and Multitask Methods for Secondary Pharmacology Prediction with Cross-Panel Validation

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Background and Purpose: The prediction of secondary pharmacological effects of small molecules is a critical aspect of drug discovery, aiding in the identification of potential off-target effects. In this study, we systematically evaluated the performance of both single and multitask methods using diverse machine learning algorithms. Single-task methods, including k-Nearest Neighbors, Random Forest, Gradient Boosting, and Support Vector Machines, were compared against multitask methods such as Bayesian matrix factorization (Macau) and a Graph-Convolutional Neural Network (GCNN). **Methods:** The dataset comprises over 3,000 proprietary small molecules screened at Genentech in a standard secondary pharmacology assay panel that covers a wide range of off-targets (>40 targets/assays considered in this study) with variable hit rates. To evaluate the influence of public domain data, bioactivity data was pooled from ChEMBL target records. In addition, we performed cross-panel comparisons using Novartis's published Secondary Pharmacology Database (SPD) and Eurofins's SafetyScreen Panel, alongside Genentech internal

data, to assess concordance and model portability across public and private sectors. Single- and multitask regression models were trained and validated on both proprietary and combined proprietary and public datasets using standard cross-validation and time-split validation methods, and were benchmarked against historical internal controls and reference compounds. **Results:** Our findings reveal that the performance of these methods depends on the nature of the task (i.e., assay) and data distributions, such as the fraction of active ($pIC50 \geq 5$) compounds. Overall, multitask methods demonstrated superior performance, especially in data-rich tasks. Even more notably, multitask learners improved recall for tasks with fewer active compounds. On the other hand, as expected, single-task methods exhibited higher precision due to the skewness of the $pIC50$ distribution toward inactive compounds. The addition of public domain data improved model performance overall and increased cross-source portability. **Conclusions:** Together, these findings suggest that multitask learning may help alleviate class-imbalance problems and improve rare event detection. Furthermore, public-private data integration can help make better predictions especially in data-poor or data-imbalanced tasks. In most cases, multitask methods were best suited to take advantage of data-rich tasks and transfer information to more data-poor ones. However, single-task methods remain generally competitive. In safety assessment, these insights are used to refine predictive modeling strategies and ultimately facilitate more informed decision-making in early drug development.

PS 4356 Investigating the mechanisms of emerging and legacy contaminants bioconcentration in fish tissues using machine learning models

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Background and Purpose: The rapid increase in the production and usage of anthropogenic organic chemicals inevitably results in their release into the environment. These contaminants can bioaccumulate in aquatic animals and pose potential threats to human health. Surface water is a major reservoir for the release of anthropogenic chemicals. These contaminants could be absorbed, distributed, metabolized, or excreted across different tissues in aquatic animals and pose harm or even death to the aquatic animals. Although existing models such as QSAR (Quantitative Structure-Activity Relationship) and CART (Classification and Regression Trees) have been used to predict key mechanisms, they either focused on a set of pre-selected physicochemical descriptors or had low prediction accuracy. This study aimed to leverage multiple feature representations and advanced machine learning models to improve the prediction of bioconcentration mechanisms. **Methods:** We used a published Bioconcentration factor (BCF) dataset with 763 chemicals categorized into three classes of fish bioconcentration mechanisms: (1) inert chemicals that accumulate in lipids, (2) chemicals interacting with tissues, and (3) chemicals undergoing metabolism or elimination. We tested four types of input features: (1) physicochemical descriptors; (2) RDKit-generated molecular features from chemical SMILES; (3) molecular fingerprints; and (4) a combination of molecular features and physicochemical descriptors. We trained and fine-tuned multiple machine learning models, including Gradient Boosting Decision Trees (GBDT), Support Vector Classifier (SVC), Logistic Regression (LR), and Random Forest (RF), and compared their predictive model performance. We further developed a webtool based on model performance and user convenience. **Results:** GBDT model achieved the best predictive performance among all tested models, with an accuracy of 0.899 ± 0.024 and a recall of 0.811 ± 0.064 using physicochemical features. RDKit-derived descriptors with the addition of logKow and logBCF had competitive performance with 0.859 ± 0.026 accuracy and 0.756 ± 0.037 recall. LogBCF and logKOW were the most influential physicochemical features in classifying the chemicals' bioconcentration mechanism, followed by RDKit descriptors such as Chi1n and VSA_Estate1. Molecular fingerprints had poor prediction performance in this dataset. Recall was prioritized to minimize misclassification of highly bioaccumulative chemicals. We successfully built a webtool where users only need to input a chemical's CAS number and logKOW value to obtain a predicted bioconcentration mechanism class from our combined feature set of molecular features and physicochemical descriptors. **Conclusions:** This study demonstrates that machine learning models, particularly GBDT, can accurately classify fish bioconcentration mechanisms. Physicochemical descriptors offered the highest predictive value, while RDKit-derived features provided complementary performance advantages and ease of automated feature retrieval. Our web-based prediction tool facilitates rapid, accessible prediction of chemical bioconcentration mechanisms, supporting chemical risk assessment and regulatory decision-making in ecotoxicology.

PS 4357 Examining Lead Emissions in the Computers/Electronics Industry and Their Relationship with Socioeconomic and Health Outcomes

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Background and Purpose: With the rapid advancement of artificial intelligence (AI) and the growing global demand for electronic products, the computers and electronics industry has expanded substantially in recent years. Lead emissions from this sector may exacerbate existing socioeconomic and health disparities, particularly

in industrialized and urbanized regions. This study aimed to examine temporal and spatial trends in lead emissions from the computers and electronics industry across the United States between 2010 and 2024 and to investigate their relationship with socioeconomic and health indicators. **Methods:** Lead emission data from the U.S. Environmental Protection Agency's Toxics Release Inventory (TRI) were extracted and analyzed for the years 2010-2024 for computer and electronics manufacturing facilities. Spatial maps were generated annually to identify spatial and temporal patterns. Socioeconomic variables such as household income, age, education, race, population size, and insurance coverage were extracted using U.S. Census data at the county level for matched years. We used unsupervised t-SNE and UMAP visualization to explore patterns in socioeconomic outcomes within high and low emission county groups. **Results:** Lead emissions showed substantial temporal and spatial heterogeneity, peaking in 2012 at 9272 pounds, compared with a mean of 4,646 pounds across the remaining years. Emissions were concentrated in urban and suburban regions, with notable hotspots in Allen County (IN), Oakland County (MI), and Madison County (AL). Disproportionately high emissions were observed in Middlesex (MA), Alameda (CA), and Hillsborough (FL). High-emission counties such as Jackson, MO, and Madison, AL had a median age of 37.7 years, a median household income of \$58,654, and a bachelor's degree attainment rate of 15.8%. In contrast, low-emission counties, including Anderson, TN, and El Paso, CO, had a higher median age of 43.4 years, a median household income of \$54,290, and a lower bachelor's degree completion rate of 11.1%. **Conclusions:** This study highlights lead emissions from the electronics manufacturing sector and their uneven distribution across U.S. regions, with notable concentrations in specific urban and industrial regions. Further work will be done using mortality data from CDC WONDER to quantify the spatiotemporal influence of lead emissions on disease-specific death rates. Our findings underscore the need for continued monitoring and regulation of heavy metal emissions to mitigate environmental and health inequities associated with industrial growth.

PS 4358 Leveraging Targeted Gene Sets and Neural Networks for Zebrafish Transcriptome Extrapolation in High-Throughput Toxicogenomics

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Background and Purpose: Zebrafish (*Danio rerio*) are a powerful vertebrate model for developmental toxicology and chemical safety assessment, yet large-scale transcriptomics in zebrafish remains limited by cost and data heterogeneity. Targeted transcriptomics offers a cost-effective alternative, but extrapolation methods tailored to zebrafish have not been systematically developed or evaluated. While the S1500+ platform is widely used for toxicogenomics research with rat, mouse, and human cell lines as model systems, its use in zebrafish has been limited due to lack of bioinformatics approaches for analysis of such data. To that end, we sought to (i) curate a large zebrafish transcriptomic training data resource, and (ii) evaluate multiple extrapolation strategies to reconstruct unmeasured transcriptome-wide expression profiles for data originating from the zebrafish-specific reduced representation gene set ("S1500+"). **Methods:** We assembled 14,924 zebrafish RNA-Seq samples covering 21,930 genes across 1,246 studies. Using the S1500+ gene subset (3,062 genes), we trained and tested three extrapolation approaches: principal components regression (PCR), a locally weighted extension (PCR+), and a neural network mixture-of-experts model (NN-MoE). Model performance was assessed by mean absolute error (MAE), mean squared regression error (MSRE), and weighted variants of these metrics. **Results:** Extrapolation performance using the baseline approach was strongly influenced by tissue and developmental context, with within-tissue models outperforming cross-tissue models. Errors were lowest when training and testing were conducted within the same tissue or between developmentally related tissues. Both PCR+ and NN-MoE improved upon the baseline PCR approach, with NN-MoE reducing average MAE by ~20% and MSRE by ~17%. Importantly, extrapolation remained reliable for the majority of genes, even when restricting predictions to those below a practical MAE threshold. **Conclusions:** We demonstrate that targeted transcriptomics can be effectively extended to zebrafish, enabling robust transcriptome-wide extrapolation at reduced cost. The NN-MoE method provided the most substantial gains, highlighting the value of non-linear and ensemble modeling in heterogeneous datasets. These results establish a scalable framework for zebrafish toxicogenomics and suggest that accuracy will continue to improve with larger, better-annotated datasets, paving the way for broader application in chemical safety assessment.

PS 4359 Learning Aging and Disease related Epigenetic Trajectory for Type 1 Diabetes

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Background and Purpose: Type 1 diabetes (T1D) is a chronic autoimmune disease that leads to insulin deficiency due to the destruction of β cells in the pancreas, and patients often exhibit signs of accelerated aging. Emerging evidence suggests that epigenetic modifications play a critical role in T1D; however, the continuous trajectory of epigenetic changes related to T1D remains largely undiscovered. To

address this, we developed a novel mixed-effects neural ODE model that learns aging and disease related epigenetic trajectory for T1D from longitudinal DNA methylation data. **Methods:** The proposed model incorporates a population level age-modulated fixed effect, a disease group effect, and an individual level random effect to separate universal aging patterns from disease-specific methylation trajectories. The study uses a peripheral whole blood DNA methylation dataset consisting of 87 healthy control and 87 T1D cases with up to five visits each. To reduce dimensionality, we identified 200 age related CpGs, 285 T1D related CpGs, and 250 individual features using random forest models. Model performance was evaluated through 5-fold cross validation and compared to an RNN baseline. **Results:** The mixed-effects neural ODE achieved better performance compared to the baseline models with RMSE = 0.24 and R^2 = 0.97, demonstrating its ability to efficiently learn population dynamics while incorporating group and individual differences. Predicted trajectories for a T1D-associated CpG showed distinct patterns between T1D and control cases. **Conclusions:** The study proposed a novel mixed-effects neural ODE model that learns continuous epigenetic trajectories for longitudinal DNA methylation data while distinguishing disease specific patterns from universal aging related trajectories. While effective, the approach may be limited by the use of a targeted CpG panel and simplified dynamic assumptions. Future work includes comparison with other sequence models and biological interpretation of selected CpG sites.

PS 4360 Advancing Molecular Design: Leveraging Language Models for Modular De Novo Compound Generation via LlamaSMILES

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Background and Purpose: Molecular design traditionally involves several cycles of generation, evaluation, and optimization. This trial-and-error approach is time-consuming and costly. *De novo* design of chemicals based on *in silico* methods and machine learning faces challenges in flexibility, efficiency, and synthetic feasibility. Here, we present LlamaSMILES, an innovative approach for training large language models (LLMs) to generate Simplified Molecular Input Line Entry System (SMILES) strings fine-tuned to facilitate multi-parameter optimization. **Methods:** The model was pretrained on large public chemical databases, including PubChem, ZINC, and DSSTox, to learn general molecular representations. After pretraining, the base model was fine-tuned on specific tasks or properties of interest, such as non-toxicity, non-carcinogenicity, and other desirable molecular characteristics. The fine-tuned model was then used to generate novel molecules that met predefined criteria. Additionally, user-supplied or proprietary data were incorporated for further fine-tuning to optimize the model for specific applications or domains. This iterative pretraining and fine-tuning process enabled efficient generation of customized molecules with targeted chemical and biological properties. As a proof of concept, we employed our novel approach to design safer and more effective flame retardants (FRs). FRs are high-volume chemicals widely used in industrial and consumer products, but many existing compounds exhibit high toxicity and bioaccumulation, resulting in widespread regulatory bans. To efficiently evaluate LLM model performance, we developed a custom Quantitative Structure-Activity Relationship (QSAR) model trained on 315 publicly available FRs with a range of limiting oxygen indexes (LOI) to predict FR efficacy and retrosynthesis feasibility using ASKCOS. **Results:** The LOI QSAR model achieved an R^2 of 0.618 in a 3x5-fold cross-validation, indicating good predictive performance for limiting oxygen index. We then applied this model to a library of 2,369 newly generated, novel flame-retardant (FR) candidate molecules to predict their flame-retardant properties. The QSAR predictions indicated that over 67% of the generated molecules are likely to be FRs with LOI values > 26, with an additional ~10% predicted to be flame resistant (LOI > 25), confirming a high LOI for the majority of the novel FR molecules. Among these, 176 of the 2,369 novel FRs simultaneously met our high-efficacy criterion (LOI > 27), low-toxicity criteria (non-mutagenic and non-carcinogenic), and exhibited high retrosynthetic accessibility, as assessed by ASKCOS. **Conclusions:** Our *in silico* results show robust model performance, and we are currently evaluating the novel FRs using combustion tests. Our novel LLM-based molecular design approach is easily customizable to work on a desired set of chemical properties, lending itself to a broad range of molecular design tasks.

PS 4362 AI-Assisted Physiologically Based Pharmacokinetic (PBPK) Modeling with Transfer Learning to Predict Biodistribution of Diverse Microplastics

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Background and Purpose: Microplastics (MPs) are emerging contaminants of growing concern in environmental and human health, yet their toxicokinetic behavior remains poorly characterized due to limited experimental *in vivo* data. Physiologically based pharmacokinetic (PBPK) models can mechanistically predict tissue distribution and internal exposure; however, existing PBPK models are typically developed for specific MP types and cannot be readily extrapolated to diverse MPs due to their physicochemical heterogeneity. To address this challenge, we developed an integrated computational framework that leverages nanoparticle (NP) pharmacoki-

netic knowledge to predict MPs biodistribution using transfer learning. The goal is to establish a data-driven, AI-assisted PBPK modeling approach capable of inferring critical kinetic parameters for MPs from their physicochemical properties. **Methods:** A comprehensive NP database was established by curating biodistribution data from previous peer-reviewed studies, covering physicochemical properties including particle size, surface charge, surface coating, and core material. A multi-compartment, membrane-limited PBPK model was constructed by incorporating mechanistic features including organ-specific partition coefficients, permeability-surface area products, saturable mononuclear phagocyte system uptake with Hill kinetics, first-order release rates, and biliary and renal elimination pathways. Each NP formulation's time-concentration profile in blood, liver, spleen, kidney, lung, and tumor was fitted to optimize critical NP-specific parameters. Local sensitivity analysis identified the top 10 influential parameters governing biodistribution. Optimized parameter sets, paired with physicochemical descriptors, were used to train a deep neural network (DNN) for predicting PBPK parameters from material properties. The trained NP DNN will then serve as the source model for transfer learning, enabling fine-tuning of the network with limited MP data to predict MP-specific PBPK parameters and simulate biodistribution profiles. **Results:** The calibrated NP PBPK model accurately predicted time-concentration profiles across six organs and multiple formulations, achieving an average $R^2 = 0.65$, representing substantial improvement over common parameter approaches. Organ-specific accuracy was highest in lung ($R^2 = 0.76$, $RMSE = 4.6\%ID/g$) and tumor ($R^2 = 0.61$, $RMSE = 2.13\%ID/g$). Sensitivity analysis revealed that partition coefficients (lung, kidney), permeability-surface area products (spleen, lung, kidney), biliary and urinary elimination constants, and half-saturation constants (liver, kidney, spleen) were consistently among the top-ranked parameters. Parameter variability spanned 0.01-100 $\times$ relative to initial estimates, reflecting formulation-dependent kinetic diversity. The resulting parameter-descriptor matrix forms the foundation for machine learning-based inference of PBPK parameters for new nanomaterials and, through transfer learning, for MPs with sparse experimental data. **Conclusions:** This study establishes a mechanistic-AI hybrid framework that integrates PBPK modeling, machine learning, and transfer learning to bridge the data gap in MPs toxicokinetic. By learning transferable pharmacokinetic representations from NP, the model can infer MP-specific parameters and predict biodistribution even in the absence of *in vivo* calibration. This AI-assisted PBPK approach supports quantitative risk assessment of emerging contaminants, aligns with the 3Rs principles, and represents a scalable paradigm for predicting internal dosimetry across data-poor materials. Once verified, this framework can be extrapolated to model the biodistribution of diverse MPs with varying physicochemical properties.

PS 4363 CONNECT Knowledgebase: Cohort-Oriented Network kKnowledgebase for Enhanced Collaboration

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Background and Purpose: Environmental health cohorts have expanded substantially through recent efforts to pool diverse study populations, increasing the need for efficient summarization and integration of findings across studies. Children are uniquely vulnerable to environmental exposures, yet knowledge from existing cohort publications remains difficult to synthesize because investigators must manually review hundreds of papers and environmental-health terminology lacks harmonization. These challenges limit scalable knowledge extraction and slow evidence dissemination for early-life environmental risks. To address this gap, we are developing CONNECT Knowledgebase, a Cohort-Oriented Network kKnowledgebase for Enhanced Collaboration. CONNECT aims to streamline knowledge curation through web scraping and large language model, unify environmental-health language, and provide an interactive platform for summarizing, visualizing, and advancing discovery across population-based cohort studies. **Methods:** We developed a multi-stage AI-assisted knowledge-extraction pipeline using our previous Normative Aging Study Cohort Network (121 publications). First, we implemented automated literature extraction through Selenium-based web scraping of DOI links, applying a $\geq 3,000$ -word threshold to identify full-text articles suitable for analysis. Second, we used the GPT-3.5-turbo API with prompt-engineering strategies to extract exposures and outcomes. Extracted terms were mapped to a three-layer hierarchical taxonomy: Layer 1: broad exposure or outcome class; Layer 2: specific exposure or outcome category; Layer 3: measurement-level detail. Fuzzy string matching ($\geq 80\%$ similarity) was applied to capture formatting variants and validate semantic consistency. Third, we tested prompting approaches (e.g., zero-shot/few-shots prompting, role prompting, chain of thoughts prompting, self-consistency prompting) and tuned ChatGPT hyperparameters to improve extraction accuracy. We are currently manually curating a children's environmental health cohort knowledgebase, and our next step is to validate this AI-assisted pipeline using the newly curated dataset. This pipeline represents the foundational step for scalable development of the CONNECT Knowledgebase. **Results:** Our evaluation across 121 publications showed strong performance of the AI-assisted extraction pipeline for both exposures and outcomes. At the terms level, exposure extraction achieved 98.2% accuracy at Layer 1, 86.6% at Layer 2, and 92.8% at Layer 3. Outcome extraction showed a similar pattern, with 97.4% accuracy at Layer 1, 86.5% at Layer 2, and 80.6% at Layer 3. Paper-level evaluation, which assesses whether the model correctly identified at least one accurate term in each publication, demonstrated consistently high coverage across taxonomy layers. Exposure extraction reached 100% at Layer 1, 99.2% at Layer 2, and 98.3%

at Layer 3. Outcome extraction reached 99.2% at Layer 1, 95.8% at Layer 2, and 87.6% at Layer 3. Overall, the pipeline reliably captured high-level concepts and maintained strong performance at more granular measurement layers, supporting its use for scalable knowledge curation in environmental health cohorts. Additionally, we built an interactive website (cohortnetwork.org) to enable more convenient access to the knowledge graph and to facilitate exploration of cohort-related publications. **Conclusions:** We successfully developed an AI-assisted pipeline as the foundational first step of CONNECT, enabling systematic and scalable extraction of environmental health information from cohort publications. The pipeline demonstrated strong performance across hierarchical taxonomy layers and supports efficient knowledge graph construction. As we continue building our children's environmental health knowledgebase, future work will validate this approach in the newly curated cohort dataset and expand extraction to include additional entities such as study population and statistical link.

PS 4364 Predictive Modeling for Toxicovigilance in HIV Therapy: Leveraging Machine Learning and National Surveillance Data in Resource-Limited Settings

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Background and Purpose: In resource-limited settings, pharmacovigilance systems often lack the capacity for real-time detection of antiretroviral therapy (ART)-related toxicities such as hepatotoxicity, nephrotoxicity, and treatment-limiting adverse drug reactions. This study developed and validated a machine learning-based predictive model to identify risk patterns for ART-related complications using Malaysia's national HIV surveillance data. **Methods:** Key programmatic indicators were extracted from Malaysia's Global AIDS Monitoring (GAM) reports (2023-2024), including ART coverage, viral suppression rates, CD4 counts at diagnosis, and regional disparities in service delivery. A gradient boosting classifier was trained on demographic and clinical variables—emphasizing late ART initiation ($CD4 < 350$ cells/mm³), treatment continuity, and viral suppression status—to stratify patients into risk categories for long-term ART toxicity. Model performance was evaluated using stratified 10-fold cross-validation and independent test data. Feature importance analysis identified the strongest predictors. **Results:** Late ART initiation, suboptimal viral suppression, and regional health service gaps emerged as key predictors of ART-related toxicities. The model achieved an overall accuracy of 85% and an AUROC of 0.89, indicating excellent discriminatory ability in differentiating high- from low-risk patients. High-risk profiles clustered in Sabah, Kelantan, and underserved urban districts. The top predictors were CD4 count at entry, viral suppression at 12 months, and treatment continuity. These findings demonstrate the potential of real-world HIV surveillance data to inform predictive toxicovigilance and guide targeted follow-up strategies, particularly in resource-constrained settings. **Conclusions:** Integrating machine learning with national HIV surveillance data enables predictive toxicovigilance tools that can proactively identify patients at risk for ART-related toxicities. Such approaches are scalable, data-driven, and well-suited for resource-limited health systems, supporting early intervention, safer ART delivery, and more efficient allocation of clinical follow-up resources.

PS 4366 Safety-ome and Panels for Over 3,000 Phenotypes: a Systematic and Translational Approach Using Human Genetics and Pharmacology

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Background and Purpose: Drug candidates are often evaluated for their activities against unexpected targets (off-targets), to either prospectively flag potential hazards or to provide mechanistic insights for a given phenotype. The *in vitro* to *in vivo* translatability is critical when selecting "phenotypically consequential" off-targets. **Methods:** Human genetics and indication-based pharmacology offered unraveled insights. Enhanced natural language processing tools were applied to harness the power of large data obtained from 7 genetics and 2 pharmacology databases. **Results:** Mapping biological roles to organ systems, we curated targets implicated in 22 organ systems of safety concerns, resulting in a Safety-ome composed of over ~11,000 proteins. This is a significant expansion from the previously selected off-target proteome, whose scope include 5 organ systems. Prioritization of the large panel using expression pattern and gene conservation across species resulted in a core panel of 500 targets. Mapping biological roles obtained from the databases to more specific terms than organ systems allowed us to systematically generate over 3,000 phenotype-based panels, which can be used as gene or protein sets for issue resolution. All curated components were systematically and successfully validated using independent data source, i.e., gene expression data from the Comparative Toxicogenomics Database. All panels, together with a user-friendly App, are published to aid effective safety assessment and issue resolution with strong "translational" focus. **Conclusions:** This study integrated evidence from human genetics and pharmacology to identify crucial safety-related proteins (termed Safety-ome), as well as a small-footprint gene panel to support screening and user-friendly app. Utilizing standardized vocabulary for mapping phenotypes enables the systematic collection of gene or protein sets. These phenotype-centric panels can be used to guide safety issue resolution and provide mechanistic insights.

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Background and Purpose: Phthalates are high-production-volume chemicals widely used as plasticizers in consumer products. Phthalate exposure can disrupt ovarian function and lead to reproductive toxicity. There is an urgent need to develop new approach methodologies (NAMs) to evaluate phthalate toxicity and elucidate associated underlying toxicity mechanisms. Here, we integrated machine learning (ML) with *in vitro* screening assays to evaluate phthalate-induced reproductive toxicity by identifying associated molecular targets and pathways. **Methods:** We retrieved all available bioassay data from PubChem for a curated phthalate dataset comprising phthalates (ortho-phthalates) and their analogs (isophthalates and terephthalates), and prioritized assays suitable for toxicity evaluations and computational modeling. Quantitative structure-activity relationship (QSAR) models were developed using five ML algorithms and four types of chemical descriptors for these assays. The resulting models were applied to impute activities within the target compounds without experimental data for the selected assays, thereby constructing a bioprofile that enabled the construction of pathway models for evaluating reproductive toxicities. To ensure reliability, an in-house quantitative PCR (qPCR) assay was used to assess the phthalate-induced molecular initiating events (MIEs) and key events (KEs) by measuring expression changes in candidate genes in mouse ovarian follicles exposed to phthalates or their analogs. **Results:** QSAR models across all 56 selected assays showed good predictive performance, with an average correct classification rate (CCR) of 0.74. Fourteen top-hit assays for phthalates and analogs were prioritized, encompassing nuclear receptors, metabolic enzymes, and cellular toxicity markers, which are potential targets involved in regulating steroidogenesis, cell cycle progression, and apoptosis in ovarian follicles. Phthalates and analogs exhibited similar bioprofiles across these top-hit assays, indicating shared modes of action of reproductive toxicity. qPCR assays validated target gene expression changes consistent with predictions, enabling construction of an adverse outcome pathway (AOP) network. In this network, phthalates and analogs activate nuclear receptors such as PPAR γ (MIEs), which alters downstream gene expression (KE1), disrupts steroidogenesis and promotes granulosa cell apoptosis (KE2), and leads to estradiol reduction and hormone imbalance (KE3). These perturbations ultimately result in ovarian dysfunction and impaired fertility as adverse outcomes (AOs). The AOP network provides a mechanistic framework for evaluating reproductive toxicity of phthalate-related chemicals and other toxicants acting through the same MIEs. **Conclusions:** This study establishes a hybrid NAM that integrates ML modeling with *in vitro* assays to evaluate phthalate-induced reproductive toxicity. We identified critical molecular targets and developed an AOP network that captures the cascade from MIEs to AOs. This novel framework can be broadly applied for chemical prioritization and regulatory decision-making, ultimately protecting female reproductive health.

PS 4368 *In silico* modeling of on-target and off-target safety pharmacology of oligonucleotides

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Background and Purpose: Therapeutic oligonucleotides are rapidly expanding modalities for metabolic, cardiovascular and rare diseases, yet their clinical success is often limited by hybridization-dependent and hybridization-independent off-target effects, immune-stimulation, and on-target overdose in tissues with high exposure. Early de-risking therefore demands a mechanism-based safety assessment integrated into lead optimization. We constructed a comprehensive *in-silico* toxicology platform that combines sequence bioinformatics, machine-learning models and targeted wet-lab assays to predict and rank safety liabilities of double-stranded oligos. The workflow supports rational sequence selection, reduces animal usage and aligns with the FDA predictive toxicology roadmap. **Methods:** Four sequential modules were developed. (1) Lead sequence design: A base-walk algorithm sliding 1 nt across the start region of the gene of interest was used to generate all possible 19-23-mer siRNA duplexes; empirical rules of guide strand selection and thermodynamic asymmetry were applied for candidate filtering, and sequences containing TLR-agonist motifs were removed to avoid immune response. (2) Off-target prediction: The full sequence of the guide strand was aligned on full transcriptome using BLAST, and the seed region (positions 2-8) was specifically searched against human 3' UTRs; predicted off-target hits were filtered by degree of sequence complementarity and weighted by tissue-specific expression levels. (3) Secondary structure prediction: RNAfold-derived free energy for candidate siRNAs, siRNA-mRNA duplex, and the local secondary structure of the target mRNA at each potential binding site were compared and integrated into a machine learning model to rank candidates. (4) Experimental validation: On-target silencing efficiency was evaluated by qPCR, and transcriptome-wide off-target effects were assessed using RNA-seq. **Results:** The platform was applied to an INHBE-targeting, GalNAc-conjugated siRNA program for metabolic disease. Starting from 23 possible 19-mer duplexes, we shortlisted five leads with predicted Abs. IC50 < 0.1 nM. Genome-wide mapping identified X high-

risk off-target transcripts in liver; RNA-seq confirmed no significant suppression of these genes in hepatocytes. One sequence containing a TLR-stimulating motif was removed. **Conclusions:** Integrating sequence-based bioinformatics and ML-driven activity ranking provides a transferable safety assessment for GalNAc-siRNA therapeutics. The case study delivered a development candidate with robust hepatic potency and minimal immunostimulatory potential.

PS 4369 Predicting Target Tissues of Chemical Exposure Using Coupled Tensor-Matrix Completion

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Background and Purpose: Accurately predicting which tissues are affected by environmental chemical exposures remains challenging due to complex exposure patterns and limited toxicity data. Traditional *in vitro* and *in vivo* approaches are still essential for testing chemical toxicity. However, these methods require substantial time and resources. The existing toxicology-related biological data now make it feasible to build computational approaches that can help prioritize toxicity testing. Given the high sparsity of toxicology data, matrix completion offers a promising approach for uncovering meaningful patterns of chemical toxicity. The purpose of this study is to apply a novel algorithm, Coupled Tensor-Matrix Completion (CTMC), to predict tissue-specific toxicity by integrating tissue-specific gene expression profiles. **Methods:** Human-related chemical-gene interaction data were extracted from the Comparative Toxicogenomics Database (CTD). Chemicals annotated as "approved" drugs in DrugBank were removed. Chemical-gene pair counts were normalized and rescaled to reduce the influence of extreme values while preserving variation among low-abundance signals. The gene-gene similarity tensor included three gene similarity matrices, and one tissue-specific gene expression correlation matrix. The three gene similarity matrices were calculated with R package GOSemSim based on Gene Ontology (GO) annotations. Tissue-specific genes were identified from GTEx transcriptomic profiles, with highly expressed genes selected using an automated elbow-point detection algorithm that determines the inflection point in ranked expression values. The chemical similarity tensor incorporated two types of chemical similarities with Morgan and topological torsion fingerprints using the RDKit package. Three tissue-specific benchmark chemical gene interaction datasets were constructed for the kidney, liver, and lung, each containing the highly expressed genes of the corresponding tissue along with randomly selected genes. To evaluate algorithm performance, overall interaction values for two randomly selected chemicals, one well-studied and one less characterized in the CTD, were removed from the matrix and subsequently predicted. For each tissue, the above procedure was repeated ten times, each time with a different pair of randomly selected chemicals, to generate ten distinct datasets. Thus, each tissue was tested with 10 well-studied and 10 less characterized chemicals. The CTMC algorithm was implemented in C++ with Armadillo and OpenBLAS, enabling efficient processing of matrices containing 1,000 chemicals and 1,000 genes. **Results:** To evaluate model performance, the number of original interactions that could be recovered was first assessed. In the kidney dataset, the model recovered 97% (66/68) of tissue-specific and 96% (43/45) of non-tissue-specific interactions for 10 well-studied chemicals, and 94% (17/18) and 98% (42/43), respectively, for 10 less-characterized chemicals. In the liver dataset, recovery rates were 86% (304/354) and 80% (119/149) for well-studied chemicals, and 76% (34/45) and 100% (13/13) for less-characterized chemicals. In the lung dataset, all interactions for less-characterized chemicals were correctly predicted, while 88% (71/81) of tissue-specific and 73% (16/22) of non-tissue-specific interactions were recovered for well-studied chemicals. To characterize tissue-specific toxicity, overall chemical-gene interaction profiles were evaluated for each chemical by comparing interaction values between tissue-specific and non-tissue-specific genes. A Wilcoxon rank-sum test was conducted to compare predicted interaction scores between tissue-specific and non-tissue-specific targets. Significant differences (FDR < 0.05) in interactions with tissue-specific genes were observed for 17 of 20 chemicals in kidney and lung, and for 14 chemicals in liver, with chemicals showing higher interaction values demonstrating stronger tissue-specific effects. **Conclusions:** The results from the tissue-specific analysis not only demonstrate the CTMC method's ability to predict single chemical-gene interactions but also its strong potential for predicting tissue-specific toxicity. Benchmark analyses indicate that CTMC effectively captures chemical-gene interaction patterns across different tissues, suggesting its capability to identify tissue-specific toxic effects.

PS 4370 Mechanistic insights in toxicological risk assessment: integration of transcriptomics into the Adverse Outcome Pathways liver network

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Background and Purpose: Risk assessment of pharmaceuticals, additives and other chemicals are crucial in protecting the public from potentially hazardous substances. Though advancements have been made to elucidate toxicity of compounds in animal models and *in vitro* assays, these models often lack insights into the mechanism

that leads to toxicity and lack high-throughput capabilities, which is critical given the magnitude of compounds to be assessed. The Adverse Outcome Pathway (AOP) frames biological processes from Molecular Initiating Events through Key Events (KE) to the Adverse Outcome (AO), describing how a molecular event can lead to an Adverse Outcome at the Organ, Organism or Population level. To quantify AOPs for risk assessment, integration of omics data, in particular co-expression gene sets, would enable high-throughput quantification of KEs and reduce the need for traditional, animal-based testing of untested compounds. The aim of this research is to integrate gene co-expression network modules (WGCNA) from toxicogenomics (TXG) data in the AOP framework for liver adverse events, to quantify the potential of compounds exposure to perturb KEs. Biological relevance of the integrated modules in the context of the KEs will also be assessed to determine the performance of the integration methods. **Methods:** We analyzed TXG data from large perturbational studies of primary human hepatocytes (PHH) and rat *in vivo* liver data (both from the TG-GATES datasets), and HepG2 (Wijaya et al., 2023) and constructed specific WGCNA networks for each dataset (Callegaro et al., 2019; Callegaro et al., 2023). Then, we constructed a liver AOP network by collecting AOP data from the AOP-Wiki RDF (Martens et al., 2023) and integrated co-expression modules based on gene content of both sources as well as contextual matching of the biology by performing NLP. Finally, a case study assessed the performance and applicability of the matching within the context of liver steatosis (LS). **Results:** Transcriptomic modules into key events (KEs) are matched thanks to the combination of the several methods implied, highlighting that transcriptomic integration is achievable only by looking at gene, ontology and description content. Around 1/3 of all KEs are assigned to one or more modules, while nearly all modules from the three test systems were integrated in the AOP data, highlighting the need for better curation of several KEs. Several KEs of the liver steatosis networks are matched to multiple modules in more than one liver test system, being PHH and rat *in vivo* modules more represented than HepG2 modules. Interestingly, modules from different test systems matched to the same KE shows a large portion of non-overlapping genes, indicating that omics integration should take into account test systems specifications. **Conclusions:** By applying several orthogonal methods of integration we have delivered transcriptomic integration in the liver AOP network employing gene co-expression network analysis, gene content, ontology content and NLP-based matching. Importantly, we highlight how each species and cell type (and condition of cell growth) influences the integration, delivering different genes sets associated to KE depending on the test system. This is of crucial importance when considering the application of omics integration to AOP for quantification of KE for, for example, read-across and transcriptomic Point of Departure determination. This work is currently supported by the EFSA funded project TXG-MAP and has received funding from Partnership for the Assessment of Risks from Chemicals (PARC) (grant agreement No 101057014).

PS 4371 Tricyclic pyrone CP2 restores lipid and energy dyshomeostasis in the APP/PS1 mouse model of Alzheimer's disease

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Background and Purpose: Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the leading cause of dementia worldwide. Increasing evidence supports that AD is also a metabolic disorder, where mitochondrial dysfunction is associated with cognitive decline and disease progression. Mitochondria, therefore, represent both a source and a target of neurotoxic stress in AD pathogenesis. Recent findings demonstrated that mild inhibition of mitochondrial complex I with tricyclic pyrone compound CP2 elicits a broad neuroprotective response in early-onset AD models. The present study investigated the potential effects of CP2 to reverse metabolic dysregulation associated with late-onset AD using the APP/PS1 mouse model. **Methods:** 19-month-old female APP/PS1 transgenic mice and non-transgenic littermates were treated with CP2 or vehicle for six weeks. Brain tissues from the cortico-hippocampal region were analyzed using bulk RNA sequencing and targeted metabolomics. Transcriptomic profiling, along with systems biology approaches, was used for assessing molecular and metabolic alterations associated with CP2 treatment. We generated the first manually curated genome-scale metabolic model of the mouse brain, namely iMiceBrain, for simulating metabolic flux changes to predict metabolic shifts in control and CP2-treated conditions. This systems toxicology approach enabled assessment of CP2's effect on pathways related to energy production, lipid metabolism and redox balance. **Results:** Transcriptomic analysis of APP/PS1 mouse brains showed altered expression of *Cdkn1*, *Foxm1*, *Pik1*, and *Akt2*, indicating disrupted cellular senescence, FOXO signaling, and mitotic control, characteristic of late-onset AD. Differential expression of *Hmgcs1*, *Acaca*, *Lss*, *Sqle*, and *Slc27a1* was suggestive of alterations in cholesterol and lipid metabolism, as well as PPAR signaling. *Ldlr*, *Trem2*, and *Gfap* upregulation indicated the activation of glial cells and a link to neuroinflammation. CP2 treatment in APP/PS1 mice restored the expression levels of *Neurog1* and *Nfe2l*, suggestive of neurogenesis. Downregulation of *Lss*, *Apoa1*, and *Lcat* genes in CP2-treated APP/PS1 mice suggests a role in cholesterol homeostasis and clearance. Integrating transcriptomic data with the iMiceBrain model identified lipid and energy metabolism-related pathways, including cholesterol, bile acid, glycerophospholipids, the pentose phosphate pathway (PPP),

and β -oxidation, associated with CP2 treatment in APP/PS1 mice. **Conclusions:** Our systematic transcriptomic profiling and integrated metabolic modeling provides mechanisms of how CP2 restores mitochondrial functions and metabolic shifts in late-onset AD mouse model.

PS 4372 Performance of Different Virtual Control Group Generation Approaches

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Background and Purpose: Virtual Control Groups (VCGs) could be a potential 3R approach in toxicological study design as an ethically favorable alternative to traditional Concurrent Control Groups (CCGs). By stringent filtering of control animal data from previously conducted studies (Historical Control Data, HCD), VCGs can be generated and used instead of CCGs to determine treatment relation, if statistical integrity or biological relevance assessments are not compromised. This study investigates the development, validation, and application of VCGs in 28-day oral toxicity studies in rats. **Methods:** Using a HCD database of 185 studies performed at BASF between 2009 and 2023, four distinct approaches for generating VCGs were explored: sampling individual animals, sampling entire control groups from complete studies, domain-wise sampling based on filtered subsets of the HCD, and simulation-based modeling. The simulation approach probabilistically generates virtual control animals by leveraging endpoint-specific distribution patterns, offering a data-driven alternative to traditional control group design. Each method was applied to twelve 28-day legacy studies, and the resulting statistical results for the various endpoints in each study were compared to those derived from conventional CCGs. VCGs were generated using a multi-iteration approach to enhance statistical robustness. **Results:** Among the evaluated methods, simulation-based VCGs demonstrated superior performance in terms of flexibility, predictive accuracy, and reproducibility. However, the analysis also highlighted challenges in modeling endpoints with high intrinsic intra- and interstudy variability, like elements of the Functional Observational Battery (FOB). A reanalysis of three studies, each involving blinded analysis by subject matter experts for the investigated endpoints, found that multi-iteration VCGs generally accurately captured population characteristics. The VCG generation procedure provided a stable baseline across most endpoints, potentially making the VCGs more robust than traditional CCGs. **Conclusions:** In conclusion, the results support further investigation of the VCG concept, identification of mitigation strategies for endpoints where modeling is still challenging, and exploration of the path toward regulatory acceptance.

PS 4373 Machine Learning-Enabled Extraction and Analysis of Thyroid Endpoints from Regulatory Documents

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Background and Purpose: Thyroid disruption testing presents interpretive challenges due to the hormone's broad physiological influence on multiple organ systems. Regulatory studies are often complicated in identifying and contextualizing thyroid-related findings due to heterogeneous structure and various reporting formats of these documents. To systematically evaluate how thyroid endpoints inform regulatory decision-making, publicly available regulatory studies from the U.S. Environmental Protection Agency (EPA) and other agencies were retrospectively analyzed using a document-level machine learning pipeline. By replacing extensive manual review with reproducible automation, this approach enhances efficiency in evidence synthesis. Thyroid-relevant endpoints (e.g., T3, T4, TSH levels) were extracted and standardized into a tabularized dataset suitable for cross-study comparison. This enabled quantitative assessment of which endpoints are most frequently measured, most often statistically significant, and most influential in determining adversity and risk conclusions. **Methods:** We employed an automated pipeline that integrates document retrieval, relevance screening, structured extraction, and post-processing to build a thyroid-focused dataset from regulatory documents. Initial retrieval drew on the Google Search API alongside the EPA ChemView database, constrained by curated queries for thyroid-related language (e.g., "thyroid", "TSH", etc.). Retrieved documents were evaluated and screened for relevance programmatically. The large language model (LLM) driven extraction workflow mapped documents to structured outputs covering study metadata, chemical identifiers, and thyroid endpoints (e.g., serum hormone measures). The consolidated results were harmonized and quality-checked, producing the final searchable compendium of thyroid biomarkers for cross-study quantitative comparison. **Results:** The automated retrieval pipeline identified 6,214 unique regulatory studies of which 1,851 documents were down-selected after relevance filtering. The LLM-powered structured data extraction parsed thyroid-specific quantitative endpoints and compiled alongside study level metadata and statistical outcomes. The resulting dataset enabled cross-study comparison of thyroid endpoint frequency, directionality, and regulatory interpretation. Preliminary analyses reveal variation in endpoint frequency and interpretation across

study designs, underscoring the value of harmonized data structures for cross-study comparison. Integrating regulatory context directly within the dataset provides a unique opportunity to evaluate how thyroid data contributes to risk assessment decisions. Ongoing efforts aim to quantify endpoint-specific variability and its impact on adversity determinations. **Conclusions:** This work demonstrates the feasibility and value of using machine learning based extraction to systematically compile and analyze thyroid-related endpoints from regulatory documents. The resulting work not only streamlines evidence synthesis but also enables quantitative evaluation of endpoint variability and regulatory interpretation. By integrating structured *in vivo* toxicological data with risk assessment context, this machine learning approach establishes a framework for retrospective analyses that enhances efficiency of endocrine-related risk assessments, while aligning traditional toxicology with next-generation approaches.

PS 4374 Understanding the mechanisms behind liver vs. kidney susceptibility to fenofibrate overdose

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Background and Purpose: Fenofibrate, a peroxisome proliferator-activated receptor α (PPAR α) agonist, is widely prescribed to treat hyperlipidemia and has therapeutic potential in liver and kidney diseases. However, fenofibrate is also associated with adverse effects, including elevated creatinine and liver and kidney toxicity, although the underlying mechanisms remain unclear. In addition, how fenofibrate regulates lipid metabolism differently in the liver and kidney is not well understood. Therefore, in this study, we investigated the dose-dependent effects of fenofibrate on liver and kidney metabolism in rats, with a focus on PPAR α activation and potential mechanisms contributing to organ-specific toxicity. **Methods:** We used high-throughput transcriptomic data from 5-day rat *in vivo* studies, where rats received either vehicle control or fenofibrate at eight different concentration levels ($n = 4$ per group) daily for five consecutive days, after which liver and kidney tissue samples were collected on the sixth day for a targeted RNA sequencing analysis. We then performed differential gene expression, injury signature, pathway enrichment, and detailed individual gene comparison analyses to investigate how liver and kidney metabolism were differentially altered between the two organs. **Results:** Our results show that fenofibrate exposure significantly increased liver but not kidney weights and caused larger perturbations in the liver compared to the kidney transcriptome, with many of the changes related to PPAR α regulation. Interestingly, our study revealed that the PPAR α and RXR α genes are differentially regulated between the liver and kidney. In addition, we identified several differences between the liver and kidney in cellular and mitochondrial fatty acid transport, lipoprotein metabolism, fatty acid oxidation, branched-chain amino acid degradation, and glucose metabolism pathways. Furthermore, using our individual gene level comparison and universal toxicity gene signatures, we identified transcriptomic inflection points at which the changes in the PPAR α -mediated regulation of lipid metabolism switched from beneficial to deleterious as the fenofibrate concentration increased leading to liver injury. Our analysis revealed that fenofibrate overdose overwhelmed several mitochondrial-driven processes related to lipid metabolism in the liver, leading to redox imbalance and oxidative stress, which ultimately resulted in the development of liver injury. **Conclusions:** Using fenofibrate dose-response studies, our study identified potential differences in the PPAR α -mediated activation of lipid metabolism between the liver and kidney and elucidated potential mechanisms through which fenofibrate overdose may cause liver injury. Therefore, our study provides an improved understanding of PPAR α activation by its agonists and may guide the development of future therapeutics targeting the modulation of lipid metabolism in the liver and kidney.

PS 4375 Identifying mitochondrial bioenergetic changes and NAD⁺ modulation associated with cognitive resilience in Alzheimer's Disease

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Background and Purpose: Alzheimer's disease (AD) is marked by progressive cognitive decline and mitochondrial dysfunction. Asymptomatic Alzheimer's disease (AsymAD) individuals retain normal cognitive functions despite comparable amyloid and tau pathology. Comparison of bioenergetic changes associated with mitochondrial dysfunction has not been performed for AD and AsymAD. Molecular mechanisms underlying mitochondrial dysfunction in AD and AsymAD are important for identifying therapeutic strategies that can restore the bioenergetics in disease state. **Methods:** We performed an integrated multiomics analysis of postmortem brain tissue from ROSMAP, Mayo Clinic and Mount Sinai Brain Bank cohorts to investi-

gate mitochondrial dysfunction in AD and AsymAD. We classified the samples into three groups: AD ($n = 182$), Asymptomatic-AD ($n = 200$), and controls ($n=106$), based on Braak stage, CERAD score and Mini-mental state examination (MMSE) scores. Proteomics and single nucleus RNA sequencing data from different brain regions such as prefrontal, temporal, and hippocampus were analyzed across neuronal and glial subtypes to assess mitochondrial pathways in the study groups. We developed a machine learning model to identify important bioenergetic features that distinguish AsymAD from AD. Differentially expressed proteins were queried against DrugBank database to identify candidate small molecules that could potentially restore mitochondrial bioenergetics. **Results:** We used an integrated approach combining proteomics, machine learning, and single-nucleus RNA sequencing to identify mitochondrial genes/proteins that were differentially expressed between AD and AsymAD across cell types, brain regions, and cohorts (ROSMAP, Banner, and Mount Sinai). Proteomic analysis showed AsymAD exhibited enhanced mitochondrial metabolism including branched chain amino acid catabolism (ECHS1, BCKDHB), TCA cycle (IDH2, SUCLA2), fatty acid oxidation (CPT2, ACADSB). Upregulation of mitochondrial translation (MRPL37, TOMM40), structural proteins formation (PDHB, FIS1), and calcium ion transport (MCU, VDAC2) supporting improved energy regulation in AsymAD. DrugBank analysis identified 16 candidate drugs or small molecules, including NADH, Carvedilol, Radicolol, and L-glutamic acid, interacting with resilience-associated proteins. Most druggable proteins from our analysis showed interactions with NADH, and although NADH does not cross the blood brain barrier, our findings suggest that enhancing NAD⁺ metabolism in brain could restore redox balance and preserve mitochondrial function in AD. **Conclusions:** This systems toxicology analysis shows that cognitive resilience in AsymAD is associated with maintenance of mitochondrial bioenergetics and antioxidant defenses that prevents neurotoxic stress. The identification of proteins and NAD⁺-linked metabolic regulators provides the mechanistic basis for developing interventions focused on preventing or reversing energy failures in AD. These findings highlight the power of multiomics and machine learning approaches in identify molecular signatures of neuroprotection relevant to both chemical- and disease-induced mitochondrial toxicity.

PS 4376 Development of an Interpretable Structure-Activity Relationship Model for Predicting Hepatotoxicity in Repeated-Dose Toxicity Studies

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Background and Purpose: *In silico* toxicity prediction based on Structure-Activity Relationship (SAR) is one of the promising approaches for efficient chemical safety assessment. However, utilization of SAR models for complex toxicity endpoints such as organ and systemic toxicity observed in repeated exposure to chemicals is limited. For regulatory acceptance and use of SAR models and their predictions, interpretability as well as predictive performance is important. In this study, we developed machine learning-based SAR models for hepatotoxicity observed in repeated dose toxicity (RDT) studies in rats and selected the best model through performance evaluation. Subsequently, the atom-level visualization method based on molecular fingerprints was applied to the best model to enhance prediction interpretability. **Methods:** Data for oral RDT studies with administration periods of 28-100 days were collected from databases. Chemicals showing histopathological changes in the liver at doses $\leq 1,000$ mg/kg bw/day were classified as positive for hepatotoxicity, while chemicals showing no changes at doses $\leq 1,000$ mg/kg bw/day were classified as negative. A total of 1,269 chemicals (631 positives and 638 negatives) were collected. Then, 100 positive and 100 negative chemicals were randomly selected to form the test dataset, and the remaining 1,069 chemicals were used for model training. Four machine learning algorithms, Stochastic Gradient Descent (SGD), Random Forest (RF), Support Vector Machine (SVM), and Light Gradient Boosting Machine (LGBM) were employed to develop each predictive model. Morgan fingerprints were used as explanatory variables. Structural and physicochemical descriptors were used to define the applicability domain. For performance evaluation, leave-one-out cross-validation (LOOCV) on the training dataset and external validation (EV) on the test dataset were performed. The atom-level visualization method based on molecular fingerprints was applied to the constructed model. Analysis of contributing substructure for each prediction was conducted to confirm the enhancement of interpretability by applying the method to the developed model. **Results:** All models achieved an area under the receiver operating characteristic curve (AUROC) greater than 0.75 in both LOOCV and EV. Among them, the RF model exhibited the highest AUROC score of 0.800 in LOOCV and 0.829 in EV. Regarding other metrics, the highest sensitivity of 0.750 was exhibited by the RF model in LOOCV and 0.725 by the SGD model in EV. On the other hand, the highest specificity of 0.727 in LOOCV and 0.795 in EV was obtained by the LGBM model. Based on its balanced performance, the RF model was selected as the best model. Functional group-based analysis of the RF model revealed that the functional groups which contributed most to positive predictions were halogens, followed by nitro groups, nitriles, and azole groups. Except for nitriles, these functional groups were known as structural alerts associated with hepatotoxicity. In contrast, hydrophilic moieties such as sulfo groups, carboxylic groups, and serine side chains contributed to negative predictions. These hydrophilic groups are considered to attenuate chemical toxicity by decreasing membrane permeability and increasing excretion from the body. **Conclusions:** Predictive models for hepatotoxicity observed in rat RDT studies were developed. The best model achieved

AUROC values above 0.8 in both LOOCV and EV. The application of the atom-level visualization method enhanced model interpretability by clarifying the rationale for positive and negative predictions. This study contributes to regulatory acceptance and use of machine learning-based toxicity predictive models for complex toxicological endpoints.

PS 4377 Bridging *In Vitro* Bioactivity to Clinical Adverse Drug Events Through a Multimodal Knowledge Graph Modeling Framework

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Background and Purpose: The use of *in vitro* assay outcomes to predict clinically relevant adverse drug events (ADEs) continues to be a critical challenge in predictive toxicology. Many ADEs result from off-target binding that may not be adequately captured in experimental toxicity testing. To answer this challenge, we developed a data-driven modeling framework that correlates *in vitro* bioprofiles to clinical outcomes from the FDA Adverse Effect Reporting System (FAERS), using a multimodal knowledge graph and deep learning approach. **Methods:** Publicly available databases were integrated to construct a knowledge graph that links drugs to targets to ADEs/diseases. Beginning with over 2,000 drugs with clinical ADEs reported by FAERS, *in vitro* target activity for these drugs was retrieved from PubChem. The resulting 10,000+ drug-target activities were searched in the Comparative Toxicogenomics Database (CTD), where curated drug-disease and target-disease links from the scientific literature were retrieved. ADEs/diseases were then hierarchically mapped to the Medical Dictionary for Regulatory Activities (MedDRA). A multimodal neural network was then trained based on this integrated dataset, combining chemical structure, *in vitro* bioassay activities, and clinical outcomes to identify potential relationships between drug-target activity and observed ADEs. **Results:** The knowledge graph revealed significant target-disease correlations, including several off-target associations that aligned with known cardiac, metabolic, and hepatobiliary ADEs. The integrative multimodal models demonstrated good predictive performance, achieving AUROC scores above 0.7 across multiple ADEs. Network-based phenotypes obtained from CTD and Human Phenotype Ontology highlighted mechanistic connections linking drug-target activity to clinical endpoints, illustrating potential adverse outcome pathways. **Conclusions:** This study presents an interpretable mechanistic approach linking multimodal *in vitro* assay data to adverse clinical outcomes. By leveraging multimodal integration and knowledge graph representations, our framework enables the prediction of specific ADEs by shared target biology and chemical structures, offering a new framework to mechanistically grounded *in silico* toxicity assessments that can aid in early identification of off-target outcomes in drug discovery.

PS 4378 Integrating Chemical Perturbation Signatures for *In Silico* Toxicity Prediction

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Background and Purpose: The gene expression profiles observed when biological organisms or cells are exposed to chemical substances—known as chemical perturbation signatures—provide crucial information for predicting pharmacological effects and toxicities. However, unlike drugs, for which large-scale chemical perturbation signature datasets have been established, such datasets for pesticides and general industrial chemicals remain largely undeveloped. As a result, the development of *in silico* prediction models for these chemicals has been significantly delayed due to the lack of appropriate training data. To address this issue, we aim to establish a comprehensive information platform and develop prediction models to understand the relationships between chemical substances and biological responses by collecting gene expression data related to chemical exposure from the Gene Expression Omnibus (GEO) and constructing a chemical perturbation signature database. **Methods:** Gene expression data were collected for three species—rat, mouse, and human—targeting 2,040 general industrial chemicals whose toxicity data are registered in the AI-SHIPS Chemical Toxicity Database (AI-SHIPS ToxDB) and 380 pesticides registered in our in-house pesticide database. An automated search program extracted relevant datasets (GSEs) from GEO by identifying experimental metadata (e.g., titles and abstracts) that included the target chemicals. For the same chemical, data obtained under different conditions were treated as separate samples to comprehensively capture gene expression alterations across diverse exposure scenarios. **Results:** In the automated search phase, datasets were obtained for 11 general industrial chemicals and 15 pesticides in male rats, 164 and 152 in mice, and 37 and 162 in humans, respectively. After curation, the coverage of gene expression data relative to the AI-SHIPS toxicity dataset and in our pesticide database was approximately 3% for general industrial chemicals and 2% for pesticides in rats and mice. The collected gene expression data were statistically analyzed to identify sets of genes that exhibited expression changes in response to chemical exposure. Through this analysis, key gene sets involved in the mechanisms of chemical toxicity (chemical-associated genes) were identified. For these chemical-associated genes,

proximity evaluations were conducted with outcome-related genes on a protein-protein interaction (PPI) network. **Conclusions:** These signatures will be utilized as training data for inferring the mechanisms of action of chemical substances, grouping chemicals with similar toxicity profiles, and reconstructing exposure-related gene expression patterns. This work provides a foundation for an *in silico* evaluation system capable of predicting the toxicity of novel chemical substances based on gene expression data.

PS 4379 An AI-powered method for evaluating *in vivo* level toxicity from genomic dose-response data

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Background and Purpose: For more than a century, animal studies have been used to identify the potential adverse consequences induced by exposure to chemicals and to facilitate human health risk assessment. Recently, there has been a significant push to move from animal testing to new approach methodologies (NAMs) including transcriptomics and *in silico* methods such as AI. Genomic data based risk assessments being developed focus on establishing a transcriptomic point of departure (tPOD) for genomic level changes, however, the tPODs do not cleanly map to organ level effects and are thus more difficult to use for hazard characterization and decision making. Here we propose a different approach in which data at the genomic level is first converted to an organ level response via an AI model, then the model's predictions are used to derive a POD at the *in vivo* level. This approach allows risk assessors to continue using well-established approaches to evaluate toxicity while still taking advantage of, in terms of efficiency, performing transcriptomic studies. The objective of this project was to develop an AI-driven methodological framework that enables *in vivo* toxicity evaluation at the organ level from transcriptomic dose-response data. It is hypothesized that genomic dose-response profiles, together with key experimental context [e.g., exposure durations, species, strains, etc.] from either *in vitro* or *in vivo* studies contain sufficient information to infer *in vivo* organ-level toxicity [e.g., endpoints and response levels]. **Methods:** We trained an AI model to predict *in vivo* response levels given a chemical structure, *in vivo* exposure information, and the result of a genomic measurement. We also developed an approach to calculate a benchmark dose (BMD) from AI model predictions at specified dose levels. Since chemicals in the Open TG-GATES database have both *in vivo* data and genomic dose-response data, the database provides an excellent testing point for our methodology. This gave us a total of 150 chemicals and 120 of which were used for training and 30 of which were used for validation. The model was trained by randomly selecting a genomic measurement from the set of genomic measurements for that chemical. Model training was performed using an 80-20 train validation split and took about a day on an RTX-5090. From the model predictions, we were able to calculate BMDs for 3,880 of 3,884 datasets with a significant dose response trend. **Results:** Our preliminary results show that the AI model had a relative dose-level prediction error of 10.13%, indicating reasonable accuracy for predictions from the AI model. The calculated BMDs were also well correlated with the BMDs calculated from the *in vivo* data with an R value of 0.7294. This is similar to the correlation between tPODs and their *in vivo* counterparts, indicating our method is comparable to current genomic data based risk assessment methods regarding POD derivation. Uncertainty calibration was also strong as the 90% CI showed a coverage of 94.05% indicating only modest over-coverage. Additionally, our methodology is naturally conservative with 80.9% of predicted BMDs being lower than their observed counterpart. **Conclusions:** Our AI-powered method for using genomic data for *in vivo* level risk assessment showed strong results on Open TG-GATES data, demonstrating reasonable levels of accuracy with some other nice properties including strong uncertainty calibration and natural conservatism. This validates our hypothesis that an AI-powered approach is capable of bridging the gap between transcriptomic results and *in vivo* level outcomes. In the future, we will train more advanced models with more data likely yielding further improvements in the accuracy of the model which will lead to downstream improvements in the derived PODs.

PS 4381 Mitigating features in quinoline derivatives: an expert-review SAR analysis of indeterminate QSAR predictions

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Background and Purpose: Indeterminate QSAR predictions might hinder regulatory toxicology by leading to inconclusive conclusions. Expert review can augment *in silico* assessments and help interpret ambiguous outcomes. While QSAR models emphasize positive features and structural alerts, countervailing or deactivating features can substantially weight the overall prediction and reduce false positives. This work aims to investigate whether specific mitigating features within quinoline-containing structures hamper Michael addition and ultimately diminish mutagenic potential. **Methods:** An expert-review approach is applied to indeterminate QSAR results. An SAR-based analysis of the quinoline chemical environment is conducted, emphasizing deactivating features that may hinder activation of the quinoline moiety and prevent Michael addition. Data and (Q)SAR predictions for quinoline derivatives have been considered. Putative mitigating features have been

identified and characterised. **Results:** The SAR assessment highlighted consistent patterns where certain substituents or electronic/steric hindrance reduced likelihood of Michael-type nucleophilic attack, correlating with decreased mutagenic signal in subsets of compounds. Experimental data supported the notion that deactivating features can mitigate predicted reactivity, thereby clarifying ambiguous QSAR outcomes when integrated with expert review. **Conclusions:** Expert review remains essential when leveraging *in silico* tools for toxicology, particularly for regulatory purposes. To reduce inconclusive outcomes, the expert review with an SAR-focused evaluation of the chemical environment can provide a more nuanced, mechanism-informed conclusion. These integrated approaches help manage indeterminate QSAR predictions and support more robust decision-making in safety assessments.

PS 4382 QSAR Models to Anticipate the Degradation of Common Chemical Functional Groups

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Background and Purpose: When determining the toxicity of pharmaceuticals and other chemical products, significant consideration must go towards the presence of impurities, many of which can be formed as byproducts during synthesis. However, additional impurities can form spontaneously over time as chemicals sit in storage, even under ideal conditions. Degradation includes numerous mechanisms and can be catalyzed by several factors, such as heat, pH, oxidative species (including air), and light. The exact degradation pathway a compound will follow depends entirely on which functional groups are present and what conditions the compound is exposed to. Many common functional groups are susceptible to hydrolysis, including esters, amides, thioesters, halides, and more. Hydrolysis generally occurs by one of two mechanisms: base-catalyzed and acid-catalyzed. In a basic environment, an electropositive atom, such as the carbon attached to a halogen, is attacked by a nucleophilic hydroxide ion. In an acidic environment, free H^+ ions protonate an electronegative atom, such as the oxygen in an ether. Both mechanisms generally result in the molecule being split into an alcohol and a second group that varies based on the parent functional group. Some groups are equally susceptible to both acid- and base-catalyzed hydrolysis while others are only susceptible to one. **Methods:** Hundreds of hydrolysis rate constants for various species have been collected over the past century, which can be used to construct a robust Quantitative Structure Activity Relationship (QSAR) model to predict the hydrolysis rates of new compounds. If there is enough data for a particular functional group, a statistical model can be built. Statistical models are created using an in-house code that extracts structural fragments from each molecule and then looks for any correlation between these fragments and the rate of hydrolysis, expressed as $\log(k)$. If data is more limited but still sufficiently varied and systematic, an expert rule-based model can be built. The rules focus on the environment on either side of the hydrolyzed bond, checking for substructures that can either alter the charge or are bulky enough to block an ion from bonding. Determining the magnitude of the effects of these fragments requires data collected under similar experimental conditions, particularly solvent and temperature. **Results:** Both methods are powerful for predicting the rate of hydrolysis, provided that sufficient data is available. For groups, such as esters, where hundreds of molecules have published data, the statistical models were very reliable for predicting the rate, with the ester model having a RMSE of 0.77 and R^2 of 0.91. Expert rule-based systems could be built for most functional groups but were particularly useful for those with datasets too small to build a statistical model. For esters and amides, the RMSE was 0.63 and 0.41, respectively, with an R^2 of 0.86 and 0.92, respectively, showing a modest improvement over the statistical ester model. A benefit of the rule-based models is that the rules could be reused between select similar functional groups, such as esters and amides or thiols and hydroxyls, with minimal adjustments. For example, while oxygen will generally only form two bonds, nitrogen will make at least three, meaning that an amide can have either a hydrogen or an additional third branch to consider. The broad applicability of the rules simplified the creation of expert rule-based models and also helped to confirm the rules' robustness. **Conclusions:** The similarity across most hydrolysis mechanisms made building accurate QSAR models relatively straightforward, despite the wide range of functional groups to be covered. Where enough data was available, a robust statistical model was built that could reliably predict the rate of hydrolysis. For functional groups that had not been studied as thoroughly, expert rule-based systems were derived from the data that was available, leveraging the similarity between certain functional groups to expand the rule set when possible. Using these models, both the structure and prevalence of hydrolyzed impurities can be anticipated so that they can be included in a chemical's risk assessment and mitigated, if necessary.

PS 4383 ToxMet: a web-tool for the analysis of toxicogenomic data using genome-scale metabolic modeling

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Background and Purpose: Chemical-induced organ toxicity is routinely assessed using rat transcriptomic data collected at various timepoints and dose conditions, and understanding its underlying mechanisms relies on analyzing changes in gene expression using various statistical methods, such as gene set enrichment analysis, dose-response modeling, or identification of genes whose expression correlates with injury phenotypes. Recently, genome-scale metabolic models (GSMs) have been used to provide a systems-level understanding of gene expression changes by correlating them with organ metabolism and injury phenotypes. However, most of the algorithms that integrate gene expression with GSMs require familiarity with MATLAB or Python programming, making them less accessible for users without computational experience. Furthermore, these algorithms' results cannot be readily visualized in the context of metabolic networks. Therefore, we developed a user-friendly web-application that integrates transcriptomic data with a rat GSM to understand changes in organ metabolism and predict potential metabolites for targeted biomarker discovery. **Methods:** GSMs represent the entire metabolic network of a cell or organism, including all the metabolic reactions, the genes catalyzing the reactions, and the relationship between the genes and reactions in a computational framework. In our web-application, we implemented the rat GSM contents as explorable tables for the users, with additional information related to genes, reactions, and metabolites provided via external links. For transcriptomic data integration, we implemented two well-validated computational algorithms related to flux balance analysis, i.e., Pheflux and TIMBR, which predict alterations in organ metabolism at the metabolic reaction level and plasma metabolite alterations, respectively. **Results:** Here, we introduce ToxMet, a user-friendly web-application that integrates transcriptomics to predict and visualize chemical-induced metabolic perturbations in rat tissues using toxicogenomic measurements and GSMs. ToxMet takes rat transcriptomic data as input and predicts chemical-induced changes in metabolite secretions and metabolic pathway activity, providing prediction results as interactive and downloadable tables, scatter plots, and network visualizations. The scatter plots provide a summary of changes across the subsystems and major pathways, which helps identify the most altered pathways and their direction of alteration (increase or decrease in activity) with respect to controls. The network visualizations provide details of reactions and metabolites contained within the subsystems, which can help trace changes across reactions when analyzed in conjunction with the algorithms' predictions. Users can download the raw results for other downstream analyses or to generate plots in other formats and can search and visualize the rat GSM network for the presence of metabolites, reactions, genes, and subsystems of interest. Finally, we demonstrate ToxMet's applicability by predicting known toxicity mechanisms using publicly available toxicogenomic data. **Conclusions:** ToxMet is a free and easy-to-use web-based software that can predict metabolic alterations from gene expression data. ToxMet's predictions provide novel insights into the metabolic mechanisms of chemical-induced toxicity and can aid in the discovery of biomarkers and therapeutics.

PS 4384 Supporting source-to-outcome assessment in toxicology through data interoperability

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Background and Purpose: Environmental Health Science (EHS) is an inherently interdisciplinary field that studies how environmental factors, both natural and human-made, affect human health. This field integrates concepts from biology, chemistry, toxicology, epidemiology, and public health to understand environmental risks that can lead to disease or adverse health outcomes. Source-to-outcome (S2O) analyses can provide insight into risks and mitigation strategies associated with environmental exposures but require integration of multiple EHS data streams. Data integration can provide evidence-based support to inform risk assessment and decision making, but the practicality of data integration across EHS fields can be challenging because the current landscape is largely fragmented. Domains such as toxicology and exposure science have heterogeneous and inconsistent data formats, nuanced methodologies, and varied reporting practices. Integrating mechanistic data from across these domains is challenging because datasets end up siloed, and the metadata that can be critical for understanding how to appropriately understand and use measurements may not be available. This work aims to advance infrastructure for integrated analyses across EHS domains by leveraging standards and terminologies to promote data interoperability, findability, and reusability. We highlight a use case that develops a source-to-outcome computational model to support standards development across EHS domains. We emphasize model construction, preliminary results, and application to advance integrated analyses. **Methods:** We present a pilot use case that spans the S2O continuum and focuses on $PM_{2.5}$ exposure and decreased lung function to exemplify the complexity of linking real-world environmental exposure

data to health impacts. We used the Aggregate Exposure Pathway (AEP), and Adverse Outcome Pathway (AOP) frameworks as a scaffold to describe the S2O continuum and guide our work, linking them together to form the continuum. The exposure component of the use case employs a synthetic population, air quality data, and variation in demographic factors to simulate the AEP influencing PM_{2.5} intake during wildfire events. The biological component leverages AOP networks to represent mechanistic relationships through AOP Bayesian Network (AOPBN) modeling. This methodology provides a probabilistic structure for integrating evidence streams and evaluating the impacts of data integration on predictive confidence. We used the multiple-path particle dosimetry model (MPPD) to simulate lung deposition resulting from exposures, linking the exposure and outcome components through dosimetry. **Results:** We present preliminary results of exposure, dosimetry, and outcome modeling. Exposure modeling results include predicted daily PM_{2.5} exposure from forest fires in synthetic individuals, simulated through RTI's Synthetic Population Modeling system. We demonstrate variability across time and across individuals in the population, and highlight differences in exposure among census demographic groups. Particle dosimetry modeling results from MPPD are provided, accounting for physiological factors such as age and sex. We present network construction and preliminary results from AOPBN analyses, including probabilistic predictions of decreased lung function given input dosing conditions. **Conclusions:** Our use case demonstrates how standardized, interoperable data ecosystems that span the S2O continuum can support comprehensive risk assessments and inform on human health outcomes. We show that data interoperability is key to the future of mechanistic analyses in EHS, including S2O analyses, for helping to assemble information into meaningful knowledge. We emphasize that interoperability goes beyond compatibility of units and extends to making data searchable and easily processed by computational tools, as well as ensuring that contextualizing information that informs on the appropriate interpretation of data is available. We emphasize that building tools that anticipate data reuse and standardize the capture of this information is essential, and highlight opportunities for improved interoperability. This work is funded by the National Institute of Environmental Health Sciences under award number 1R24ES036127.

PS 4385 Statistical Evaluation of DrugMatrix and Open TG-GATEs for Construction of an Integrated Toxicogenomics Resource

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Background and Purpose: DrugMatrix (US) and Open TG-GATEs (Japan) are two of the largest publicly available toxicogenomics datasets, each containing thousands of microarray profiles across multiple tissues, dose levels, and chemical exposures. Because animal studies are costly, and constrained by ethical and scalability limitations, maximizing the utility of existing datasets is essential for developing predictive models that can support future toxicological risk assessment. The objective of this study is to determine whether DrugMatrix and Open TG-GATEs possess compatible gene-expression characteristics after being processed under a unified normalization pipeline, thereby evaluating the feasibility of integrating the two datasets into a single training resource. **Methods:** Raw Affymetrix CEL files containing probe-level intensity measurements were processed using the Robust Multi-Array Average (RMA) algorithm, including convolution background correction, quantile normalization, and median-polish summarization, to reduce technical noise and platform-specific variability. Biological replicates were averaged to generate one expression profile per treatment condition. Cross-dataset compatibility was evaluated by comparing descriptive statistics, global expression distributions under the matched tissues and experimental modes. Kernel density estimation (KDE) was applied to obtain smoothed probability density curves for gene-expression values. Graphical diagnostics were used to assess distributional concordance: Probability-Probability (P-P) plots were used to compare the cumulative distribution functions of the two datasets, assessing whether they assign similar proportions of genes to each expression range. Quantile-Quantile (Q-Q) plots evaluated agreement of expression intensities at matched quantile levels, assessing whether the datasets share comparable dynamic ranges after normalization. These distributional diagnostics quantify whether the two datasets share comparable expression scales, tail behavior, and overall shaping after unified normalization. **Results:** RMA normalization produced highly comparable global expression patterns across the two datasets. DrugMatrix and TG-GATEs exhibited similar expression ranges (min $\approx$ 2.4-3.1; max $\approx$ 14.5-15.2), interquartile ranges ($\approx$ 3.1-3.5), median values ($\approx$ 5.4-6.7), and standard deviations ($\approx$ 2.1-2.3). These matched comparisons demonstrate that gene-expression measurements across the two platforms operate on the same numerical scale. Density curves from both sources displayed the characteristic bimodal distribution common in toxicogenomics microarray datasets, and the relative ordering of tissues by median expression was consistent across platforms. Probability-Probability and Quantile-Quantile comparisons further confirmed distributional agreement: both plots showed near-linear alignment across the central distribution, with mild tail deviations. These findings show that platform-specific variation is effectively reduced under the unified preprocessing pipeline. **Conclusions:** The consistency of the distributional properties observed between DrugMatrix and Open TG-GATEs after uniform preprocessing provide evidence that large-scale integration of the two datasets is feasible from a statistical standpoint. A merged dataset would expand chemicals, dose, and temporal coverage, which could serve as a more informative training foundation for toxicogenomics prediction models.

The integration supports ongoing efforts to reduce reliance on animal studies by enabling more accurate, data-driven inference of transcriptional responses to chemical exposures.

PS 4386 BioBricks-OKG: An Open Knowledge Graph Infrastructure for Integrating Chemical Safety and Toxicology Data

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Background and Purpose: Regulatory agencies face mounting pressure to evaluate chemical safety using alternative methods while maintaining rigorous scientific standards. Critical barriers include inconsistent chemical nomenclature across databases, ambiguous test method descriptions, and fragmented toxicity endpoint definitions that complicate systematic evidence evaluation. These data quality and interoperability issues are essential challenges in computational approaches and read-across methodologies for regulatory submissions. BioBricks-OKG was developed in partnership with NICEATM to address these fundamental data infrastructure challenges by creating a harmonized knowledge graph that standardizes chemical identifiers, test methods, and endpoint definitions across major toxicology databases, thereby supporting evidence-based regulatory decision-making and facilitating transparent, reproducible chemical risk assessments. **Methods:** BioBricks-OKG implements ontology-driven harmonization of chemical safety data from authoritative toxicology sources, including EPA CompTox, ToxCast, Tox21, ICE (Integrated Chemical Environment), CTD, and AOP-Wiki, as well as more general biological and chemical databases such as PubChem and ChEBI. Many of these datasets are extracted as tabular data that are versioned using the BioBricks system, which are then transformed and mapped to entities that integrate multiple datasets. Chemical substances are harmonized using PubChem CIDs as common identifiers to link chemical entities across databases. The ontology approach utilizes existing standard ontologies from the OBO Foundry and controlled vocabularies employed by biological databases. In combination, this allows for creating a workflow where the source data for data integration are reproducible and there is provenance tracking for knowledge graph assertions. **Results:** BioBricks-OKG successfully harmonized chemical identifiers across major toxicology databases by mapping diverse nomenclature systems to PubChem CIDs, resolving inconsistencies that previously prevented cross-database queries. The knowledge graph enables complex regulatory queries that were previously impractical due to variations in database schemas and inconsistent identifier use. Specific examples include: identifying all substances classified as GHS Category 1 carcinogens with available ToxCast high-throughput screening data and linking chemicals to relevant adverse outcome pathways based on mechanistic assay results. Each part of the overall knowledge graph is available as KG bricks modeled in RDF with serialization in standard formats, as well as in pre-indexed HDT format for immediate local queries after downloading. In addition, KG bricks can be loaded into any number of triple-store servers to support efficient cross-graph querying over the network using SPARQL. By combining all these data sources into a shared schema, the data source can also be queried via an LLM front-end, allowing for various applications, such as natural language querying or as a source for retrieval-augmented generation of toxicology reports. **Conclusions:** BioBricks-OKG directly impacts toxicology practice by providing infrastructure that enables regulatory agencies to systematically evaluate chemical safety using integrated evidence from multiple sources. This addresses a documented need identified by NICEATM and regulatory stakeholders for improved data interoperability in alternative method evaluations. The harmonized knowledge graph reduces technical barriers to implementing read-across approaches and weight-of-evidence assessments required for regulatory submissions. This infrastructure supports ICCVAM's mission to promote scientifically valid alternative methods, accelerating the transition away from animal testing while maintaining rigorous standards for human health protection. The open-source, version-controlled design provides sustainable infrastructure adaptable to future NAM data types and evolving regulatory needs.

PS 4387 Human liver microtissue gene networks enable mechanisms-based chemical safety assessment

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Background and Purpose: Next generation risk assessment (NGRA) is crucial for understanding the potential risks posed to human health by chemical or drug exposures. Within the NGRA framework, various new approach methods (NAMs) have been developed as alternative of animal studies. Human liver microtissues (hLiMT) are a physiologically relevant liver model, cultured in 3D spheroids and are composed of human primary hepatocytes and non-parenchymal cells, including Kupffer and endothelial cells. High-throughput transcriptomics of these spheroids after chemical exposures have the potential to derive mechanism-based transcriptional points of departure for chemical safety assessment. **Methods:** Human liver microtissues were cultured in a high-throughput 384-wells format and exposed to 56 chemicals, cytokines or growth factors at three concentrations (five-fold dilutions), not exceeding 15% cytotoxicity. Samples were collected at 8, 24 and 168 hours after exposure and

analyzed using targeted whole transcriptome sequencing (TempO-Seq), followed by weighted gene co-expression network analysis (WGCNA) to identify modules of co-regulated genes. **Results:** Transcriptome analysis of 500 unique treatments in hLiMT yielded over 250 liver spheroid specific gene co-expression networks, which are visualized in the interactive R-shiny based TXG-MAPr platform. These modules cover genes involved in various key biological processes, including cellular stress responses, inflammation, metabolism, cell cycle regulation and translation. Most of these modules were preserved in other liver cultures, like primary human hepatocytes. Known reference compounds, like tunicamycin, diethyl maleate and cytokines, activate expected stress response modules for unfolded protein response, oxidative stress and inflammation, respectively, confirming the mechanistic relevance of the gene networks in liver spheroids. Uploading and integration of external data from liver toxicant exposure further demonstrated the potential to link module activity with liver adverse outcome pathways (AOPs). **Conclusions:** The hLiMT TXG-MAPr tool provides a comprehensive, mechanism-based framework for chemical hazard identification and characterization. It enables comparative interpretation of transcriptomic responses, supporting industry and regulatory stakeholders in applying NAMs for next-generation chemical safety assessments.

PS 4388 Integrated Multiomic analysis of arsenic-induced perturbations to the pluripotency network of mouse embryonic and epiblast-like stem cells

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Background and Purpose: Arsenic contamination in water impacts the health of up to 220 million individuals worldwide. Despite arsenic's status as one of the most well-studied toxicants, the mechanisms underlying arsenic's carcinogenicity following *in utero* exposure remain poorly understood. The current, most prevalent theory for arsenic's carcinogenicity centers on S-adenosyl methionine (SAM), a key metabolite that serves as the cell's primary methyl donor. Following exposure, cells methylate arsenic to facilitate its excretion from the body, leading to a depletion of SAM that is thought to lead to global hypomethylation and, eventually, cancer. However, our preliminary evidence in murine embryonic stem cells (ESCs) and epiblast-like stem cells (EpiLCs) shows that the SAM hypothesis fails to explain arsenic's etiology in germ cells. The ESC and EpiLC models are especially informative to understanding the impacts of arsenic *in utero* as they represent two key, differentially methylated stages of embryonic development. Furthermore, studies have shown that arsenic impacts the ability of stem cells to maintain pluripotency, suggesting a potential avenue for long-term impacts following short-term *in-utero* exposure. **Methods:** Our study leverages multi-omics data (bulk RNA-seq, RRBS, metabolomics, and Chip-Seq) to better understand the multiple mechanisms through which arsenic impacts the pluripotency network of mouse ESCs and EpiLCs. Cells were exposed to arsenic for 48 hours before analysis. The datasets were analyzed separately using k-means clustering and ontological analyses of differential features to identify key components impacted by arsenic exposure. These datasets were then integrated using MOFA (multi-omics factor analysis) and regulatory network construction to investigate pluripotency network-specific impacts. **Results:** Integrated analysis reveals clusters that showcase cell type differences between ESCs and EpiLCs, and both cell-type specific and general perturbations caused by arsenic on multiple cellular levels. CpGs in ESCs became hypermethylated on average while EpiLCs become hypomethylated, contrary to the general trend of arsenic inducing global hypomethylation in most cell types. Bulk RNA-seq data showed clusters of genes similarly dysregulated in both cell types related to mitochondrial function and cell cycle regulation. ESC-specific perturbations show that arsenic pushes ESCs towards a partial loss of naïve pluripotency. Cluster dynamics and results of rescue experiments indicate that the dysregulation of histone post-translational modifications triggers cascading effects on DNA methylation and gene expression, leading to aberrant differentiation control. **Conclusions:** This study underscores the relationship between epigenetics and genetics in regulating the pluripotency network. Our analysis reveals that arsenic partially disrupts the pluripotency network while preserving key features of cell identity. These early embryonic disruptions can inform lifetime disease risk assessment. The multi-omic approach employed in this study provides several critical advantages over single-platform analyses. The integrated analysis identifies shared and cell-type-specific variance across molecular layers, allowing us to distinguish primary arsenic targets from secondary downstream effects. This comprehensive molecular profiling strategy establishes a framework for understanding how environmental toxicants disrupt complex biological networks during critical developmental windows.

PS 4389 Metabolism of Selected N-Nitrosamine Drug Substance-Related Impurities: Insights from the MUTAMIND Project

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Background and Purpose: N-Nitrosamines are mutagenic compounds and often potent carcinogens. When they occur as residual impurities related to active pharmaceutical ingredients, known as N-nitrosamine drug substance related impurities (NDSRIs), their hazard depends on chemical context, in particular the envi-

ronment around the N-NO moiety. Understanding the mechanisms underlying their metabolism is crucial, as it is closely linked to the carcinogenic potential of these compounds. The MUTAMIND SC05 project aims to experimentally elucidate the major metabolic sites of NDSRIs that govern their activation and deactivation pathways. Within this project, *in silico* methods are initially being used to explore these pathways and guide subsequent *in vitro* metabolism experiments in the project. **Methods:** Computational tools for metabolism prediction (Metasite from Molecular Discovery) were applied to seven representative NDSRIs (N-nitroso-bethahistine, N-nitroso-duloxetine, N-nitroso-ketamine, N-nitroso-propranolol, N-nitroso-sertraline, N-nitroso-trimetazidine, and N-nitroso-varenicline) to identify probable sites of metabolism, predict reactive metabolite formation, and evaluate competing activation and detoxification pathways. These compounds were selected to represent diverse structural classes and metabolic scenarios. **Results:** *In silico* metabolism predictions identified the cytochrome P450 (CYP) isoforms most likely involved in NDSRI metabolism. Some isoforms (e.g., CYP1A2, CYP2C19) were highlighted as key contributors to α -hydroxylation of the nitrosamine moiety, a critical step in metabolic activation. N-nitroso-trimetazidine showed high selectivity toward a single isoform, while N-nitroso-varenicline was predicted to be a poor CYP substrate, consistent with experimental data. For most NDSRIs, the less substituted α -carbon was the preferred site of metabolism; however, in N-nitroso-propranolol, the more substituted α -carbon was favored, likely due to electronic effects from the adjacent hydroxyl group, consistent with the CPCA classification of the β -hydroxyl group as deactivating. The analysis distinguished activation driven by chemical reactivity from interactions within the CYP active site. For example, the three methoxy substituents of N-nitroso-trimetazidine appear to act as deactivating features, steering CYP-mediated oxidation toward these groups rather than the α -carbons, likely due to both their favorable electronic properties and spatial orientation in the active site. A similar trend is observed for N-nitroso-varenicline, where the α -positions are less favorable sites for CYP-mediated oxidation compared to other more distant parts of the structure. The detoxification pathways were also analyzed to assess potential competing Phase II detoxification mechanisms (e.g., those mediated by glutathione S-transferases or sulfotransferases). Most NDSRIs were predicted to undergo detoxification reactions, particularly glutathione conjugation with a high probability and similar to that of at least one CYP isoform. However, N-nitroso-ketamine, detoxification pathways were predicted as less probable than CYP-mediated α -hydroxylation. **Conclusions:** The analysis indicates a complex interplay between electronic and steric factors at the α -carbons and other sites of metabolism of the molecule, influencing chemical reactivity, CYP-mediated activation and competing enzymatic detoxification pathways. The computational results identified key CYP isoforms and metabolism routes likely involved in the activation and detoxification of NDSRIs, offering valuable insights to guide experimental *in vitro* metabolism investigations of the compounds in this project.

PS 4390 Unified Transcriptomic Cell Atlas of Human Neural *In Vitro* Systems

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Background and Purpose: Human stem cell-derived neural organoids, spheroids, and 2D co-culture systems are foundational new approach methodologies (NAMs) for studying brain development and disease, yet an integrated, high-resolution transcriptomic characterization of these distinct platforms is lacking. Such an atlas is critical for benchmarking model fidelity and standardizing their use throughout drug discovery and development pipelines. As such, we sought to use single-cell RNA sequencing (scRNAseq) to extensively profile the transcriptome of an array of *in vitro* models. Our goal is to build a unified transcriptomic cell atlas of different *in vitro* neural systems and to leverage this atlas to expand the utilization of NAMs within pharmaceutical research and development. **Methods:** We performed scRNAseq on *in vitro* models derived from human induced pluripotent stem cells. This profiling spanned multiple culture dimensions, differentiation protocols, cell donors, and time to yield a comprehensive database. **Results:** Our analysis resolved hundreds of thousands of cells, including multiple neuronal subtypes across brain regions, neural progenitor cells at various stages of differentiation, and major glial and non-neuronal lineages. A direct transcriptomic comparison revealed a general induction of stress and hypoxia-related genes in 2D co-culture systems compared to 3D models. **Conclusions:** We provide the first and largest unified single-cell atlas of diverse neural *in vitro* models. This resource provides a foundational resource to evaluate the physiological limitations of different NAMs, directly informing the selection of appropriate models for pharmacological studies.

PS 4391 **Bayesian Evaluation of a Physiologically Based Pharmacokinetic (PBPK) Model to Characterize Size-Dependent Biodistribution of Microplastics in Mice**

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Background and Purpose: Microplastics (MPs) are emerging environmental contaminants that can cross biological barriers and accumulate in multiple tissues. Due to their diverse physicochemical characteristics, including particle size, surface charge, and surface modification, the mechanisms governing their *in vivo* behavior are complex and difficult to predict. Conventional modeling approaches are often insufficient to capture these variations. This study applied a Bayesian evaluation of a physiologically based pharmacokinetic (PBPK) model to quantify the size-dependent biodistribution of MPs in mice and to characterize uncertainty across different datasets and particle sizes. **Methods:** Experimental biodistribution data (% injected dose, %ID) for MPs ranging from 20 nm to 5 μ m were compiled from multiple published *in vivo* studies covering major organs (liver, kidney, spleen, lungs, gastrointestinal tract, and urine). A multi-compartment PBPK model implemented in *mrgsolve* was designed to simulate the processes of absorption, systemic distribution, and elimination. The model explicitly included kinetic processes for gastrointestinal transport, tissue uptake, reticuloendothelial system (RES) sequestration, and excretory clearance. These kinetic rates were first fitted deterministically and then refined through Bayesian Markov Chain Monte Carlo (MCMC) calibration using the Differential Evolution Adaptive Metropolis (DRAM) algorithm to generate posterior parameter distributions. A Bayesian meta-analysis was subsequently applied to quantify size-dependent variability in these kinetic processes, enabling the model to extrapolate across a continuum of MP sizes. **Results:** The Bayesian PBPK model accurately captured the biodistribution of MPs across all size groups and studies, achieving $R^2 > 0.93$ with 85 % of observed data within a two-fold error range. Model evaluation indicated that the majority of data points in the independent evaluation dataset fell within the 95 % credible intervals (CIs) of model simulations. Posterior predictive checks and forest plots confirmed the model's ability to reproduce observed tissue concentrations with reliable uncertainty bounds. Sensitivity analysis identified gastrointestinal absorption, RES sequestration, and systemic clearance as dominant factors controlling size-dependent behavior. **Conclusions:** This Bayesian evaluation of a PBPK model provides a robust and quantitative framework for assessing microplastic biodistribution and propagating uncertainty across particle sizes. Incorporating meta-analytic estimates of size variability allows the model to predict tissue distribution for a broad range of microplastic sizes, supporting mechanistic interpretation, cross-study comparison, and health risk assessment of micro- and nanoplastics in biological systems

PS 4392 **Integrating Artificial Intelligence to Advance Chemical and Carcinogenic Risk Assessment in Medico-Legal QME Practice**

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Background and Purpose: Evaluating carcinogenic potential for structurally diverse chemicals remains challenging using traditional toxicologic approaches, particularly when exposures involve mixtures, aromatic hydrocarbons, or low-dose chronic profiles frequently encountered in medico-legal QME assessments. Advances in artificial intelligence (AI) and machine learning provide opportunities to integrate mechanistic toxicology with chemical structure features and public hazard classifications. Published tools such as the RASAR read-across system and machine-learning models applied to genomic instability signatures demonstrate how open toxicology resources can support evidence-based carcinogenicity evaluation. This project examined whether AI-driven modeling, using *only* publicly available chemical datasets, could generate mechanistically interpretable predictions relevant to carcinogenic hazard assessment within QME practice. **Methods:** A dataset of chemicals was compiled entirely from public toxicology resources, including PubChem structural data, RDKit-derived physicochemical descriptors, structural alerts from the OECD QSAR Toolbox, and carcinogenicity classifications from IARC and the NTP Report on Carcinogens. Three descriptor classes were extracted: (1) molecular features such as molecular weight, hydrogen-bond donor count, and aromaticity; (2) structural alerts associated with genotoxic or DNA-reactive mechanisms; and (3) hazard groupings derived from established carcinogenicity lists. Gradient boosting, random forest, and a simple neural network were evaluated as modeling approaches to predict carcinogenicity-relevant signatures. Interpretability was assessed using feature-importance methods, and representative chemicals were examined to evaluate biological plausibility and alignment with known mechanistic pathways. **Results:** Across modeling approaches, chemically and biologically meaningful predictors consistently emerged. Aromatic ring count, hydrogen-bond donor number, and DNA-reactivity alerts contributed most strongly to separating known carcinogens from low-toxicity comparator chemicals. For example, benzene contains one aromatic ring, whereas higher-potency polycyclic aromatic hydrocarbons possess three or more, a pattern reflected in the importance of aromaticity-related descriptors. Model interpretability analyses highlighted mechanistic drivers corresponding to recognized carcinogenicity pathways, including aromaticity-linked genotoxicity, electrophilic reactivity, and other structural alerts commonly used in regulatory toxicology. In representative examples, the models consistently assigned higher carcinogenicity-related scores

to compounds historically classified as carcinogenic, demonstrating alignment between computational outputs, chemical structure, and established hazard classifications. **Conclusions:** AI-based toxicologic modeling using publicly available chemical datasets can provide mechanistically transparent insights that complement traditional carcinogenic hazard evaluation. Integrating structural alerts, physicochemical features, and established classifications supports evidence-based toxicology and enhances interpretability in medico-legal and occupational settings. These findings illustrate the feasibility of using open data and accessible machine-learning methods to support carcinogenicity assessment within QME practice. Future work will incorporate larger public datasets (e.g., ToxCast and Tox21) and expand mechanistic coverage to strengthen alignment with regulatory read-across frameworks used in contemporary toxicology.

PS 4393 **SMILES to brain: predicting blood-brain barrier permeability of environmental chemicals.**

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Background and Purpose: The blood-brain barrier (BBB) plays a critical role in protecting the central nervous system by tightly regulating the passage of circulating molecules into the brain. While the BBB is highly selective, multiple environmental toxicants, such as commercial and industrial chemicals, pesticides, microplastic associated additives and emerging pollutants, have been shown to penetrate the BBB and accumulate in neural tissue. Such contaminants may contribute to neurotoxicity and disease risks. Yet, current knowledge of the BBB permeability for environmental chemicals remains limited. Most existing datasets and predicting models are developed primarily for pharmaceuticals and do not adequately represent the structural diversity or physicochemical properties of environmental toxicants. In addition, testing chemical BBB permeability is labor intensive, time consuming and highly costly, requiring specialized *in vitro* models or animal studies that are impractical to scale across thousands of untested chemicals. Therefore, the critical data and methodologies gaps of BBB permeability studies of environmental neurotoxins persist. For instance, machine learning models to accurately assess the BBB permeability of chemicals of environmental concern are still lacking. To address these gaps, in this study, we developed a machine-learning framework to predict chemical BBB permeability and strengthen mechanistic interpretation of neurotoxicity screening. **Methods:** We leveraged a parallel artificial membrane permeability assay for brain membranes (PAMPA-BBB) dataset of 106 compounds of environmental concerns to develop machine learning models to predict their permeability. Multiple models including random forest, gradient boosting machine, and neural networks using various molecular fingerprints and molecular descriptors were developed. A nested 5-fold cross-validation was used for model evaluation and hyperparameter tuning. Various model evaluation metrics such as accuracy, AUROC, specificity, and sensitivity were used. Feature-importance and cumulative-importance analyses were applied to identify important molecular sub-structures and properties. **Results:** Our preliminary results showed that random forest achieved the highest accuracy (0.793 +/- 0.064) and AUROC (0.69 +/- 0.081) with high specificity (0.939 +/- 0.061) but low sensitivity (0.286 +/- 0.181), indicating strong identification of low permeability compounds while underpredicting high permeability ones. Gradient boosting showed slightly lower accuracy (0.783 +/- 0.077) and AUROC (0.679 +/- 0.098) with improved sensitivity (0.419 +/- 0.075). The MLP classifier had the highest sensitivity (0.514 +/- 0.029) but lower accuracy (0.727 +/- 0.138) and specificity (0.782 +/- 0.155) reflecting a tradeoff between detecting high permeability compound and overall predictive performance. Feature importance analysis revealed that features associated with lipophilicity, polar surface area, and hydrogen-bonds donors were among the strongest predictors of BBB penetration, in line with mechanistic expectations. **Conclusions:** Our preliminary results demonstrated that machine learning models can effectively predict the BBB permeability of environmental chemicals, addressing a critical gap in neurotoxicity risk assessment. Using a PAMP-BBB dataset of 106 environmental relevant compounds, random forest, gradient boosting, and MLP classifiers were developed leveraging molecular fingerprints and descriptors. The MLP classifier achieves best overall performance. Future work will focus on improving model sensitivity and performance with imbalanced dataset. Collectively, these results underscore the value of integrating machine learning approaches into BBB permeability studies, providing a scalable, cost effective and informative strategy to prioritize environmental chemicals for neurotoxicity evaluation and support public health decision making.

PS 4394 **Filling Gaps in Toxicity Data of Pesticides with OrbTox - a repository of multidomain experimental data and predictive models**

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Background and Purpose: The new approach methodologies including *in silico* methods (read across and QSAR models) are gaining importance for hazard and risk assessments, especially as the regulatory and economic emphasis is on reducing reliance on traditional animal testing. The genotoxicity assessment of pesticides and

their metabolites is important to assess their harmful effects on the environment and human health. However, for a large number of pesticides/biocides limited or no data exists from six commonly employed regulatory genotoxicity assays. We have recently developed and validated predictors of Ames test outcome in OECD-recommended strains with and without metabolic activation. Herein we describe using these QSAR models for filling gaps in the bacterial mutagenicity assessments for pesticides and identifying structural features responsible for mutagenicity. **Methods:** Publicly available CCRIS database which contains over 80K Ames test records was harmonized to compile a cleaned data set of over 6K unique compounds with experimental details compliant with the OECD Test Guidelines # 471. QSAR model to predict Ames test results in each of the ten Strain-S9 combinations was developed using Saagar descriptors to fill gaps in the compounds not tested in all ten Strain-S9 combinations to create a unified modeling data set that guarantees a compound labeled Neg in Ames test is Neg by all ten Strain-S9 models. The final QSAR model, Unf(All), was used (via OrbiTox) for predictions of bacterial mutagenicity of new chemicals. 4826 unique pesticide substances collated from 18 public pesticide/biocide databases by Foil *et al.* were used to find gaps in genotoxicity evaluation by overlapping substances with data from six commonly used six genotoxicity assays. Substances missing Ames test evaluations were processed through OrbiTox. Among the 4826 pesticides, 1592 were found to be Ames Neg, 382 Ames Pos, and 2852 without Ames test evaluation. **Results:** In 3x5-fold cross validation, the final Unf(All) model returned AUROC, Kappa, PPV, NPV, Sensitivity, and Specificity, respectively, 0.951, 0.769, 93.7%, 82.1%, 89.3%, and 89.1%. Structures of these 2852 compounds with missing Ames test evaluation were processed through the Unf(All) Ames model installed in OrbiTox². Due to structural reasons, e.g., inorganics, organometallics, etc., 172 compounds could not be processed. Additional 156 isomeric structures with InChI key identical to the corresponding 2D SMILES were excluded. Model's applicability domain (AD) check rendered 156 predictions unreliable. The acceptable predictions were labeled Neg (1655), Ind (418, Indeterminate), and Pos (336), respectively, based on predicted probability of being an Ames Pos <0.4, 0.4 to 0.6, >0.6. Analyses of the structures of compounds predicted Pos or Neg revealed several substructures predominantly present in Ames Pos and Neg compounds. For example, meta-dinitrobenzene substructure had Pos:Neg distribution of 44:0 and aliphatic or nitro group had Pos:Neg distribution of 97:6. Similarly, substructures with predominant Neg:Pos distribution include delta lactones and cyclohexane ketones with 56:0 distribution and tetra alkyl methane with 130:3 distribution. Additional substructures with differing distributions in Ames Pos and Neg compounds will be presented in the poster. **Conclusions:** The robust QSAR Ames mutagenicity predictor derived from harmonized experimental data compliant with OECD Test Guideline 471, given its accuracy in cross-validation and test sets, is an excellent resource for filling gaps in data for untested compounds. The Saagar descriptors used for building QSAR models allow visualization of structural features likely to render a compound Ames Pos or Neg. The Ames mutagenicity predictor described here and over hundred other QSAR models of various toxicity endpoints are available in OrbiTox, which is free for non-commercial use.

PS 4395 ChemHydra: A Novel AI-Driven Pipeline for ADME, Physicochemical, and Toxicity Endpoint Prediction Integrating Advanced Chemical Characterization

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Background and Purpose: As data from New Approach Methodologies (NAMs) rapidly expand alongside modern artificial intelligence (AI), there is a clear need for an updated QSAR pipeline that integrates cutting-edge AI approaches to model and benchmark complex toxicity, physicochemical, and ADME endpoints. To meet this need, our team has developed a comprehensive, Python-based cheminformatics pipeline, ChemHydra, engineered to generate robust, predictive, and interpretable QSAR models that comply with all OECD principles. Our methodology advances the traditional workflow by focusing on rigorous chemical preprocessing, comprehensive descriptor generation, flexible modeling with robust benchmarking, and detailed model interpretation. **Methods:** ChemHydra is built on three advanced steps. The first is rigorous chemical standardization, which is optimized by processing large-scale databases (e.g., PubChem, DSSTox, ChEMBL); we use an in-house salt list to remove counter-ions, SMARTS-based reactions to standardize complex chemical states, and a proprietary method to assign correct protonation states. This is followed by comprehensive descriptor generation, where we integrate a diverse set of molecular descriptors, including 1D/2D/3D properties, custom substructure descriptors, Saagar, and vector-based embeddings from pre-trained SMILES-based language models. Finally, ChemHydra features flexible modeling and benchmarking, integrating over 40 machine learning (e.g., XGBoost, Random Forest) and deep learning (e.g., MLP, Transformers, Multitask Learning) algorithms. This suite allows us to benchmark a full range of models, selecting the optimal architecture for any given dataset. All models are paired with local interpretation methods to fulfill OECD Principle 5 as well as standard QMRF report. **Results:** We benchmarked our pipeline on large, publicly available datasets for physicochemical, ADME, and toxicity endpoints, with performance compared to established models like ChemProp, OPERA, and Vega. Our generated models consistently performed comparably to or outperformed these benchmarks. For physicochemical properties, we are able to predict logP and the Henry's Law constant using a Saagar descriptor and an XGBoost approach with high performance ($R^2 = 0.92$ and $R^2 = 0.93$, respectively, in 5-fold

cross-validation). For ADME property modeling of plasma protein binding, our deep learning model (utilizing Saagar descriptors and LLM embeddings) achieved an R^2 of 0.76. We also predict the biotransformation rate constant with an R^2 above 0.9 using a deep learning approach. Finally, for toxicity endpoints such as carcinogenicity, a consensus model using Saagar descriptors predicted mammalian carcinogenicity with an AUROC of 0.971. Furthermore, for mutagenicity, a multitask deep learning model applied to Ames data achieved a mean balanced accuracy (B-Acc) of 0.85 across all strains. **Conclusions:** We present an integrated, modern QSAR pipeline that builds more accurate and interpretable models by leveraging advanced AI and novel descriptors. This pipeline has led to the development of over 100 models for ADME/Toxicity endpoints that are also accessible on our OrbiTox web platform (<https://orbitox.org/>).

PS 4396 Systematic Evidence Mapping of Per- and Polyfluoroalkyl Substances (PFAS): A Large Language Model-Based Toxicokinetic Database

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Background and Purpose: Per- and Polyfluoroalkyl Substances (PFAS) are synthetic compounds ubiquitously present in the environment and living organisms, including humans. Understanding their toxicokinetic (TK) profiles is critical for exposure and human health risk assessment. The objective of this study was to develop a comprehensive PFAS TK database by leveraging large language models (LLMs) to systematically identify, extract, and compile concentration-time and tissue distribution data from published scientific literature. **Methods:** A systematic PubMed search (1980-August 2025) identified TK studies using terms related to pharmacokinetics, absorption, distribution, metabolism, excretion, toxicokinetics, and biodistribution, combined with species identifiers and 578 PFAS compounds curated by the U.S. EPA's Center for Computational Toxicology and Exposure. Full texts of eligible studies were processed with the GPT-oss-120b large language model to extract structured data on compounds, species, organs, exposure routes, and biological matrices. Extracted data were consolidated, deduplicated, manually validated, and integrated into a relational database supporting query and visualization by chemical, species, tissue, and TK parameter. **Results:** A total of 888 articles were screened, and 874 records covering 578 PFAS compounds were included. After manual verification for relevance to PFAS TK, 639 records were retained. Among these, perfluorooctanoic acid (PFOA) (n = 458), perfluorooctane sulfonic acid (PFOS) (n = 427), and perfluorononanoic acid (PFNA) (n = 222) were the most frequently reported compounds. Studies primarily involved humans (n = 280), rats (n = 199), fish (n = 138), and mice (n = 129). The most represented biological matrices were liver (n = 378), kidney (n = 238), and serum (n = 189). The resulting database enables systematic comparison of PFAS TK profiles across species and tissues to support data-driven modeling and risk assessment. **Conclusions:** This study demonstrates the feasibility of applying LLMs to systematically extract and organize PFAS TK data from scientific literature. The curated database integrates 639 curated records covering 578 PFAS compounds, species, and organs, providing the most comprehensive PFAS TK evidence resource to date. This LLM-assisted framework enhances data accessibility and supports evidence-based exposure and human health risk assessment for PFAS.

PS 4397 Leveraging continuous, real-time air quality monitoring in agricultural settings to improve health and economic outcomes

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Background and Purpose: Agriculture contributes to a high percentage of air pollutants globally. Air pollution in agricultural settings arise from pesticide and fertilizer usage, burning practices, as well as livestock and manure management. Aerosolized pollutants from these sources pose significant challenges to farmers, consumers, and ecosystems — not only through their direct effects but also by persisting in the environment for extended periods. A major challenge in measuring agricultural air pollution is capturing its highly variable, short-lived emissions from diffuse sources like fields. Pollutant levels shift rapidly with weather, soil, and farming practices — making reliable measurement difficult. **Methods:** We developed a high-resolution solar-powered internet of things (IoT) device integrated with remote sensing and real-time data aggregation capabilities. Through a U.S. Fulbright Scholar Award, we are currently collecting continuous, longitudinal data on parameters such as particulate matter (PM_{2.5}, PM₅ and PM₁₀), volatile organic compounds (VOC), temperature, humidity and NO_x in rice fields in Sri Lanka through the entire cultivation cycle from land preparation to post-harvest. **Results:** Our proof-of-concept testing studies indicate technical feasibility for long-term (>90 days) uninterrupted air quality monitoring and remote sensing. Preliminary data on pollutant levels during the first cultivation cycle will be presented. Machine learning and advanced data analytical models will be applied to make predictions on air quality events. These data will also be aligned with crop yield data as well as human, occupational and ecological health

outcomes data. **Conclusions:** Using these technologies for continuous, real-time air quality monitoring can help improve multiple health and economic outcomes in agricultural settings.

PS 4398 Chemical Effects in Biological Systems (CEBS): A Curated Toxicology Database with Extensive Data, Integrated Datasets, and User-friendly Interface.

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Background and Purpose: The Chemical Effects in Biological Systems database (CEBS) contains extensive data from toxicology studies in support of the Division of Translational Toxicology (DTT) at the National Institute of Environmental Health Sciences (NIEHS), along with data from other studies of environmental health relevance. It encompasses a wide range of *in vivo* and *in vitro* data types at the individual animal/subject level, summary level, and conclusion level. CEBS incorporates active internal databases into a central data repository and provides high quality curated datasets with rich standardized metadata to better serve the scientific community. This effort includes harmonizing legacy terms and metadata with current standards and aligning terms to OBO (Open Biological and Biomedical Ontology). These curated datasets are updated with modern data from new research efforts, while novel datasets, like individual animal data for genetic toxicology studies, are also being made available through CEBS. **Methods:** Not applicable. Abstract is for non-laboratory or non-field Studies. **Results:** CEBS grants public access to data from over 10,000 studies for 12,750 test articles (chemicals, environmental agents). The CEBS user interface features a single search bar to query test articles, study identifiers, and other keywords related to study metadata and publications. Search results are consolidated onto dedicated pages for each test article, including study data, protocol, conclusions, publications, datasets, and links to related articles and projects. Data are also organized by endpoint into data collections and are accessible through a filterable and downloadable application at <https://cebs-ext.niehs.nih.gov/datasets/>, as well as in flat file format for computational analysis and AI applications at <https://doi.org/10.22427/NTF-DATA-110-021-001-000-6>. CEBS also offers guided applications for exploring and analyzing specific data types, such as gene expression, pathology (<https://cebs.niehs.nih.gov/organsites/>), etc. High-interest datasets, including TOX21 data, studies containing both genomic and apical endpoints with benchmark dose (BMD) analysis, and expression data, are readily accessible. **Conclusions:** Not applicable. Abstract is for non-laboratory or non-field Studies.

PS 4399 Exploring ToxCast: Enhancing Public Access to Targeted Bioactivity Screening Data

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Background and Purpose: Building confidence in new approach methods (NAMs) for prioritization and hazard characterization requires bioactivity data to be accessible and easily interpretable. The US Environmental Protection Agency Toxicity Forecaster (ToxCast) program makes *in vitro* medium- and high-throughput screening assay data publicly available for thousands of chemicals. The ToxCast pipeline (tcpl) is an open-source R package that stores, manages, curve-fits, and visualizes data while populating the linked MySQL database, invitrodb. Tcpl is released in versions at the Comprehensive R Archive Network (CRAN), available here: <https://CRAN.R-project.org/package=tcpl>. Vignettes describing an introduction to tcpl, database structure, assay annotation, processing, and data retrieval are available for users seeking additional details on the functionality of tcpl. The latest invitrodb release (v4.3, August 2025) includes data from 29 different assay sources, including the Toxicology in the 21st Century (Tox21) federal consortium, 717 assay platforms with 1,647 normalized assay readouts across a diverse biological space with over 500 mapped gene targets for 10,487 unique chemicals. Ongoing work is focused on augmenting and enhancing how ToxCast data can be accessed to address toxicology research questions for a diverse user group. **Methods:** The agile nature of the ToxCast project allows for integration and interoperability with other software applications. To accommodate recent software and database releases while supporting a growing ToxCast userbase, accessibility has been expanded within existing tools via updates to the CompTox Chemicals Dashboard (CCD, <https://comptox.epa.gov/dashboard/>), Computational Toxicology and Exposure Application Programming Interfaces (CTX APIs), and database downloads. **Results:** For a new user, the CCD presents a view of potency and relative efficacy metrics across ToxCast endpoints within the bioactivity module for chemicals of interest. Herein, users can sort, filter, and export results. In CCD v2.6, feature updates associated with invitrodb v4.2 include new visualizations, export options, and integration of analytical data to inform chemical applicability domain for *in vitro* screening. Users with more customized ToxCast data needs, or with a need to use tcpl for their own data processing, may wish to set up a personal instance of invitrodb and interact with the data via tcpl. The CTX Bioactivity API is the default connection for tcpl, allowing users to avoid large data downloads by accessing invitrodb programmatically via an API. APIs provide a read-only solution for users who require more flexibility than the CCD can provide. Additionally, comprehensive

assay documentation aligned with the OECD Guidance Document 211 has been released for all 1647 ToxCast endpoints, which improve ToxCast targeted bioactivity data interpretability. Visit the Exploring ToxCast Data webpage for more information. **Conclusions:** ToxCast provides a standard for consistent and reproducible data delivery for diverse, targeted bioactivity assay data with readily available documentation and a unified open-source software approach. Recent software and database updates expand the data delivery options. These updates enable use of ToxCast data within an integrated data landscape by a diverse group of users for a myriad of toxicology applications. *This abstract does not necessarily reflect U.S. EPA policy.*

PS 4400 Leveraging Machine Learning for Efficient Prediction of Acute Inhalation Toxicity of Environmental Chemicals

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Background and Purpose: Assessing the acute inhalation toxicity of chemicals, indicated by median lethal concentration (LC50) values, is key for evaluating human health risks from exposure to environmental chemicals. Traditional *in vivo* testing methods are often lengthy and require significant resources, highlighting the need for developing computational models that can predict toxicity for a wide array of chemicals efficiently. **Methods:** In this research, we introduce a regression model based on machine learning to predict acute inhalation LC50 values in units of ppm and mg/L. We utilized a dataset consisting of 612 chemicals with experimentally determined 4-hour LC50 values as the training set. The Python RDKit library was employed to compute 210 molecular descriptors for each chemical, covering various physico-chemical properties. Initially, 15 machine learning algorithms were assessed for their predictive capabilities. The Extra Trees algorithm showed the most promising results, and further enhancements were made through recursive feature elimination (RFE), optimization of data transformation, and hyperparameter tuning to refine the model's accuracy. Furthermore, we evaluated the model's applicability domain using a chemical library of 47,768 environmental chemicals and the K-Nearest Neighbor method to assess its practical utility. **Results:** Two separate models were developed to predict LC50 values in ppm and mg/L. The final model for ppm achieved an R² of 0.619 and a root-mean-square error (RMSE) of 0.873, while the mg/L model achieved an R² of 0.538 with an RMSE of 0.904. Both models demonstrated reasonable applicability domains based on the selected threshold. **Conclusions:** The regression model based on machine learning presented in this study demonstrates significant accuracy and efficiency in predicting LC50 values. It provides a scalable and effective approach suitable for high-throughput toxicity screening. By incorporating machine learning, this model can streamline toxicological evaluations and lessen reliance on experimental testing, thereby aiding regulatory and safety efforts.

PS 4401 ChatAOP: AI-Driven Multi-omics Framework for Revealing New Modality Tox Mechanisms in Adverse Outcome Pathways

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Background and Purpose: The Adverse Outcome Pathway (AOP) framework plays an important role in organizing toxicological information and enabling early risk mitigation during drug development. Increased integration of multi-omics datasets with advanced AI methodologies will enhance AOP generation and derisking of new molecular entities in nonclinical contexts. **Methods:** Herein, we developed ChatAOP, an AI-driven framework designed to predict and prioritize mechanisms underlying new molecular entities through the integration of diverse data sources. The platform combines multi-omics datasets; curated relationships among proteins, diseases, and adverse effects, existing information from the AOP-Wiki, and human protein-protein interactions from the STRING v11 database. This integration enables localization of molecular initiating events, key events, and corresponding outcomes to specific tissues and cell types. Additionally, ChatAOP incorporates a prompt-based interface to provide an intuitive, user-friendly experience for users. **Results:** To validate the performance of the proposed ChatAOP framework, we utilized in-house multi-omics data from patient populations to uncover specific toxicological mechanisms across different stages of chronic disease progression. This analysis identified more than 300 differentially expressed genes DEGs associated with distinct disease states and generated a list of potential Adverse Outcome Pathways AOPs linked to toxicological liabilities. Specifically, the analysis uncovered AOPs linking key mechanistic pathways to distant organ toxicities: 1) ROR2 to Diabetic Nephropathy via podocyte effacement and glomerular basement membrane degradation, 2) IL6 to Aortic Dissection via the inflammatory/remodeling cascade and loss of structural integrity, and 3) THRB to Neurodevelopmental Disorders via impaired neural circuit formation. **Conclusions:** The proposed ChatAOP framework represents a promising tool for safety target identification, hypothesis generation for potential safety mechanisms, and the discovery of novel biomarkers and alternative therapeutic indications for disease states.

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Background and Purpose: The Safe and Sustainable by Design (SSbD) framework aims to assess the holistic impact of a chemical throughout their life cycle. However, current impact assessment methodologies mostly operate in isolation, lacking integration of human and ecotoxicity mechanistic insights with environmental, social, and economic factors. To address this gap, this study developed a unified, mechanistically grounded framework that strengthens holistic understanding of chemical impacts within SSbD by expanding the Adverse Outcome Pathway (AOP) framework with environmental and socioeconomic pathway structures. The work specifically assessed how these novel Impact Outcome Pathways (IOPs) could enable more coherent cause-effect reasoning across different types of impacts relevant to chemical design. **Methods:** Established cause-effect connections from life cycle assessment and socioeconomic literature were extracted to develop pathway structures analogous to AOPs. These connections, formalised as Key Events (KEs), were used to build an interconnected network consisting of mechanistic pathways for environmental, health, social, and economic outcomes, termed Impact Outcome Pathways (IOPs). A natural language processing (NLP) approach was applied to link mechanistic events from AOP-Wiki entries with the established IOP KEs, thereby identifying cross-dimensional Key Event Relationships (KERs) in which safety-related KEs connect to environmental or socioeconomic KEs. In addition, a proof-of-concept case study is planned to evaluate whether integration of NAM-derived mechanistic data into the IOP network provides more holistic, decision-relevant information than conventional SSbD assessment. **Results:** The resulting network contains over 450 nodes (KEs) and 650 KERs. Shared KEs connect both within individual environmental, health, social, and economic domains and across domains, highlighting cross-dimensional influences for integrated assessment. NLP-based mapping captured connections between AOP KEs and IOPs that can be represented either as direct KERs or as modulating factors. Cross-dimensional KERs were observed between all domains, illustrating how highly interconnected chemical impacts become when described through detailed mechanistic pathways. **Conclusions:** The IOP framework provides a coherent basis for enhancing SSbD by embedding mechanistic toxicology into broader sustainability assessment. This approach supports more integrated and holistic evaluation of chemical impacts by linking AOP-structured, NAM-derived evidence directly to environmental, health, social, and economic outcomes.

PS 4403 Evaluation of a comprehensive and flexible behavior analysis tool for neurotoxicity testing in larval zebrafish (NeuroBEAT)

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Background and Purpose: There are hundreds of thousands of chemicals in commerce, yet less than 150 chemicals have been tested for their potential to harm the developing nervous system using OECD-approved test guidelines. This data gap largely reflects the absence of OECD-approved alternative methods for developmental neurotoxicity (DNT) testing. Zebrafish larvae have emerged as a promising alternative model for DNT testing. Zebrafish behavior in response to various light or acoustic stimuli can be used as a functional readout for neurodevelopment. However, progress in this field is hindered by the lack of standardized, user-friendly analysis pipelines capable of visualizing automated behavioral data, statistically comparing treatment groups, and deriving toxicologically relevant metrics such as AC₅₀ values. Here, we introduce a novel and adaptable analysis pipeline which can cover a wide range of commonly used light-dark transition assay designs, as well as new human-relevant endpoints, including non-associative habituation which is a conserved form of learning and memory retention. **Methods:** Zebrafish motor activity was quantified using a video-based tracking system (Δpixels/second). Timeseries data comprised of distance moved over time (phase data, habituation data) and immediate startle responses to light or acoustic stimuli (1 sec post-stimulus) were analyzed. Timeseries data involved repeated measures of the same larva and were analyzed using generalized additive mixed effects models (GAMMs). Startle responses consisted of single measurements per larva and were modeled using linear mixed effects models (LMMs). For GAMM-modeled endpoints, the Fréchet distance between fitted splines was used to capture differences between treatment groups and controls regardless of effect direction. Concentration-response data for all endpoints were competitively fitted using 10 different curve-fitting models included in the US EPA tcpl v3.0 pipeline. Toxicologically relevant metrics were extracted from the winning model, some of which, including AC₅₀ and scaled top values, were used for comparison with *in vitro* ToxCast assays. **Results:** Both GAMMs and LMMs delivered accurate results and captured diverse behavioral responses in zebrafish behavior including altered locomotion, altered startle responses and habituation learning. R²-values ranging from 0.6-0.85. AC₅₀ values derived from concentration-response models aligned well with *in vitro* ToxCast assays data and in some cases suggest enhanced sensitivity. **Conclusions:** We developed a novel, adaptable pipeline to analyze automated zebrafish behavior data. We used a range of example chemicals causing different behavior effects to demonstrate the robustness and flexibility of our mixed effects modeling strategies. For all endpoints tested, concentration-response analyses produced toxicologically meaningful metrics independent

of effect direction. Overall, implementation of a flexible and standardized analysis pipeline could enhance confidence in using zebrafish behavioral data to predict the neurotoxic potential of environmental chemicals and pharmaceutical compounds.

PS 4404 Multi omics integration of transcriptional and metabolic responses to DILI compounds across human liver models

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Background and Purpose: Cellular stress responses rely on coordinated transcriptional programs where co-expressed genes support specific functions. Yet, transcriptomics captures only one regulatory layer. Post-transcriptional mechanisms—including mRNA stability, translational control, and protein modifications—further shape outcomes, meaning gene expression changes do not always predict metabolic consequences. Integrating transcriptomics with metabolomics provides a more complete mechanistic view by linking regulatory networks to biochemical effects. Drug-induced liver injury (DILI) remains a major cause of drug failure and withdrawal. Mechanistic understanding in human-relevant systems requires quantitative frameworks connecting molecular initiating events (MIEs) to adverse outcomes. This study integrates targeted transcriptomics and metabolomics across three human liver models to characterize DILI mechanisms and evaluate the sensitivity and reproducibility of omics-based points of departure (PODs) for risk assessment. **Methods:** Three Class A DILI compounds—nitrofurantoin, diclofenac, and ketoconazole—were profiled in primary human hepatocytes (PHHs), HepG2 cells, and hiPSC-derived hepatocyte-like cells (HLCs). Targeted transcriptomics used the TempO-Seq platform, and broad targeted metabolomics applied a high-throughput LC-MS/MS panel covering ~250 metabolites across 13 classes. HepG2 cells were analyzed in time-course (3-48 h, two doses) and dose-response (five to six concentrations, 24 h) formats, while HLCs and PHHs were assessed in 24 h dose-response series. Differentially expressed genes showed clear time- and dose-dependent patterns and were projected onto the PHH Toxicogenomics Mapper (TXG-MAPr) framework containing 398 functionally annotated gene modules. This module-based approach reduced data complexity and improved biological interpretability, enabling cross-system comparison. Spearman correlations between metabolites and module eigen-gene scores identified strongly associated metabolite-gene network pairs. For example, oxidative stress activation corresponded to negative correlations between glutathione (GSH) depletion and modules enriched for NRF2-driven (modules 144, 337) and ATF4-regulated (module 295) genes. Benchmark dose (BMD) modeling of 24 h dose-response data yielded transcriptomic (tPOD) and metabolomic (mPOD) points of departure. Module-level tPODs were the median BMD of genes within enriched modules (≥3 genes covering ≥5% of membership). Individual metabolite BMDs were defined as mPODs. For correlated metabolite-gene pairs, tPODs and mPODs were compared to determine which omics layer responded at lower concentrations and assess POD reproducibility across systems. **Results:** Correlation-based integration successfully linked metabolic shifts with transcriptional network activation across all liver models, demonstrating the broad metabolomics panel's utility in different contexts. Time- and dose-dependent responses confirmed conserved activation of oxidative stress, ER stress, and mitochondrial dysfunction modules across systems, though magnitudes varied. Omics-based POD modeling showed that comparing mPODs and tPODs for correlated metabolite-gene networks identified the more sensitive regulatory layer, providing insight into cross-system consistency and endpoint uncertainty. **Conclusions:** This integrative multi-omics framework aligns metabolic and transcriptional changes across human liver systems, linking metabolite alterations to activation or repression of defined gene networks. It enhances interpretability of high-throughput NAM data and supports quantitative evaluation of sensitivity and reproducibility across PHH, HepG2, and HLCs. By comparing tPODs and mPODs, this study establishes a foundation for selecting the most sensitive omics layer for protective POD derivation, advancing mechanism-based safety assessment in next-generation chemical testing strategies.

PS 4405 Genome-scale CRISPR Screening Identifies C18orf32 as a Novel Regulator of Lipid Metabolism Mediating PFOA-induced Liver Toxicity

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Background and Purpose: Perfluorooctanoic acid (PFOA) remains a public health concern due to its persistence in the environment, despite global production bans. Epidemiological, animal, and *in vitro* studies have consistently linked PFOA exposure to liver toxicity characterized by lipid dysregulation; however, the molecular mechanisms underlying these adverse effects in humans remain poorly understood. Conventional correlative approaches cannot establish causal gene-phenotype relationships, highlighting the need to identify functional genetic determinants of PFOA-induced lipid perturbations. **Methods:** To address this gap, we employed an integrated functional toxicogenomics framework combining CRISPR screening (target gene identification), target validation, and assessment of target-derived cellular and

molecular phenotypes. **Results:** From a genome-wide CRISPR screen in HepG2/C3A human liver cells, we identified 319 candidate genes (140 sensitive and 179 resistant) that modulate PFOA toxicity. Among these, *C18orf32*—a lipid droplet (LD)-associated protein and the top resistant candidate—was selected for further mechanistic investigation. Targeted knockout (KO) of *C18orf32* conferred marked resistance to PFOA, accompanied by reduced LD accumulation under exposure conditions, suggesting a functional role of *C18orf32* in lipid dysregulation. To elucidate molecular mechanisms underlying the resistance, we conducted systematic transcriptomic profiling of WT, *C18orf32* KO, WT+PFOA, and *C18orf32* KO+PFOA cells. *C18orf32* KO caused extensive gene expression reprogramming, with over one-third of coding genes differentially expressed. Notably, lipid metabolic pathways including cholesterol metabolism, PPAR signaling, fatty acid metabolism, and peroxisomal functions—known mediators of PFOA-induced hepatotoxicity—were significantly downregulated in *C18orf32* KO cells. **Conclusions:** Collectively, our results identify *C18orf32* as a previously unrecognized regulator of hepatic lipid homeostasis and a key molecular determinant of PFOA-induced lipid dysregulation and liver toxicity in humans.

PS 4406 Tire antiozonants 6PPD and its quinone derivative effects on *Saccharomyces cerevisiae*

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Background and Purpose: N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine or 6PPD is a tire coating that prevents the degradation of rubber bonds. When introduced to atmospheric ozone, 6PPD oxidizes to 6PPD-Quinone (6PPD-Q). Both 6PPD and 6PPD-Q have been found in road water runoff, dust particles, and various rubber products. The introduction of 6PPD into the environment has also led to a deadly impact on the coho salmon population among other aquatic species. The discovery of the toxicological effects of 6PPD and its quinone derivative is new and not thoroughly understood. Understanding the underlying mechanism in various model organisms is essential to further our knowledge base and help establish regulations on the use of these chemicals. **Methods:** 6PPD and 6PPD-Q were tested for toxicity in *Saccharomyces cerevisiae* yeast. The yeast proliferation was determined after 6 hours using spectrophotometry. Mitochondrial staining was also employed to visualize 6PPD and 6PPD-Q's effect on the mitochondrial structure and function. Furthermore, RNA sequencing was completed for both 6PPD and 6PPD-Q to analyze the genes, pathways and proteins effected by each chemical. **Results:** The yeast cell viability showed a stepwise decrease when exposed to 6PPD. When completing the same experiment for 6PPD-Q the optical density for the yeast increased in a step wise manner when exposed to 100, 50, and 25 μ M of 6PPD-Q. Mitochondrial staining was completed for 6PPD to validate the decrease in cellular growth. The fluorescence intensity of treated cells compared to the non-treated control showed a significant decrease indicating a reduction in mitochondrial activity. RNA sequencing was completed for both 6PPD and 6PPD-Q. For both treatments oxidative phosphorylation KEGG pathway genes were downregulated. 6PPD caused an upregulation in pyruvate metabolism while 6PPD-Q caused an increase in starch and sucrose metabolism. **Conclusions:** 6PPD and 6PPD-Q differentially effect *Saccharomyces cerevisiae* through various pathways. Future studies will focus on mitochondrial staining for 6PPD-Q and rtPCR to validate the RNA sequencing data.

PS 4407 The cytotoxic effect of Trichloroethylene (TCE) involves both the extrinsic and intrinsic apoptosis pathways via potential binding of TCE to Calmodulin and Fas receptor in human neuronal cells

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Background and Purpose: Trichloroethylene (TCE) is a common environmental pollutant. Environmental exposure to TCE is associated with a variety of neurological effects which was supported by findings that TCE affected the mitochondria in human nerve cells, causing degeneration. There is a relatively small body of evidence supporting the connection between TCE and apoptosis. This study is to determine the role of TCE in apoptosis in neuron cells/tissues and the potential target(s) of TCE. **Methods:** TCE was obtained from Sigma-Aldrich, pre-dissolved in 50 μ L of DMSO and added to 10 mL of culture medium. Human neuronal cells (HTB-11) were cultured per ATCC guideline and treated with TCE of different concentrations (0.5 μ M, 5 μ M and 50 μ M) for 24 hours. To assess environmental effects, ganglia were isolated from blue mussels (*Mytilus edulis*) and exposed to the same concentrations of TCE. According to the manufacture's instructions (Sigma-Aldrich), cell viability was tested in MTT assays and cell membrane permeability was tested in LDH assay for cytotoxicity levels. Caspase 3, caspase 8 and caspase 9 activity were measured using the ApoAlert caspase fluorescent assay kit (Clontech). All experiments were repeated 3 times. P-values, calculated from two-tailed student t-tests and one-way ANOVA. Molecular docking was performed to identify potential binding target molecules and intermolecular interaction. Chemical structure of TCE was obtained from PubChem compound database. A total of 23 distinct PDB coordinates of 9 different protein compounds from the apoptosis pathway were downloaded from Protein Data Bank. Software PyRx was used for virtual screening of ligand-receptor interactions.

AutoDock Vina and AutoDock 4.2 were used as docking software. For visualization and analysis of the docked results, Discovery Studio Visualizer software was used. **Results:** In HTB-11 cell line, there was a dose-response relationship between TCE and cell viability, with survival rate of 89% ($p < 0.05$), 82% ($p < 0.05$) and 70% ($p < 0.01$) at 0.5 μ M, 5 μ M and 50 μ M of TCE respectively. In *M. edulis*, ganglia tissues exhibited $59\% \pm 3.7$ dead cells at 50 μ M compared with $14\% \pm 2.1$ in controls ($p < 0.001$). LDH assay showed that TCE caused a small and negligible damage to cell membrane ranging from 0.7% to 1.9% ($p > 0.05$). To investigate the possible role of TCE in apoptosis pathway, caspase assays were performed. At 0.5 μ M, 5 μ M, and 50 μ M of TCE, caspase 3 activity in HTB-11 cells increased by 15% ($p < 0.05$), 28% ($p < 0.01$), and 4% ($p < 0.05$), respectively. To determine the involved pathway, caspase 8 and caspase 9 activities were performed as caspase 8 mostly works in the extrinsic pathway and caspase 9 in the intrinsic pathway. In HTB-11 cells, there was a dose-dependent induction of both caspase 8 and caspase 9 activities by TCE. At the highest level of 50 μ M of TCE, caspase 8 and caspase 9 activities increased by 443% ($p < 0.01$) and 164% ($p < 0.01$) respectively. Molecular docking experiments that the two most stable ligand-receptor complex were calmodulin (PDB ID 3ewt) with binding energy of -4.2 kcal/mol and Fas receptor (PDB ID 3ezq), with binding energy of -3.9 kcal/mol, both of which have been shown to be critically involved the extrinsic apoptosis pathway (Fas receptor) and the cytochrome c/mitochondria-mediated intrinsic pathway (Calmodulin) although the Calmodulin is less established than Fas receptor. **Conclusions:** The results demonstrated that the cytotoxic effect of TCE involves both intrinsic and extrinsic apoptosis pathways in human neuronal cell line and marine invertebrate ganglia. The apoptotic effect is likely mediated through a novel mechanism in which TCE directly binds to calmodulin molecule and Fas receptor. As TCE is found to be associated with diseases of nerve system, the present study could potentially lead to development of targeted therapeutic strategies.

PS 4408 Concentration-dependent induction of proliferation and oxidative stress by phthalates (DEHP and MEHP) in RWPE-1 prostate epithelial cells

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Background and Purpose: Epidemiological studies suggest a strong association between phthalates exposure and proliferative diseases in the prostate including prostate cancer. Previous studies have also shown increased proliferation of prostate cancer cells via phthalate exposure. Increased cell proliferation is a hallmark of carcinogenesis. However, whether phthalates can cause increased proliferation in normal prostate epithelial cells is not known. Therefore, the objective of this study was to evaluate the effects of both parent compound (DEHP) and its primary metabolite (MEHP) on the proliferation of normal prostate epithelial cells. The role of oxidative stress and the molecular mechanism through which these phthalates exert their effects on these cells was further investigated. **Methods:** RWPE-1 cells, an immortalized prostate epithelial cell line derived from normal prostate epithelium, were exposed to the phthalate di-2-ethylhexyl phthalate (DEHP), and its active metabolite mono(2-ethylhexyl) (MEHP) separately at various concentrations (0.1, 1, 10, and 100 μ M) for 24 and 72 h. Cell viability was assessed using MTT assay and oxidative stress was assessed using DCFH assay. Cell cycle via flow cytometry was used to further confirm the effects of these phthalates on cell proliferation. **Results:** RWPE-1 cell exposure to DEHP and MEHP at concentrations of 0.1, 1, 10, and 100 μ M, resulted in a dose-dependent decrease in cell viability. There was also a significant increase in ROS production across all respective treatments for both DEHP and MEHP. Using flow cytometry there was an observed agonist and antagonist dose-dependent relationship between cell cycle arrest and cellular proliferation for the respective treatments. **Conclusions:** This study, for the first time, revealed that phthalates can induce proliferation at lower concentrations whereas growth inhibition/cytotoxicity occurred at higher concentrations in normal prostate epithelial cells. Cell cycle analysis further confirmed the increased proliferation observed by MTT assay. A concentration-dependent increase in ROS production as well as an increase in population of S phase cells due to phthalate exposure, and attenuation of these effects by antioxidant NAC, further suggests the role of oxidative stress on proliferation and cytotoxicity in RWPE-1 cells. To further analyze the effects of these phthalates at the molecular level, RNA and proteins have already been collected and are currently being analyzed to determine effects on gene expression, inflammatory cytokines, and growth signaling pathways including estrogen/androgen receptor-dependent signaling.

PS 4409 The Effects of Diisononyl Phthalate and its Primary Metabolite on Non-malignant Colonocytes

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Background and Purpose: Diisononyl phthalate (DiNP) is a commonly used plasticizer in a variety of PVC based products, including food packaging. Estimates of average human exposure to DiNP fall between 1-2 μ g/kg/bw/day, with the 95th percentile of exposure estimated between 5-10 μ g/kg/bw/day. DiNP is readily hydrolyzed in the gastrointestinal tract to its primary metabolite mono-isononyl phthalate

(MiNP), however, MiNP is not further metabolized until it reaches the liver. Because the gastrointestinal tract is the primary route of exposure to these phthalates and previous research has primarily focused only on DiNP, it is important to understand the effects of both DiNP and MiNP on colonic epithelia. **Methods:** Young adult mouse colonocyte (YAMC) cells were exposed to varying concentrations of DiNP or MiNP between 0.01 μ M and 100 μ M in multiple assays to assess cell growth, proliferation measured via 5-bromo-2-deoxyuridine (BrdU) incorporation, apoptosis measured via caspase 3/7 release, and gene expression measured via RTqPCR. **Results:** Reductions in cell number were observed after 72 hours at DiNP concentrations of 10 μ M and 100 μ M, and MiNP concentration of 100 μ M. A reduction in cell proliferation was also seen at 100 μ M of each phthalate. Interestingly, a reduction in apoptosis was seen at the 100 μ M concentration of each phthalate. There were also significant changes in mRNA expression in genes related to cell cycle regulation and apoptosis. **Conclusions:** Both DiNP and MiNP decrease cell number, proliferation, and apoptosis in nonmalignant mouse colonocytes. There were also significant changes in gene expression at an environmentally relevant dose (0.01 μ M). Our results suggest that these phthalates can alter colonic cell physiology in an *in vitro* model that we have shown to closely mimic colonocyte response to toxicants *in vivo*. Ongoing analyses to better understand these changes include assessing the potential cytotoxicity of DiNP and MiNP, their potential effect on cell senescence, and their effect on cell cycle progression.

PS 4410 Deconjugation of Polychlorinated Biphenyl Sulfates to Hydroxylated PCBs by Anaerobically Cultured Mouse and Human Gut Microbiota

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Background and Purpose: Polychlorinated biphenyls (PCBs) are persistent environmental contaminants linked to neurotoxicity, hepatotoxicity, endocrine disruption, and cancer in humans. The gut microbiome is increasingly recognized as a critical player in xenobiotic metabolism, and it displays wide variations across individuals due to differences in human genetics, diet, lifestyle, and exposures. We hypothesize that variations in individual susceptibility to PCB toxicity may be partly due to differences in gut microbiome composition and function, which can influence the metabolism of PCBs. To test this hypothesis, the present study cultured fecal microbiomes in anaerobic conditions that mimic the gut environment. The aim was to investigate the metabolism of PCB sulfates, which are major metabolites produced during the hepatic phase II conjugation and detoxification processes, into hydroxylated PCBs (OH-PCBs), which are typically more toxic. The liver metabolizes PCBs through Phase I oxidation to hydroxylated metabolites, followed by Phase II conjugates the metabolites with sulfate to form water-soluble conjugates that are excreted. **Methods:** Anaerobic incubations were performed in a 48-well plate using a Whitley DG250 Anaerobic Workstation at 37 °C. Two groups of human fecal microbiome communities were diluted in phosphate buffered saline containing L-cysteine as a reducing agent and resazurin as an oxygen indicator (50 mg fecal content/mL anoxic PBS). To determine the necessity of microbiome in PCB metabolism, large intestinal content from germ-free or conventional C57BL/6 wild-type mice (n=3 per group) were used. The human and mouse fecal microbiomes (1 mg/mL) were incubated with either dimethyl sulfoxide (DMSO; control), or a mixture of PCB3, 11, 25, and 52 sulfates (0.71 mM each, totaling 2.83 mM) in DPBS buffer for 6 hours. The reactions were then collected and quenched with formic acid to stop enzymatic activity. Analysis of PCB metabolites was performed using an Agilent 1290 Infinity II UPLC system coupled to a SCIEX Triple Quad 7500 mass spectrometer. **Results:** Both human and conventional mice microbiomes enzymatically hydrolyzed PCB sulfates. Higher chlorinated PCB sulfates were metabolized more readily. Gut microbiota from male conventional mice showed greater conversion of PCB sulfates to OH-PCBs than those from females, and human microbiomes metabolized PCB sulfates more extensively than mouse microbiomes, with clear interindividual variability linked to microbial community composition. To identify tractable microbial targets to interrupt this reactivation, we performed a bioinformatics survey of gut sulfatases and their anaerobic sulfatase-maturing enzymes (anSMEs). We compiled 2,161 nonredundant sulfatases, which were highly sequence-diverse, and 245 anSMEs with conserved radical-SAM/SPASM motifs that mapped to the Unified Human Gastrointestinal Protein catalog with >99% representation. Alignment of translation-initiation regions (~30 to +18 nt) revealed a tight 9-nt start-site consensus for anSMEs but substantial divergence across sulfatases, indicating that anSMEs are more amenable to pan-antisense targeting, whereas discrete sulfatases in dominant taxa will require strain-specific designs. Guided by these patterns, antisense PNA-CPP candidates are now being designed and prioritized for anaerobic *ex vivo* testing, with the goal of transiently suppressing desulfation and limiting the conversion of PCB sulfates to OH-PCBs while preserving community structure. **Conclusions:** Our study demonstrates that the gut microbiome is a critical driver of PCB sulfate metabolism, with clear interspecies, interindividual, and sex-dependent differences in the conversion of PCB sulfates to more toxic OH-PCBs. By coupling anaerobic *ex vivo* incubations with a targeted bioinformatics survey of sulfatases and anSMEs, we identify microbial sulfatase pathways as actionable nodes for intervention and establish anSMEs as particularly attractive candidates for pan-antisense inhibition. These findings lay the groundwork for non-antibiotic, precision strategies to dampen xenobiotic reactivation in the gut and ultimately mitigate PCB-associated toxicity.

PS 4411 Spatial Transcriptomic Mapping of Toxicity Differences Between Benzo[a]pyrene and an Alkylated Derivative

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Background and Purpose: Polycyclic aromatic hydrocarbons (PAHs) are a ubiquitous class of petro- and pyrogenic-derived contaminants characterized by at least two fused aromatic rings. Many PAHs are known to act through the Aryl Hydrocarbon Receptor (AHR), which acts as a transcription factor responsible for activating downstream genes primarily responsible for metabolizing xenobiotics. Current regulatory practices often group PAHs by their parent compound, though functional group additions to the parent can result in differential toxicity. Of these derivatives, alkylated forms are among the most environmentally abundant. A developmental screen of Benzo[a]pyrene (BaP), one of the most studied PAHs to date, and 6 alkylated derivatives revealed differential toxicity. Of these, only 8-methylbenzo[a]pyrene (8MBaP) produced significant adverse morphological outcomes. These endpoints emerge at 72 hours post fertilization (hpf) at low nominal concentrations of 0.133 μ M. At similar concentrations and developmental timepoints, BaP produced no morphological abnormalities, yet both BaP and 8MBaP have previously been shown to act through AHR2 in zebrafish. This shows that the addition of a single methyl group to BaP significantly impacts *in vivo* toxicity while activating the same receptor. To begin to understand what might be driving these differential responses to BaP and 8MBaP exposures, we combined previously collected, phenotypically anchored bulk RNA sequencing with spatial transcriptomics. **Methods:** Specific pathogen-free wild-type 5D strain zebrafish from the Sinnhuber Aquatic Research Laboratory were used for all studies following IACUC-approved guidelines. At 8 hours post fertilization (hpf), embryos were plated in 96-well plates and exposed to 1% DMSO, 13.3 μ M Benzo[a]pyrene, or 13.3 μ M 8MBaP via an HP D3000e digital dispenser and kept in the dark. At 72 hpf, larvae were euthanized on ice, arranged in 10x10 mm cubes of Optimal Cutting Temperature compound, flash-frozen, and sectioned using a cryotome. Two sagittal sections of tissue were placed on Curio-Seeker™ spatial transcriptomic slides with two adjacent H&E. cDNA was generated, followed by library preparation, bead purification, and QC. Samples were sequenced using a NovaSeq 6000 with a read depth of 2 billion reads per tile. The Latchbio bioinformatic pipeline was used to align and match associated bead barcodes, with Seurat being used to identify unique clusters of cells. Spatial visualization was performed using the Excelxgene application, comparing larvae that had similar spatial orientation and representation of cell and tissue types. **Results:** AHR2 related gene transcripts, such as *cyp1a*, *cyp1b1*, *cyp1c1*, *sult6b1*, and *slnr* were all overexpressed in both exposed groups. In all cases, the 8MBaP exposed larvae had higher fold change induction compared to that of BaP. Spatial analysis revealed that these transcripts had increased expression in the liver, whereas 8MBaP had increased expression in the liver and cartilage-containing cells lining the trunk. Transcripts such as *foxq1a* and *pigm* were highly expressed in 8MBaP exposed larvae, especially in craniofacial cartilage. Other genes uniquely overexpressed in the 8MBaP-exposed larvae were isolated to liver cells such as *arg2*, *pck1*, and *agxtb*. Shared under-expressed genes between BaP and 8MBaP were *Krt4* and *Krt15* in the cartilage and skeletal muscle spanning the trunk, with *ctyl1* being uniquely under-expressed in 8MBaP. **Conclusions:** The spatial transcriptomics showed that AHR2 activation was present in both BaP and 8MBaP, but the magnitude and spatial distribution differed, in congruence with previous bulk RNA sequencing data. Cartilage cells appear to be highly responsive to 8MBaP than to BaP especially with respect to canonical AHR2 genes and genes associated with cellular development, cell migration, and membrane stability. Liver transcripts from 8MBaP-exposed larvae showed an increase in inflammatory stress response and metabolic dysfunction. Transcripts involved in cytoskeletal and epithelial structure were all under-expressed throughout muscle and cartilage cells, suggesting dysregulated tissue development. This data suggests 8MBaP causes increased dysregulation of various genes associated with drug metabolism, cell migration, cellular structure, and stress, with a particular susceptibility in cartilage-containing cells throughout the entirety of the larvae.

PS 4412 Toxicity Profiling of Polycyclic Aromatic Hydrocarbon Mixtures in Primary Human Bronchial Epithelial Cells

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Background and Purpose: Individuals are commonly exposed to polycyclic aromatic hydrocarbons (PAHs); a class of environmental contaminants composed of benzene rings derived from incomplete combustion of natural and manufactured organic material. There is strong evidence supporting that PAHs are known to cause detrimental effects such as lung cancer, irritation, and difficulty breathing. Individuals are predominantly exposed to PAHs in complex mixtures in the environment; however, limited toxicity data exists for PAH mixtures, as well as many of the less commonly studied PAHs in environmental samples. This study examines and compares cytotoxicity, oxidative stress, and mitochondrial membrane potential among four PAH mixtures and their 25 derived chemical components originating from air and water samples obtained near sources of PAH exposure at wildfires and contaminated creosote sites. We hypothesize that toxicity of the environmentally relevant PAH mixtures is reflective of toxicity of the individual components due to chemical interactions and the potential for additive response. **Methods:** Primary human bronchial epithe-

lial cells (HBEC) were treated with mixtures and their individual components in a dose-response (0-600 μ M) for 24 hours and then assessed for cytotoxicity by release of lactate dehydrogenase (LDH), oxidative stress by formation of reactive oxygen species (ROS) with 2',7'-dichlorofluorescein diacetate (DCFDA), and mitochondrial membrane potential. **Results:** Overall, toxicity was observed in a chemical-specific manner across dose and endpoint contributing to the response of PAHs mixtures. **Conclusions:** These studies are the first to assess toxicity of these environmentally-relevant PAH mixtures in HBEC and contribute to our understanding of how PAHs contribute to toxicity in environmental mixtures.

PS 4413 Sex-Specific Regulation of Hepatic Cytochrome P450 Enzymes by Arsenic Trioxide in Mice: Insights into Drug Metabolism and Hepatotoxicity

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Background and Purpose: Arsenic trioxide (ATO) is a chemotherapeutic agent used in acute promyelocytic leukemia, but its clinical application is limited by dose-dependent hepatotoxicity primarily driven by oxidative damage and changes in hepatic gene expression. CYP enzymes play a key role in the metabolism of many therapeutic agents, and alterations in their activity can impact drug efficacy and safety. Moreover, sex-based differences in CYP expression influence hepatic drug metabolism, susceptibility to tissue injury, and cancer progression. To elucidate sex-specific hepatic responses to ATO, we investigated the expression patterns of key CYP in male and female C57BL/6 mice following ATO treatment. **Methods:** Male and female C57BL/6 mice received a single intraperitoneal dose of ATO (8 mg/kg). Hepatic CYP mRNA levels were quantified at 6- and 24-hours post-treatment, and protein expression was assessed at 24 hours. **Results:** After ATO exposure, both sexes showed significant upregulation of CYP1A1 mRNA expression. Female mice exhibited pronounced suppression of CYP2C, CYP3A, CYP4A, and CYP4F isoforms; notably, CYP4A10 and CYP4A14 decreased in females while increasing in males. Several CYP2J isoforms demonstrated sex-specific regulation, with CYP2J5 and CYP2J6 suppressed in females, whereas CYP2J9, CYP2J11, and CYP2J13 showed induction. These findings highlight strong sexual dimorphism in hepatic CYP regulation following ATO treatment. **Conclusions:** ATO induces marked, sex-specific modulation of hepatic CYP enzymes, impacting pathways critical for drug metabolism and hepatotoxic risk. The data highlight the importance of considering sex-based biological differences in optimizing ATO dosing regimens and reducing adverse effects in cancer patients. Future studies should further explore mechanistic pathways underlying these differences to enhance personalized therapy.

PS 4414 Single-Cell Multiomic Insights into Zinc Modulation of Cadmium and Arsenic Effects During Lung Alveolarization

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Background and Purpose: Prenatal exposure to environmental heavy metal contaminants, including cadmium (Cd) and arsenic (As), has been shown to disrupt fetal lung development, a critical period characterized by extensive cellular diversification. Zinc (Zn) supplementation is an increasingly explored mechanism for reducing mortality and supporting development in premature infants given its essential role in diverse cellular activities, including oxidative stress response and interaction with other metals. Our previous work identified Zn-dependent proteins as molecular targets for Cd and As, implicating Zn-related pathways in heavy metal-induced developmental lung defects. In this study, we investigated the mechanistic interplay between Zn, Cd, and As in developing mouse lungs to elucidate how Zn modulates single-cell epigenomic and transcriptomic responses to heavy metals during alveolarization, thereby revealing cell type-specific molecular mechanisms underlying its potential protective effects. **Methods:** We collected single-cell multiome (scATAC-seq + scRNA-seq) data on three post-natal day P9 lungs from mice prenatally exposed to drinking water dosed with 250 ppb each of CdCl₂ and NaAsO₂ (CdAs), 250 ppb each of CdCl₂, NaAsO₂, and ZnC₄H₆O₄ (CdAsZn), or control. We analyzed 18,178 nuclei across conditions. Pre-processed data were integrated via weighted-nearest neighbor analysis, visualized using uniform manifold approximation and projection (UMAP), and clustered using the Leiden algorithm. Cell clusters were manually annotated using established marker genes. Differentially expressed genes (DEGs) were identified for each cell type using the Wilcoxon rank sum test in Seurat. **Results:** We identified 18 cell clusters through integrated scRNA- and scATAC-seq analysis which were broadly categorized as epithelial, mesenchymal, immune, and endothelial cells. Epithelial cell sub-types included alveolar type 1 (AT1), alveolar type 2 (AT2), airway secretory (Club) cells, and ciliated airway cells. CdAs and CdAsZn treatments induced largely overlapping transcriptomic alterations in epithelial cells when compared to control, indicating that Zn supplementation only partially mitigates the effects of heavy metal exposure. Genes upregulated by both treatment conditions were related to actin cytoskeleton reorganization (*Sema3e*), the integrin superfamily (*Itgb6*), and cell adhesion (*Ctnnd2*) (*BH-adjusted p-value* < 0.05). Despite overlapping transcriptional changes, distinct Zn-dependent effects were found in specific epithelial cell types. In AT1 cells, CdAsZn treatment downregulated several cytoskeleton and migration related genes that were upregulated by CdAs, including

genes linked to cell adhesion (*Cadm1*, *Magi3*), cellular reshaping (*Shroom3*), actin filament network formation (*Ctnna1*), and migration (*Pde1c*, *Nisch*). In AT2 cells, Zn mitigated CdAs-induced downregulation of structural scaffold genes (*Camk1d*, *Flna*, *Ahna*) and upregulation of others (*Nckap5*, *Pde4d*). Zn also attenuated CdAs effects on genes involved in vascular growth (*Vegfa*), RNA processing, and translation (*Sf1*, *Eef2*, *Nxf1*). With respect to cell-type markers, Zn reversed the CdAs-induced upregulation of *Scgb1a1* (Club cell marker) and reduced *Sftpc* expression (AT2 marker). Compared to CdAs, Zn also downregulated *Malat1* in both AT2 and immune cells, a long non-coding RNA controlling cellular proliferation and angiogenesis in lung cancer. Ongoing analyses based on chromatin accessibility and transcriptional kinetics reconstruct and compare single-cell differentiation trajectories across exposures. **Conclusions:** In this exploratory analysis, CdAs treatment altered expression of genes involved in cellular morphology, differentiation, and adhesion in lungs undergoing alveolarization, a developmental stage characterized by extensive tissue remodeling, cell migration, and vascular expansion. While Zn supplementation did not fully prevent these cellular responses, it modulated and attenuated CdAs effects on structural and motility-associated genes in epithelial cells. As this developmental stage corresponds to a critical window of lung maturation in preterm human infants—often associated with bronchopulmonary dysplasia and impaired alveolar growth—these findings highlight Zn as a partial modulator of heavy metal-induced risks for preterm infant lung disease.

PS 4415 Investigating mechanisms of chlorpromazine protection against cadmium-induced toxicity in cardiomyocytes

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Background and Purpose: Cadmium (Cd) is a heavy metal that induces oxidative stress and causes a variety of adverse health outcomes, including damage to the cardiovascular system. Cigarette smoking is a common source of chronic low-dose Cd exposure, and individuals diagnosed with schizophrenia have a high incidence of cigarette smoking (75-90%). This population also experiences markedly elevated cardiovascular mortality, dying 10-20 years earlier than the general population. Antipsychotics are the cornerstone of pharmacologic treatment for schizophrenia, yet little is known about interactions between antipsychotics and heavy metals. While antipsychotics are often assumed to exacerbate oxidative stress, emerging evidence including our preliminary studies suggests that chlorpromazine (CPZ), a first-generation antipsychotic, has antioxidant effects. Since oxidative stress is thought to be a major mechanism of Cd toxicity, we hypothesized that CPZ would protect against Cd-induced cellular toxicity by mitigating oxidative stress in cardiomyocytes. **Methods:** Immortalized human cardiomyocytes (AC16 cell line) cultured in 96-well plates were exposed to cadmium chloride (CdCl₂, 10 μ M - 30 μ M) with or without CPZ (4 μ M). Two experimental designs were used: (1) co-treatment, where cells were exposed to CdCl₂ and CPZ simultaneously, and (2) pre-treatment, where cells were treated with CPZ for 24 hours before exposure to CdCl₂ alone. Cells were tracked with live microscopy imaging and analyzed for growth and cell death. To explore potential mechanisms for this protection, we conducted RNA-sequencing on AC16 cells treated with CdCl₂, CPZ, or vehicle control for 48 hours. **Results:** In both approaches, CPZ protected cells from Cd-induced cytotoxicity, as shown by improved cell growth and decreased cell death. In contrast, haloperidol (another first-generation antipsychotic) did not confer protection, suggesting specific protective properties of CPZ. Transcriptomic profiling showed that Cd strongly induced metallothioneins, while CPZ altered the expression of genes implicated in key pathways related to cell survival and oxidative stress. Ongoing studies are quantifying biomarkers of oxidative stress and assessing the activity of candidate pathways and perturbation of candidate genes. **Conclusions:** These findings provide initial evidence that CPZ can protect against Cd-induced cardiomyocyte toxicity and highlight the importance of studying co-exposures in toxicology, not only to better model the complex interactions between environmental and pharmacological agents, but also as a step towards informing clinical decision-making based on individual risk factors.

PS 4416 Novel Insights into Antimicrobial's Cetylpyridinium Chloride Mechanism of Mitochondrial Toxicity

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Background and Purpose: Cetylpyridinium chloride (CPC) is a widely used antimicrobial implicated in mitochondrial toxicity comparable to canonical mitochondrial toxicants such as carbonyl cyanide m-chlorophenyl hydrazone. A quaternary ammonium compound (QAC), CPC possesses a permanently charged nitrogen head and a hydrophobic hydrocarbon tail. Estimates suggest CPC levels in human blood are ~0.6 μ M from personal care product and food exposures. Low-concentration CPC crosses the blood-brain barrier, suppresses immune cell signaling, and is toxic to eukaryotes. CPC induces mitochondrial deformation, a phenomenon linked to debilitating human diseases such as Parkinson's and Alzheimer's. This correlation indicates the importance in understanding the mechanisms behind CPC-induced mitotoxic-

ity. Our lab published CPC interference with two vital endpoints of cellular respiration: acute (1.5 hours) exposure severely diminishes both ATP production (IC_{50} 1.7 μ M RBL mast cells; 1.2 μ M primary human keratinocytes) and oxygen consumption rate (1.75 μ M IC_{50} in RBLs). Recent work demonstrates CPC (0.5 μ M) suppresses electron flow (in permeabilized RBLs) from several TCA cycle intermediates including malate and succinate, suggesting inhibition of Complexes I, II, or downstream components directly, though data has thus far indicated malate dehydrogenase is unaffected. Additional inquiry therefore led us to investigate the direct effects of CPC on Complexes I, II, III, and IV in isolated bovine mitochondria. Additionally, our lab previously discovered CPC inhibition of specific phosphorylation events, leading us to the hypothesis that CPC may also alter signaling involving adenosine-monophosphate activated protein kinase (AMPK). AMPK is a central protein of the AMPK/mTOR (mechanistic target of rapamycin) signaling pathway that, through downstream members, such as 4E-BP1 (eukaryotic translation initiation factor 4E-binding protein 1), regulates mitochondrial dynamics. Since CPC deforms the mitochondria, CPC effects on the phosphorylation status of members of the AMPK/mTOR pathway were therefore investigated. Work is underway to elucidate the precise biochemical mechanism(s) by which CPC inhibits these critical cellular processes. **Methods:** The activity of the mitochondrial complexes were probed via colorimetric plate reader assays in the presence of varying concentrations of CPC using endogenous and exogenous terminal electron acceptors. Western blotting was used to quantify CPC's impacts on the phosphorylation of key members of the AMPK/mTOR pathway and compared to controls. **Results:** The data show that CPC significantly disrupts mitochondrial Complexes I (at 5 μ M) and IV (at 10 μ M; more potent than cyanide) during a 60-minute exposure, but doesn't directly affect Complexes II or III, revealing novel mechanisms of toxicity for QACs. Additionally, the findings provide evidence that CPC (5 μ M) induces alteration of the phosphorylation of key members, including 4E-BP1 of the AMPK/mTOR pathway involved in mitochondrial dynamics. **Conclusions:** CPC is an antimicrobial that people are widely exposed to, yet our research has revealed that consumer relevant doses of CPC target specific components of the ETC and critical proteins in the AMPK/mTOR signaling pathway, revealing novel biochemical mechanisms underlying CPC mitochondrial toxicity.

PS 4417 The Pesticide Propargite Induces and Exacerbates Ferroptotic Toxicity: Implications for the Pathogenesis of Parkinson's Disease

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Background and Purpose: Pesticides such as acaricides can negatively impact human health through various cellular mechanisms. Propargite, an organosulfur acaricide used worldwide on crops, has been linked to toxicity in dopaminergic neurons and an increased risk of developing Parkinson's disease (PD). While existing research has preliminarily identified dopaminergic neurotoxicity relevant to PD, the mechanisms underlying this neurotoxicity remain unclear, highlighting a significant gap in the literature. Ferroptosis—a specific type of cell death caused by dysregulated phospholipid peroxidation—has been recognized as a mechanism involved in neurotoxicity and neurodegenerative diseases such as PD. This study tested the hypothesis that propargite induces and sensitizes cells to ferroptosis. **Methods:** Complementary cell-based and free assays were utilized to evaluate the potential effects of propargite on cellular toxicity and ferroptosis. Classical inducers, such as erastin and RSL3, were used to activate ferroptosis, while liproxstatin-1 was utilized as a standard ferroptosis inhibitor. Additionally, Z-VAD-FMK was used to inhibit apoptosis. Cytotoxicity was assessed by measuring cell viability with a calcein-AM assay, and cell death was evaluated using a lactate dehydrogenase (LDH) release assay. The impact of the compounds on lipid peroxidation was investigated using a C11-BODIPY 581/591 assay. The RhoNox-1 assay was used to measure intracellular labile iron levels, and the monochlorobimane assay was utilized to assess intracellular glutathione levels. Furthermore, a cell-free ABTS assay and a newly established combined iron- and arachidonic acid-induced C11-BODIPY oxidation assay were used to evaluate the potential radical-trapping antioxidant (RTA) activity of anti-ferroptotic compounds. **Results:** We found that propargite, tested at concentrations ranging from 0.625 to 20 μ M, is highly toxic to immortalized HT-22 mouse hippocampal neurons and N27 rat dopaminergic neurons, with IC_{50} values of 0.76 μ M and 0.64 μ M, respectively. Our research demonstrated that propargite specifically induces ferroptosis in both HT-22 and N27 neurons. This was evidenced by the significant protective effect of the ferroptosis inhibitor liproxstatin-1 against propargite toxicity, while the apoptosis inhibitor Z-VAD-FMK did not provide any detectable protection. Lipid peroxidation assays showed that propargite increased the C11-BODIPY signal in a dose-dependent manner, and this heightened lipid peroxidation was significantly ameliorated by liproxstatin-1. Using both cell-based and cell-free assays, we identified 4-hydroxyindole as a new ferroptosis inhibitor, and this inhibitor also rescued cells from propargite-induced toxicity, further supporting the notion that propargite specifically induces ferroptosis. Additionally, we found that a non-toxic dose (500 nM) of propargite sensitizes HT-22 neurons to ferroptosis. By elucidating the underlying mechanism, we determined that propargite significantly decreases glutathione levels, which explains how it induces and sensitizes cells to ferroptosis. **Conclusions:** This study is the first to identify ferroptosis as a neurotoxic mechanism associated with the pesticide propargite. Propargite not only induces ferroptosis but

also makes neurons more susceptible to it, likely due to decreased glutathione levels. This research advances our understanding of how pesticides cause neurotoxicity. Additionally, the study shows that ferroptosis inhibitors can effectively counteract propargite-induced neuronal toxicity. Targeting ferroptosis represents a promising approach for neuroprotective therapies that could mitigate neurodegeneration associated with pesticide exposure in incurable PD.

PS 4418 Role of Advanced glycation end products and their receptors on myoblast senescence *in vitro* and regeneration retardation of aging muscles *in vivo*

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Background and Purpose: The global population is rapidly aging, leading to a significant increase in age-related diseases in the coming decades. Muscle dysfunction is a prevalent chronic condition among older adults, posing significant public health challenges. One of the key age-related changes in muscle tissue is the accumulation of advanced glycation end products (AGEs). Previous studies have reported that AGEs are highly associated with muscle dysfunction, particularly in diabetes. However, the relationship between AGEs and muscle aging remains unclear. This study aimed to investigate the effects of AGEs on muscle repair function through both cellular and aging/senescence animal models. **Methods:** Mouse C2C12 myoblasts were cultured and used as an *in vitro* model. A D-galactose-accelerated senescence/aging mouse model (male C57BL/6J mice) was induced and used as an *in vivo* model with or without AGEs inhibitor aminoguanidine treatment (oral for 0.1 g/kg/day). Mice received subcutaneous injections of D-galactose at a daily dose of 1 g/kg for 4 consecutive weeks. Analyses included immunoblotting, senescence-associated β -galactosidase staining, hematoxylin and eosin (H&E) staining, immunohistochemistry (IHC), a glycerol injection-induced muscle regeneration experiment, and rota-rod muscular endurance trials. **Results:** In the cell model, non-cytotoxic concentrations of AGEs (25 and 50 μ g/mL) induced cell senescence and inhibited myogenic differentiation in C2C12 myoblasts, as evidenced by senescence-associated β -galactosidase staining and H&E staining ($p < 0.05$, $n \geq 3$). These effects of AGEs could be reversed by treatment with a neutralizing antibody against the receptor for AGEs (RAGE; 5 and 10 μ g/mL; $p < 0.05$, $n \geq 3$). In the D-galactose-accelerated senescence/aging mouse model, IHC staining showed substantial AGEs accumulation and RAGE expression in the muscles, which were reduced by treatment with the AGEs inhibitor aminoguanidine (0.1 g/kg, orally for 4 weeks; $p < 0.05$, $n = 3-6$). Muscle regeneration was assessed via glycerol injection into the tibialis anterior muscle. Early regenerating myofibers, characterized by central nuclei, were observed 5 days post-injury. At this time point, muscular endurance (as measured by rotarod latency) in the D-galactose-accelerated aging group was significantly shorter than in the control group, and was significantly improved by aminoguanidine treatment ($p < 0.05$, $n = 6$). The muscle regeneration/repair capacity was significantly impaired and the myogenesis markers Pax7 and MyoD expression were reduced in senescence/aging mice, both of which were restored by aminoguanidine treatment ($p < 0.05$, $n = 6$). **Conclusions:** These findings suggest that the AGEs/RAGE axis promotes myoblast senescence, inhibits their differentiation, and may impair muscle repair capacity in aging animals. AGEs may act as a risk factor for impaired myogenesis/muscle regeneration due to the promotion of premature cellular senescence. However, further studies are required to elucidate the detailed underlying mechanisms.

PS 4419 Employing a High-Throughput Screen to Identify Human Pregnane X Receptor Antagonists

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Background and Purpose: The xenobiotic-sensing nuclear receptor, pregnane X receptor (PXR), regulates drug metabolism and clearance. Recently, PXR has been associated with modulating cell proliferation, apoptosis, immune response, and energy homeostasis. Pinpointing compounds that can change PXR activity is necessary to prevent drug-drug interactions, determine compounds which generate toxicity, and locate compounds for further development towards therapeutic usage. **Methods:** Here, we have screened the National Center for Advancing Translational Sciences Pharmacologically Active Chemical Toolbox library, which consists of 5,099 unique pharmacologically active synthetic and naturally derived small molecules using a HepG2-CYP3A4-hPXR cell line to identify PXR antagonists. Based on efficacy, potency, and novelty, a group of potential PXR antagonists were selected for further studies including pharmacological shift assays, mRNA gene expression experiments, and molecular docking analysis. **Results:** From the primary screening, 94 compounds were identified as potential PXR antagonists and 66 were confirmed in the second run of confirmation experiments. From these, twenty potential PXR antagonists were selected to a more in-depth analysis to confirm their PXR antagonism. GSM2, JNJ-31020028, fusidic acid, and PU-H71 inhibited CYP3A4 mRNA expression in HepaRG cells, a physiologically competent cell line. Also, GSM2 and fusidic acid acquired the pharmacological shift results necessary to affirm PXR antagonism. These two compounds were also able to be docked into the PXR ligand

binding domain suggesting their ability to bind. **Conclusions:** GSM2 and fusidic acid have been identified as novel PXR antagonists. These two compounds should be further investigated to identify useful information regarding possible drug-drug interactions or other toxic consequences. Potential therapeutic effects may also be a target of interest for these newly classified PXR antagonists.

PS 4420 Development of Spatial Transcriptomics Methods for Integrating Molecular Changes with Pathologic Findings: Proof of Concept Studies with Kidney Toxicants in Rat and NHP

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Background and Purpose: Transcriptomic assays are being leveraged to evaluate the potential safety liabilities of preclinical drug candidates, and to understand the molecular mechanisms of toxicities observed during drug development. However, it can be difficult to deconvolute and associate gene expression changes in highly heterogeneous bulk tissue samples with spatially resolved histopathological findings. Single cell resolution spatial transcriptomics can bridge this gap by assessing genome-wide RNA expression changes within the anatomical context of histopathology slides, providing deeper insights into the molecular mechanisms of observed pathology for the derisking of preclinical drug candidates. **Methods:** As a proof of concept for the utility of spatial transcriptomics for mechanistic toxicology, we generated single cell spatial RNA-seq (Visium HD) data on kidney samples from NHPs and rats treated with the tubular toxicants Tobramycin and Cisplatin, respectively, as well as from rats treated with the glomerular toxicants Doxorubicin and Puromycin. **Results:** We first assessed the performance of the human and mouse probe sets on NHP and rat samples, respectively. We next used unsupervised clustering and well-established transcriptomic markers to identify major cell types in the kidney. H&E images were used to identify necrotic proximal tubules and glomeruli. Differential gene expression analysis was conducted between treated and vehicle control cells of the same type, and between normal and necrotic cells, to characterize treatment effects and transcriptional changes linked to pathological lesions. Cell type-specific molecular changes revealed pathways modulated with drug-induced kidney toxicity, distinguishing glomerular from tubular toxicants. **Conclusions:** These proof-of-concept studies are being used to demonstrate the utility of transcriptomics for illuminating spatially-resolved molecular mechanisms associated with histopathology findings. The onset and progression of glomerular and tubular toxicity is known to involve the activation of a number of different pathways across multiple cell types in the kidney, not only those specific to the glomeruli and tubules, respectively. Assaying cell-specific transcriptional profiles and their associations with anatomical manifestations of drug-induced pathophysiology in the kidney or other tissues, greatly advances mechanistic understanding of toxicity to support drug development as well as the potential development of novel biomarkers for risk assessment and monitoring.

PS 4422 Transcriptomic Characterization of Birth Defects-Associated Genes During Early Organogenesis

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Background and Purpose: Early organogenesis is a critical developmental window susceptible to environmental and genetic disruptions. Disruption of this process can give rise to functional and structural birth defects (BDs). Although most causes of BD remain unknown, genetic factors alone or in combination with environmental exposures are estimated to account for ~50% of cases. An informative strategy for investigating teratogenesis is the analysis of human genetic disorders that phenocopy teratogen-induced embryopathies. In this context, although pathogenic variants in specific genes have been associated with BD in humans and rodents, the temporal and cell-type-specific expression patterns of these genes within developmental networks remain poorly understood. To address this gap, we applied a multi-scale approach, integrating a systematic literature review with single cell transcriptomic and systems biology analysis to map the embryonic expression of genes previously linked to birth defects and to identify novel correlates potentially associated with teratogenicity. **Methods:** The literature review was conducted on PubMed using Medical Subject Headings terms related to BD and genetic predisposition to diseases. Only systematic reviews and meta-analyses conducted in humans and rodents were included. Single cell RNA sequencing (scRNA-seq) datasets were obtained from human (GSE157329, 7 embryos, Carnegie Stages 12-16) and rat (Li et al. in preparation, 32 embryos, Gestational Days 10-12), representing comparable developmental stages of early organogenesis. Data processing and analyses were performed in R, with systems biology analyses conducted using STRING v.12.0 and Cytoscape v.3.10.0. **Results:** From the literature review, 510 studies were retrieved, and following screening, 147 were included in the present study. Through these studies, we identified 426 candidate genes across human and rodent models that are associated with some of the most prevalent structural birth defects: 36 linked to cortical defects, 14 to neural tube defects, 129 to musculoskeletal defects (e.g. limbs, cranium, others), 93 to heart defects, 89 to oral clefts, 27 to genitourinary defects, 12 to gastrointestinal defects, and 23 to multiple of these BD. Functional

enrichment of these genes highlighted their key role on embryonic developmental and morphogenesis, as well as in WNT, Hippo, mTOR signaling pathways. mRNA profiles of human and rodent embryos revealed that ~90% (n=397 in human, and 374 in rats) of our candidate genes exhibited differential expression across the developmental stages. More than 85% of these candidate genes (n=368) exhibited conserved expression. scRNA-seq data from human embryos were partitioned into 19 clusters and annotated as specific cell types, whereas rat embryonic samples were classified into 35 clusters. Overall, genes associated with specific types of BD were expressed in cell types related to the development of the corresponding organs or systems. Primordial germ cells exhibited high expression levels of the candidate genes. Some genes exhibited broader, more ubiquitous expression across multiple cell populations (e.g. associated with cytoskeleton organization, such as *ACTG1*, *CALD1*, *TUBA1A*; or with transcriptional regulation, *CITED2*, *NONO*, *SON*, *SMARCA4*, or multiple BD, *FGFR1*, *RFC1*). Interaction networks were independently created and analyzed for each type of BD, identifying highly correlated genes (FDR < 0.01). Furthermore, key genes integrated into candidate genes networks emerged as potentially involved in the etiology of birth defects. **Conclusions:** Our findings demonstrate the conserved regulation of genes associated with BD across species in a temporal and cell-type-specific manner, and highlight novel network-based correlates, providing a framework to study genes and pathways that, when disrupted by environmental factors, can potentially lead to BD. Ongoing work aims to explore in depth the cell-type-specific expression patterns of these genes across species, as well as their co-expression relationships with other potentially important genes involved in the embryo development, whose disruption may lead to birth defects.

PS 4423 Placental Nrf2-regulated gene expression in a rat model of pre- and gestational fluoride exposure

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Background and Purpose: Fluoride (F⁻), a naturally occurring element in earth's crust, can be found in groundwater and reach concentrations that pose health risks in several regions worldwide. It has been documented that F⁻ can induce oxidative stress through the production of reactive oxygen species. During gestation, this imbalance may compromise placental function, a critical organ for fetal growth and maturation, leading to embryonic or fetal toxicity. The nuclear factor erythroid 2-related factor 2 (Nrf2) pathway regulates essential antioxidant genes involved in placental redox homeostasis. However, it remains unclear if F⁻ exposure at environmentally relevant concentrations alters the expression of these genes in the placenta. Therefore, the aim of this study was to evaluate the effect of pre- and gestational F⁻ exposure on the expression of Nrf2-regulated genes in rat placentas. **Methods:** Female Wistar rats (n= 5 per group) were assigned into three experimental groups: a control group and two F⁻ treated groups receiving 2.5 and 5.0 mg F⁻/kg/day by oral gavage from 14 days prior to mating until gestational day 19. Total RNA was extracted from placental samples and subsequently reverse-transcribed into cDNA. The expression levels of *NQO1*, *HMOX1*, *GCLC*, *GST*, *GCLM*, *TXN* and *GPX2* were quantified by real-time PCR (qPCR) and normalized to *GAPDH* expression to evaluate the activation of the Nrf2 pathway. **Results:** A significant increase in *TXN* gene expression were observed between the control group and the group exposed to 2.5 mg F⁻/kg/day (p= 0.0195). Additionally, *GPX2* expression was increased in the 5.0 mg F⁻/kg/day group compared to 2.5 mg F⁻/kg/day group (p= 0.0053). No significant changes were detected in the expression of *NQO1*, *HMOX1*, *GCLC*, *GST* and *GCLM*. **Conclusions:** These results suggest that pre- and gestational F⁻ exposure modulates placental redox systems in a dose-dependent manner. The selective activation of *TXN* and *GPX2* suggests distinct adaptive responses of the thioredoxin and glutathione pathways, without global activation of Nrf2-mediated antioxidant response.

PS 4424 Impact of RNA Binding Proteins on Nrf2 De Novo Synthesis During Oxidative Stress

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Background and Purpose: Oxidative stress plays an important role in a variety of diseases. Environmental exposure to excessive sun, cigarette smoke, air pollution, or several different types of toxins can cause an increase in oxidative stress. Oxidative stress has been shown to inhibit global protein translation. Increasing evidence supports the fact that selective protein translation occurs under oxidative stress. Among the selectively translated proteins are Nrf2, a transcription factor involved in the antioxidant response, and other components essential for cell survival or damage repair. **Methods:** To better understand the protein translation machinery under oxidative stress, tandem mass tag isobaric labeling quantitative proteomics was performed on ribosomes isolated by ultracentrifugation from either control cells or cells briefly exposed to oxidative stress in culture. Results from the proteomic analysis were confirmed via western blot. **Results:** The analysis identified 1871 proteins with 115 showing a significant 1.2-fold or more increase. The increased proteins

include RNA binding proteins such as DHX9, ILF3, FUBP1, YTHDF2, PABPC4, PAIRB, SMU1, and STAU2. Using western blot, these increased proteins were confirmed to have a higher association with ribosomes in HeLa cells treated with 100 μ M H₂O₂ for 1 hour. Using a biotinylated 5'-UTR of Nrf2 mRNA, DHX9, FUBP1, and YTHDF2 showed an increased binding affinity to Nrf2 mRNA during oxidative stress, while PAIRB and ILF3 did not bind to Nrf2 mRNA. Blocking DHX9, ILF3, FUBP1, YTHDF2, or PABPC4 with siRNA was able to prevent H₂O₂ from inducing Nrf2 protein synthesis. PAIRB, SMU1, or STAU2 siRNA had no effect on Nrf2 translation. DHX9 and PABPC4 were shown to have increased association with the complete 80S ribosome after treatment, while YTHDF2 was shown to have increased association with the 40S ribosome. **Conclusions:** The data supports that ribosomes undergo remodeling through changes in RNA binding protein association due to oxidative stress. While some of these changes mediate selective protein translation to relieve oxidative stress, the functional significance for others remains to be known.

PS 4425 CYP2E1 Influences GLO1 Abundance and Methylglyoxal Detoxification Across Multiple Organs

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Background and Purpose: Cytochrome P450 isoform 2E1 (CYP2E1) is a highly conserved monooxygenase enzyme involved in xenobiotic and endogenous metabolism and implicated in multiple liver pathologies, including alcoholic and non-alcoholic liver disease. CYP2E1 is known to produce toxic metabolites from ethanol, acetaminophen, and several pollutants. In addition to these established roles in xenobiotic transformation, CYP2E1 also contributes to formation of the toxic metabolite methylglyoxal (MGO) from the ketone body acetone. MGO is a precursor to advanced glycation end products (AGEs), which promote oxidative stress and inflammation. Detoxification of MGO occurs primarily through the glyoxalase pathway, with glyoxalase-1 (GLO1) functioning as the rate-limiting enzyme under transcriptional control of NRF2. Incidental observations in CYP2E1-knockout (KO) mice suggested altered GLO1 expression, prompting investigation into the relationship between CYP2E1, GLO1, and MGO. **Methods:** Male and female CYP2E1-KO mice and strain matched wild-type (WT) controls were evaluated for GLO1 expression across liver, kidney, heart, and small intestine. GLO1 protein levels were quantified using automated western blotting, and transcriptional changes were evaluated by RNA sequencing. Tissue MGO concentrations were measured by LC/MS, and untargeted liver metabolomics was used to assess downstream metabolites, including S-D-lactoylglutathione, the enzymatic product of GLO1 activity. **Results:** CYP2E1 deletion caused a consistent and significant ($p < 0.05$) reduction in GLO1 protein expression across all tissue types tested, including liver, kidney, heart, and small intestine, indicating a systemic regulatory impact. In all tissues, GLO1 levels were reduced to around half or less of the levels in WT animals. Liver metabolomic data from CYP2E1-KO mice showed decreased S-lactoylglutathione that is consistent with the decrease in GLO1 expression (about 50% of wild-type levels, $p < 0.05$). Lastly, preliminary LC/MS data has shown a 2.5-fold increase in MGO within CYP2E1-KO livers ($p < 0.01$). **Conclusions:** These findings reveal a previously unrecognized role for CYP2E1 in supporting glyoxalase pathway function and regulating GLO1 expression across multiple organs. Loss of CYP2E1 reduces GLO1 abundance and disrupts MGO metabolism, indicating that CYP2E1 may influence cellular redox balance through NRF2-dependent signaling. Overall, this work identifies CYP2E1 as a potential modulator of MGO-driven stress, with implications for metabolic dysfunction, diabetic complications, alcoholic and non-alcoholic liver disease, and other pathologies linked to MGO accumulation.

PS 4426 Sulfenyl Aziridines as Stereoselective Covalent Destabilizing Degradors of the Oncogenic Transcription Factor MYC

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Background and Purpose: Although MYC is a significant oncogenic transcription factor driver of cancer, directly targeting MYC has remained challenging due to its intrinsic disorder and poorly defined structure, deeming it "undruggable." Whether transient pockets formed within unstructured regions of proteins can be selectively targeted with small molecules remains an outstanding challenge. We discovered that spirocyclic oxindole-based sulfenyl aziridines can stereoselectively modulate MYC levels and thus inhibit MYC transcriptional activity in cells. **Methods:** We developed a stereochemically paired spirocyclic oxindole aziridine covalent library and screened this library for degradation of MYC. We used molecular biology and chemoproteomic approaches to study our molecule and also optimized our hit compound to generate a more potent, selective, and durable MYC degrader. **Results:** We identified a hit covalent ligand, KL2-236, bearing a unique sulfenyl aziridine warhead, that engaged MYC as a pure MYC/MAX protein complex, and in cancer cells to destabilize MYC, inhibit MYC transcriptional activity and degrade MYC in a proteasome-dependent manner through targeting intrinsically disordered C203 and D205 residues. Notably, this reactivity was most pronounced for specific stereoisomers of KL2-236 with a diastereomer, KL4-019, that was largely inactive. Mutagenesis of both C203 and D205 completely attenuated KL2-236-mediated MYC degradation. **Conclusions:** Our results reveal a novel ligandable site within MYC and indicate that certain intrinsically

disordered regions within transcription factors, such as MYC, can be interrogated by isomerically unique chiral small molecules, leading to destabilization and degradation.

PS 4427 From Start to Finish: Casein Kinase 2 Negatively Regulates G_{αs}-GPCR Signaling in Multiple Ways

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Background and Purpose: Casein Kinase 2 (CK2) is a constitutively active serine/threonine protein kinase composed of 2 catalytic subunits (α or α') and 2 regulatory subunits (β). The catalytic and regulatory subunits have unique functions and targets, including pertinent regulators of G_{αs}-GPCR signaling. Namely, CK2 α directly phosphorylates ABCC1 and PKA (leading to activation and inhibition, respectively), whereas CK2 β represses G_{αs} signaling. Herein, we explored the contribution of CK2 to G_{αs} signaling in human airway smooth muscle (HASM) cells, which are the primary cause of airway narrowing in asthma. **Methods:** For these studies, we used a model of cultured primary HASM cells isolated from adult tissue. We measured transcript expression of CK2 (CSNK2A1, CSNK2A2, CSNK2B) and the β 2-adrenergic receptor (ADRB2) using a previously publicly available RNA-Seq dataset or RT-qPCR. Protein expression of CK2, P-T²⁴⁹ ABCC1, P-VASP, and P-HSP20 were determined by immunoblot. Protein-protein interactions were assessed by co-immunoprecipitation and activation of G_{αs} by Bioluminescence Resonance Energy Transfer (BRET) biosensor. To measure cellular stiffness of living cells on a single-cell level, we used optical magnetic twisting cytometry (MTC). **Results:** We first established the presence of CK2 transcript and protein in HASM derived from 8 donors. Co-immunoprecipitation confirmed that CK2 α physically associates with ABCC1 and PKA-Ril under homeostatic conditions. The phosphorylated form of T²⁴⁹ (the reported CK2 phospho-site) of ABCC1 is present under basal conditions. However, inhibiting CK2 α using siRNA did not decrease phosphorylation of T²⁴⁹, suggesting a different residue is involved in the constitutive regulation of ABCC1. We then tested the effect of 4,5,6,7-tetrabromobenzotriazole (TBB), a pharmacological CK2 inhibitor, on G_{αs} mobilization. TBB increased G_{αs} mobilization, supporting that CK2 reduces available G_{αs}. To test the functional impacts of CK2, we measured phosphorylation of VASP and HSP20 (markers of PKA activation) and cellular stiffness. TBB increased P-VASP and P-HSP20 in a time- and dose-dependent manner and evoked cell relaxation in a dose-dependent manner. CK2 α -targeting siRNA similarly decreased cell stiffness, indicating CK2 α increases HASM basal tone. Inhibiting PKA using H89 did not affect TBB-evoked cell relaxation; this implicates a PKA-independent mechanism. Of note, we also found that prolonged (24 h) TBB treatment upregulated transcript expression of ADRB2 by >5 fold. **Conclusions:** These data support that CK2 α functions as a constitutive multi-nodal inhibitor of G_{αs}-GPCR signaling; CK2 acts on G_{αs}, PKA, ABCC1, and receptor transcription. Therefore, CK2 may be involved in the pathological mechanical properties of airway smooth muscle of asthma patients and is an exciting potential druggable target.

PS 4428 Mechanistic Insights into Species-Specific ABC Transporter Regulation by Acetaminophen in the Intestinal Epithelium

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Background and Purpose: Preliminary findings from our laboratory indicate that apical ABC transporters (P-gp, Mrp2, and Bcrp) in the small intestine may be modulated post-transcriptionally following acetaminophen (APAP) administration in mice. Given that several studies have reported regulation of these transporters by specific microRNAs (miRNAs) under various physiological and pathological conditions, we conducted a miRNA microarray analysis to identify potential miRNAs involved. This study aims to explore the underlying regulatory mechanisms governing intestinal transporter expression after APAP exposure and to compare these findings with a human intestinal cell line model. **Methods:** Male C57BL/6 mice were administered APAP (400 mg/kg, i.p.). Distal section of the small intestine was collected 6 hours after treatment, and miRNA expression profiles were analyzed using Illumina microarrays. An *in silico* analysis was then performed by integrating data from DIANA microT-CDS, miRDB, TargetScan, and TarBase to predict miRNA-mRNA interactions. Differentially expressed miRNAs were further evaluated for their potential mRNA binding using MIENTURNET with miRTarBase-supported interactions. mRNA expression of miRNA-targeted genes involved in the post-transcriptional regulation was measured by RT-qPCR. To compare these *in vivo* findings with human intestinal cells, Caco-2 cells were exposed to 10 mM APAP for 24 h to assess mRNA and protein expression of ABC transporters and identified miRNA targets were assessed. **Results:** miRNA sequencing cluster analysis revealed that 12 miRNAs were differentially expressed in the APAP-treated group out of a total of 709 detected miRNA species ($P < 0.01$). However, *in silico* target prediction analysis did not identify any of these miRNAs as potential regulators of apical ABC transporters at the post-transcriptional level. Following the analysis performed in MIENTURNET, we identified an increase of Igf2bp2, Hpk3, and circHpk3 in APAP-intoxicated mice. In contrast, when

the effects of APAP were examined in a human intestinal cell line, post-transcriptional regulation was observed for MRP2 and BCRP, but not for P-gp. Additionally, no changes in the expression of Igf2bp2 or circHIPK3 were observed in the human cells. Comparative analysis between mouse and human miRNAs revealed that two of the differentially expressed mouse miRNAs, mmu-miR-6240 and mmu-miR-106a-5p, lack human homologs. These miRNAs were predicted to target the 3'UTR region of Igf2bp2. Two predicted binding sites for mmu-miR-6240 were identified in the 3'UTR region of Igf2bp2 at positions 2559-2565 and 3584-3590, along with three predicted sites for mmu-miR-106a-5p at positions 2884-2890, 3669-3675, and 3830-3836. These sites may contribute to species-specific post-transcriptional regulation observed in mice. **Conclusions:** Post-transcriptional regulation of MRP2 and BCRP was observed in both mouse and human intestinal cell lines; however, species-specific regulation of P-gp was noted. Although miRNA sequencing and *in silico* analyses indicated that none of the dysregulated miRNAs were predicted to modulate ABC transporters directly, we observed an increase in the expression of circHIPK3 and the RNA-binding protein Igf2bp2. These molecules are known to interact and may form a complex capable of binding to the 3'UTR regions of target genes, thereby influencing their post-transcriptional regulation in mice. This effect was not observed in human, as the expression levels of these complex components did not significantly change following APAP exposure in human cells. Notably, two mouse-specific miRNAs, mmu-miR-6240 and mmu-miR-106a-5p, were identified as potential candidates contributing to the species-specific post-transcriptional regulation of P-gp through the circHIPK3-Igf2bp2 complex, because several predicted binding sites for these miRNAs are present in the 3'UTR region of Igf2bp2. These sites may underline the species-specific post-transcriptional regulation observed in mice, although further studies are warranted to confirm this regulatory mechanism.

PS 4429 Role of GDF15 in asparaginase-induced food aversion and liver steatosis.

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Background and Purpose: Asparaginase is a chemotherapeutic enzyme used to treat pediatric and adult forms of acute lymphoblastic leukemia (ALL). Its enzymatic activity depletes circulating asparagine and glutamine, triggering the Integrated Stress Response (ISR) an adaptive signaling pathway, which induces the transcription of many genes, including *Gdf15*. GDF15 contributes to anorexia and weight loss by suppressing appetite. Weight loss during asparaginase exposure is linked to hepatic steatosis and toxicity in mice, especially in obesity. Previously, we reported that asparaginase elevates circulating GDF15 in mice and this correlated with reduced body weight. A secondary analysis of an unpublished dataset from lean and obese mice fed either low-fat (10% fat by kcal) or high fat (60% fat by kcal) diets during asparaginase exposure also showed an inverse relationship between hepatic *Gdf15* mRNA with body weight change ($p < 0.001$) and food intake ($p = 0.0036$). Based on these findings, we hypothesized that genetic deletion of *Gdf15* would rescue asparaginase-associated food aversion and body weight loss. **Methods:** To test this hypothesis, global *Gdf15* knockout (*Gdf15*KO) mice and their wild type controls (WT) were intraperitoneally injected with pegylated asparaginase (1.5 international units per gram body weight) or equivalent saline vehicle ($n = 24$ mice; 6 males and 6 females per group). Daily body weight and food intake were recorded for four consecutive days after injection, and biospecimens (serum, liver) were collected on the last day. **Results:** Asparaginase exposure in WT mice significantly reduced food intake ($p = 0.0363$), and body weight $\sim 8\%$ ($p = 0.0021$) and increased hepatic *Gdf15* mRNA levels, as expected. In contrast, *Gdf15*KO mice maintained normal food intake ($p = 0.6465$) and were resistant to asparaginase-induced weight loss (body weight change $\leq 4\%$). We noted that asparaginase-induced activation of the hepatic ISR and liver ISR target genes in *Gdf15*KO mice were similar to WT mice, indicating that systemic loss of GDF15 did not alter other aspects of the hepatic ISR to asparaginase. Importantly, liver triglyceride accumulation was rescued in asparaginase-exposed *Gdf15*KO mice. **Conclusions:** Overall, these data suggest GDF15 is a crucial mediator of asparaginase-associated food aversion and protects against body weight loss and associated liver steatosis by rescuing normal intake.

PS 4430 Apoptotic Bodies Derived from HIV- and Alcohol-Exposed Hepatocytes Amplify Inflammation and Programmed Cell Death Pathways in Liver Parenchymal Cells.

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Background and Purpose: Liver disease remains a leading cause of morbidity in people living with HIV (PLWH), particularly among those who consume alcohol. While HIV and ethanol independently promote hepatocyte injury, their combined mechanisms of toxicity especially regarding apoptotic body (AB) formation and intercellular propagation of cell death remain poorly defined. This study aimed to determine how HIV and alcohol synergistically activate multiple programmed cell death pathways in hepatocytes and to assess whether ABs mediate secondary inflammation and hepatocyte injury. Understanding these pathways may reveal novel therapeutic targets to prevent alcohol-accelerated liver disease in PLWH. **Methods:**

Huh7.5-CYP2E1 hepatocytes were treated with HIV-1 ADA and an acetaldehyde-generating system (AGS) containing yeast alcohol dehydrogenase, 50 mM ethanol, and NAD to mimic chronic alcohol metabolism. Apoptotic bodies (ABs) generated from co-exposed cells were isolated and introduced (1:3 ratio) into naïve and AGS-pretreated hepatocytes at defined kinetic time points. Apoptosis was evaluated by cleaved caspase-3 and PARP cleavage; necroptosis by RIPK3 and phosphorylated MLKL; pyroptosis and inflammasome activation by cleaved gasdermin D, cleaved caspase-1, NLRP3, IL-1 β , and IL-18. Reactive oxygen species (ROS) and lactate dehydrogenase (LDH) release were measured to assess oxidative stress and necrosis. Pathway specificity was validated using inhibitors: Z-VAD-FMK (apoptosis/AB release), Necrostatin-1 (necroptosis), and VX-765 (pyroptosis). **Results:** Dual exposure to HIV and AGS significantly increased apoptotic markers (cleaved caspase-3 and PARP cleavage). Engulfment of these ABs by naïve AGS pretreated hepatocytes induced secondary apoptosis (caspase-3 and cleaved PARP activities) and upregulated pro-inflammatory cytokines IL-1 β and IL-18 ($p < 0.01$). Elevated p-MLKL and cleaved gasdermin D levels confirmed activation of necroptotic and pyroptotic pathways. Both ROS generation and LDH release increase in the pretreated treatment group compared to naïve treatments. Application of Z-VAD-FMK, Necrostatin-1, and VX-765 each reduced corresponding pathway markers by 50-70%, validating specificity of the observed responses. **Conclusions:** This study demonstrates that HIV and alcohol synergistically induce hepatotoxicity through the simultaneous activation of apoptotic, necroptotic, and pyroptotic cell death pathways. Importantly, apoptotic bodies act as vectors of injury propagation, transferring death signals to neighboring hepatocytes and amplifying inflammation. These findings highlight AB-mediated intercellular signaling as a critical toxicological mechanism underlying alcohol-exacerbated liver injury in HIV infection. Targeting AB formation or uptake may therefore represent a novel therapeutic strategy to mitigate viral-alcohol co-toxicity and prevent progression to end-stage liver disease in vulnerable populations.

PS 4431 Quantum Dots Interaction with Cytoskeletal Proteins via Experimental Observations and Computational Predictions.

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Background and Purpose: Quantum dots (QDs) are biocompatible nanoparticles with a unique range of optical and fluorescent properties, making them a promising medical diagnostic tool for applications in cell labeling, bioimaging, and drug administration. However, their biological integration has been hindered by QDs' ability to non-specifically interact with the cells and intracellular proteins. As such, this study investigates QDs' interaction with cytoskeletal proteins such as actin and α -actinin, through biochemical and computational approaches to unravel the consequences on cytoskeletal dynamics. **Methods:** We conducted spin-down assays to examine the effects of QDs on actin polymerization and depolymerization as well as the interaction between QDs and actin in the presence of α -actinin. Next, we performed fluorometric assays to monitor pyrene-labeled actin polymerization and depolymerization over time in the presence of varying concentrations of QD, followed by quenching between QDs and α -actinin. We are currently working on performing comparative molecular docking studies using AutoDock Vina and Boltz-2 to model QD binding with proteins. Furthermore, a custom generative AI model is being developed to predict novel ligands capable of interacting with cytoskeletal proteins to compare their binding profile with those of surface-functionalized QDs. **Results:** Our spin-down experiment showed that high concentrations of QD hinder actin polymerization and promote depolymerization. Furthermore, our spin-down assay also demonstrated the ability of QD to cause bundling of actin filaments. However, in the presence of both α -actinin and high QD concentration, actin bundling does not occur. This indicates an interaction between QD and α -actinin, which was confirmed by the quenching assay. In addition, our fluorometer assay revealed that high concentrations of QD inhibit actin polymerization while lower concentrations stimulate polymerization. Preliminary docking studies revealed potential binding sites for QDs on actin and α -actinin. **Conclusions:** Our study provided novel insights on how QDs disrupt cytoskeletal integrity and cause toxicity. The integration of molecular docking and AI-driven ligand prediction provides a framework to identify binding surfaces and potential drug discovery and nanoparticle design. Understanding these molecular interactions between QDs and proteins is essential to maximize their potential in biomedical applications while mitigating any side effects.

PS 4432 SERPINA3 Upregulation Marks Fine Particulate Matter-Induced Impairment of Osteogenic Differentiation

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Background and Purpose: Air pollution, particularly particulate matter (PM), poses a significant public health risk, with established links to respiratory disease, cancers, and cardiovascular diseases. Emerging evidence suggests that PM exposure is a risk factor for poor adult bone health, such as osteoporosis. Additionally, prenatal PM exposure has been associated with adverse birth outcomes, including increased risk for skeletal defects, though the underlying mechanisms remain unclear. Osteogenesis, the formation of bone, is governed by a complex network of epigenetic

mechanisms, microRNAs, and transcription factors that are susceptible to environmental perturbations and can manifest embryonic skeletal defects. **Methods:** We sought to investigate how PM exposure alters embryonic molecular signatures and identify potential biomarkers in human embryonic stem cells (hESCs) before cell fate commitment, potentially predisposing to skeletal birth defects. Using the H9 hESC line, we differentiated cells into osteoblasts and exposed to fine PM (PM_{2.5}) or urban dust at concentrations inhibiting differentiation by 25% (ID₂₅) or 50% (ID₅₀). **Results:** Fine PM exposure inhibited osteogenesis in a dose-dependent manner, as evidenced by reduced protein expression, alkaline phosphatase activity, matrix mineralization, and cell viability. A human molecular toxicology profiler microarray identified a distinct pattern of increased expression in the inflammation marker, *SERPINA3* in differentiating cells exposed to PM_{2.5}. Validation experiments confirmed this effect at both the transcript and protein levels. RT-qPCR analysis demonstrated a robust and reproducible upregulation of *SERPINA3* mRNA in osteoblasts exposed to FPM compared with untreated controls. Additionally, transcriptomic analysis revealed *SERPINA3* targets were differentially expressed from PM_{2.5} exposure. **Conclusions:** This increase was consistent across exposure replicates and concentrations, underscoring the reproducibility and sensitivity of the response. Given the limited understanding of *SERPINA3*'s function in bone, these results highlight the need for further molecular characterization to determine whether *SERPINA3* directly mediates or reflects PM_{2.5}-induced inhibition of osteogenesis.

PS 4433 Tracing the Evolutionary Origins of the Aryl Hydrocarbon Receptor Ligand Specificity

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Background and Purpose: The aryl hydrocarbon receptor (AHR) is an evolutionarily conserved transcription factor best known in vertebrates for mediating responses to dioxins and other environmental contaminants. While vertebrate AHRs exhibit binding to a diverse array of endo- and xenobiotic compounds, the functions of invertebrate AHR homologs remain poorly understood. To better understand the physiological roles and evolutionary trajectory of the AHR in early metazoan evolution, we investigated the ligand specificity of AHRs from invertebrate taxa representing key phylogenetic lineages. **Methods:** Chimeric fusion constructs with species-specific ligand binding domains were expressed in a one-hybrid reporter system to evaluate ligand-dependent transcriptional activity. The species analyzed represented major invertebrate lineages, including pre-bilaterians (Cnidaria-*N. vectensis*), protostomes (Nematoda-*C. elegans*; Arthropoda-*D. melanogaster*; Mollusca-*M. arenaria*), deuterostomes (Echinoderm-*S. purpuratus*), and invertebrate chordates (Urochordata-*C. intestinalis*; Cephalochordata-*B. floridae*). We screened a panel of more than fifteen compounds, including endogenous, dietary, and microbial metabolites, as well as natural and anthropogenic xenobiotics. **Results:** Multiple protostome AHRs (i.e., *C. elegans*, *D. melanogaster*, *M. arenaria*) exhibited robust, ligand-independent transcriptional activity. Several control experiments were performed to rule out experimental artifacts. Our comparative framework indicates that constitutive activation may be an ancestral or derived feature among protostome AHRs. **Conclusions:** Ongoing functional analyses and reconstructions of the ancestral protostome and pre-bilaterian AHRs are underway. By tracing the emergence and diversification of AHR ligand-binding properties across invertebrate taxa, this work provides new insights into the early evolutionary history of the AHR signaling pathway and a basis for understanding the molecular mechanisms underlying disruption by anthropogenic pollutants. [Research is supported by the National Institute of Environmental Health Sciences: R01ES035261.]

PS 4434 Early Life Microbiota Shape Metabolic Programming and Mitochondrial Resilience to Toxicants

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Background and Purpose: Exposure to environmental toxicants during critical developmental windows can alter growth and metabolic programming. While the microbiome regulates xenobiotic metabolism and host energy balance, most studies focus on post-embryonic stages, leaving microbial regulation during embryogenesis, when energetic demands and toxicant sensitivity are highest, poorly understood. Traditionally, the embryo was considered insulated from microbial influence by the chorion, but our previous work challenges this view. We show embryos raised in the absence of a microbiome during the first half of embryonic development exhibit reduced mitochondrial respiration, effects further exacerbated by xenobiotic exposure, while germ-free embryos maintain respiration. In the absence of a microbiome, lipid metabolism genes were universally upregulated alongside shifts in lipid metabolites, contrasting post-embryonic studies that report reduced lipid synthesis. These findings suggest microbial regulation of lipid metabolism is developmentally dynamic and embryonic metabolic reprogramming occurs in the absence of microbial cues. However, the mechanisms underlying these temporal effects and their consequence for toxicant sensitivity remain unresolved. Therefore, here, we mechanistically expand upon our previous findings to test the hypothesis that microbial cues are critical for mitochondrial efficiency, lipid substrate utilization, redox homeo-

stasis, and ATP production during embryonic development. **Methods:** Zebrafish (*Danio rerio*) embryos were reared under germ-free (GF) conditions or conventionalized with microbes (CV) at 6 hours post-fertilization (hpf). Embryos were exposed to either 10 μ M of benzo(a)pyrene, a potent aryl hydrocarbon receptor agonist, or a DMSO vehicle control. Endpoints were collected at 32 hpf to evaluate effects of microbial cues and BaP exposure on embryonic metabolic reprogramming and mitochondrial resilience. Molecular impacts were assessed through analysis of gene expression and metabolite abundance using our previously published transcriptomic and metabolomic datasets. Physiological consequences were determined by quantifying total ATP and ADP, while mitochondrial efficiency, glycolytic activity, β -oxidation, and redox homeostasis were measured to identify mechanisms by which microbial cues and BaP exposure alter embryonic energy production. **Results:** Although GF embryos sustained mitochondrial respiration, this occurred at reduced efficiency, indicated by increased proton leak and lower ATP production. GF embryos also exhibited decreased glycolytic activity, likely driven by triacylglycerol accumulation, which may further exacerbate ATP depletion. Consistent with this, lipid accumulation in GF embryos was accompanied by upregulation of β -oxidation. However, inhibition of β -oxidation significantly reduced respiration in CV embryos, but not GF embryos, suggesting that efficiency of utilizing lipid substrates was reduced in the absence of microbial cues. This reduced reliance on β -oxidation likely contributes to temporal accumulation of lipid stores observed under GF conditions. While BaP exposure did not alter the relationship between the microbial community and embryonic energy production, it induced a significant increase in ROS in GF compared to CV embryos and further impaired antioxidant defenses, marked by decreased catalase activity. Together, these findings indicate microbial colonization affects both metabolic efficiency and redox balance, influencing embryonic responses to environmental stressors such as BaP. **Conclusions:** We expand upon our previous findings that the surrounding microbiome plays a fundamental role in shaping energy metabolism during the earliest stages of development. Results illustrate that microbial cues are essential for maintaining energetic efficiency during embryogenesis and disruption of these cues induces metabolic reprogramming. We demonstrate the microbiome supports both aerobic and anaerobic energy production, preserves redox homeostasis, and enhances mitochondrial resilience to environmental stressors. These findings emphasize the importance of incorporating the microbiome in toxicological assessments and informs mechanistic insight to how the maternal microbiome shapes embryonic and fetal metabolic development, with implications for understanding metabolic disorders and risks of *in utero* chemical exposures.

PS 4435 Aip Mutation Reduces Sensitivity to Ahr Ligands in a Context-Dependent Manner

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Background and Purpose: Chemical contaminants, including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), are known to elicit their effects through activation of the aryl hydrocarbon receptor (AHR). Some populations of Atlantic Killifish rapidly developed resistance to high levels of these contaminants. Genome-wide association studies using these populations demonstrated that variation in the AHR interacting protein (AIP) is associated with resistance to AHR ligands such as 3,3',4,4',5-pentachlorobiphenyl (PCB 126). AIP associates with the AHR in the cytosol where it's thought to play a role in stability, ligand binding, and nuclear localization. However, AIP's exact role in AHR signaling and chemical sensitivity is poorly understood. To investigate this, two *aip* mutant zebrafish lines were utilized. Zebrafish homozygous for these mutations die at 8-10 days post-fertilization (dpf) while *ahr* knockout zebrafish exhibit no such phenotype. In addition to investigating AIP-AHR-mediated signaling and chemical sensitivity, these mutants were used to explore AIP's role beyond the AHR. **Methods:** CRISPR-Cas9 technology was used to induce a deletion in exon 2 (*aip*^{wh86} line) or exon 5 (*aip*^{wh239} line) of *aip*. Both result in an early stop codon and protein truncation. The *aip*^{wh86} line is missing 4/4 protein-interacting domains, while the *aip*^{wh239} line is missing 3/4 domains. Due to early mortality, heterozygous (+/-) adults are increased to generate offspring for experimentation. To investigate susceptibility to chemical exposure, 6-hour post-fertilization offspring of *aip*^{wh239} +/- zebrafish were exposed to a diverse set of AHR ligands. These included PCB 126, a strong Ahr2 ligand; 5-nitroacenaphthene (5-NAN), an Ahr1a ligand; and benzo(k)fluoranthene (BkF), which induces a unique caudal fin malformation called x-fin in an Ahr2-dependent manner. Embryos were exposed to a DMSO control or an Ahr ligand near the EC₅₀ concentration. At 5 dpf, the larvae were evaluated for defects by screening for 9 morphological endpoints. The number of larvae exhibiting malformations was evaluated between condition and genotype using multiple comparisons. To investigate early mortality, mRNA sequencing was performed on pooled whole larvae from both lines, and differentially expressed gene (DEG) analysis was performed. To investigate cell-type-specific changes in expression, the R package MuSiC was used to deconvolute the bulk sequencing data using a single-cell RNA-seq reference. **Results:** *aip*^{wh239} -/- zebrafish exposed to PCB 126 and BkF had fewer morphological defects overall compared to +/- and +/- animals. When considering specific morphological endpoints -/- zebrafish demonstrated some resistance to each chemical tested. Notably, 5-NAN-exposed -/- zebrafish had a lower incidence of the brain endpoint. In BkF-exposed zebrafish, there was no difference in the incidence of cranial and edema endpoints between genotypes, while -/- animals had reduced incidence of the x-fin endpoint. Unexposed

aip^{wh86} and *aip*^{wh239} larvae had 1,231 and 972 DEGs (p-value ≤ 0.05 , Log₂(FC) $\geq |1|$) in a -/- vs +/- comparison, respectively. Only 47% of the DEGs were shared between the lines. Using gene ontology analysis, biological processes involving metabolism, homeostasis, the extracellular matrix, chromosomal segregation, immune response, and bacterial defense were enriched in one line or the other, but processes shared between lines primarily involved extracellular matrix and immune response-related terms. Using MuSiC bulk RNA-seq deconvolution, changes in cell-type-specific transcript abundance were observed. **Conclusions:** We demonstrate that disruption of the *aip* gene partially protects against exposure to several Ahr ligands in a context and tissue-specific manner. Disruption also leads to large changes in gene expression and early mortality. Many of the severe phenotypes observed in *Aip* mutants are likely the result of changes in non-Ahr pathways, as Ahr null zebrafish survive into adulthood. mRNA sequencing and bulk sequencing deconvolution were used to investigate the biological processes and organ systems that are most affected by disruption of *aip* and highlight important areas for future study and validation. Supported by R01 ES033888, P30 ES030287, P42ES016465, & T32 ES007060.

PS 4436 Role of aryl hydrocarbon receptor (AhR) ligands in the regulation of microRNAs in the mouse thymus

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Background and Purpose: Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor involved in immune regulation in response to environmental toxins such as Dioxin (TCDD). Recent research highlights the role of AhR in biological processes such as immune function, inflammation, cancer development, and cell differentiation through miRNA-related epigenetic mechanisms. In this study, we investigated the effect of four AhR ligands (TCDD, resveratrol (RES), indole-3-carbinol (I3C), and 6-formylindolo[3,2-b] carbazole (FICZ) on miRNA expression in the thymus of C57BL/6 mice. **Methods:** Female mice were treated either with vehicle (VEH), TCDD (10 μ g/kg), RES (100 mg/kg), I3C (50 mg/kg), or FICZ (50 μ g/kg). On day 3, the mice were sacrificed, and thymic cells were harvested for RNA extraction and miRNA arrays. **Results:** Of 1117 miRNAs analyzed, 135 miRNAs were downregulated, and 147 miRNAs were upregulated when compared to VEH. Differential expression analysis revealed 16 upregulated microRNAs, including mmu-let-7f, miR-127, miR-142a, miR-144, miR-15b, miR-26a, miR-297a, miR-29b-1, miR-374b, miR-449a, miR-465a, miR-465b-1, miR-466a, miR-467b, miR-470, and miR-7077, and 3 downregulated miRNAs, including miR-365-1, miR-202, and miR-6970, common to all AhR ligand-treated groups. Notably, miR-let-7f and miR-142a were among the miRNAs identified, both of which have conserved sequences in humans. Predicted targets of miR-let-7f include IL33, IL7, and possibly aryl hydrocarbon receptor nuclear translocator-like 2, though further validation is needed to confirm specificity. Additionally, miR-142a may regulate the aryl hydrocarbon receptor nuclear translocator-like protein. **Conclusions:** These findings suggest a potential role for AhR signaling in miRNA-mediated immune regulation, which provides insight into molecular mechanisms underlying AhR's immunomodulatory effects. This work was supported in part by NIH grants: P30GM154631, P20GM103641, R01ES030144 and R01AI160896.

PS 4437 Role of AHR-interacting protein (AIP) variants in controlling sensitivity and resistance to dioxin-like compounds

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Background and Purpose: Humans are exposed to chemicals such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs) that cause toxicity through activation of the aryl hydrocarbon receptor (AHR). Inter-individual variation in sensitivity to effects of AHR agonists is not fully explained by variation in the AHR. A clue to other mechanisms underlying differential sensitivity to dioxin-like compounds (DLCs) has emerged from studies of fish populations with multi-generational exposure to PCBs, TCDD, and PAHs. Four populations of the Atlantic killifish (*Fundulus heteroclitus*) have independently evolved resistance to these toxicants, resulting in a >100-fold reduction in sensitivity. Genomic studies identified the AHR chaperone AHR-interacting protein (AIP/Ara9/XAP2) as the strongest candidate resistance gene, but the role of AIP in the mechanism of resistance is unknown. In addition to its role in the AHR complex, AIP is a susceptibility gene for human Familial Isolated Pituitary Adenoma (FIPA); dozens of single-nucleotide variants (SNVs) in AIP predispose to this disease. To understand the role of AIP in the toxicity of DLCs and how variation in AIP may affect sensitivity to DLCs, we are investigating AIP function in fish and mammalian cell models. **Methods:** We used RNA-seq and qRT-PCR to measure AIP expression in killifish that were sensitive or resistant to PCB126 (3,3',4,4',5-pentachlorobiphenyl). We characterized SNVs in killifish AIP sequences using genome re-sequencing data, data from RNA-seq reads from adults and embryos, and targeted sequencing of AIP in field-caught fish. To study the function of AIP variants, we transiently transfected COS-7 cells with expression constructs for AIP variants, AHR, and a luciferase reporter gene and then measured TCDD-inducible luciferase activity and nuclear localization of YFP-tagged AHR. To understand the structural implications of AIP variation, we performed homology modeling of killifish and human AIP variants, using the crystal structure of human AIP as a template. **Results:** *AIP expression:* There was no difference in AIP mRNA levels between sensitive and tolerant killifish, suggesting that altered AIP mRNA expression was not a cause of the resistance. *AIP variation:* We identified several non-synonymous SNVs in killifish AIP, including potential candidates that exhibit different allele frequencies in NBH vs. SC. Two of the variants (P219T and W224L) occur at residues conserved among humans, zebrafish, and PCB-sensitive killifish. Most killifish from the PCB-resistant NBH population have at least one of the variant residues. *AIP structure:* Homology modeling of AIP variants suggested that some of the polymorphisms could be functionally significant. Pro219 is important for the tight kink between two helices and mutation to Thr in this position can affect folding, while Trp224 seems to stabilize the loop position through hydrophobic interactions. *AIP function:* In transient transfection experiments, wild-type killifish or human AIP retained AHR in the cytoplasm and reduced both basal and TCDD-inducible luciferase activity as compared to cells without transfected AIP. Human AIP variants identified in FIPA patients or shown previously to exhibit reduced binding to AHR did not affect transactivation. In contrast, killifish AIP W224L and its human counterpart AIP W223L displayed enhanced function, retaining AHR in the cytoplasm and strongly reducing both basal and TCDD-inducible luciferase expression. **Conclusions:** The results suggest that variation in AIP protein structure can affect the sensitivity of the AHR signaling pathway. Understanding the role of AIP in controlling sensitivity to AHR agonists may shed light on the factors that determine differential susceptibility of humans to DLCs and on the role of AIP in human disease. [Supported by P42ES007381, R01ES032323, and R01ES033888.]

Background and Purpose: Mercury (Hg) is a naturally occurring element that can enter the environment through both natural and anthropogenic sources. After Hg enters the environment, it can transform into more toxic, especially neurotoxic forms, which readily bioaccumulate in the food web, raising health and environmental concerns. To study this transformation process at the basic level, specifically the reduction of mercuric mercury (HgII) to elemental mercury (Hg⁰), we examined the green algal species *Chlamydomonas reinhardtii*, which is key in Hg uptake and known to play a role in Hg cycling through its production of thiols. **Methods:** We prepared gas-tight vial cultures to observe the kinetics of gaseous Hg concentrations measured over 18 days in comparison to the controls under light and dark conditions. To compare conditions, a Lumex analyzer device measured the time to reach the maximal Hg⁰ concentration and the area under the curve. An ANOVA test has been performed to statistically test the significant differences (p-value < 0.05). **Results:** Our results showed that algae inoculated with Hg can effectively reduce Hg through volatilization. In all cultures inoculated with algae (50 μ L), peak gaseous Hg⁰ emissions were observed at a faster rate under light conditions in comparison to our controls. However, peak Hg⁰ concentrations in dark conditions were not as quick, which may point to other reduction processes performed. **Conclusions:** For future studies, we will measure the HgII concentrations in the liquid to quantify the remaining Hg in our samples. This will tell us how much Hg is transformed by the algae and how much Hg remains inside the algae, which will assist in potential mercury bioremediation efforts.

PS 4438 Investigating Mercury Transformation Potential by *Chlamydomonas reinhardtii* Algae

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Background and Purpose: Mercury (Hg) is a naturally occurring element that can enter the environment through both natural and anthropogenic sources. After Hg enters the environment, it can transform into more toxic, especially neurotoxic forms, which readily bioaccumulate in the food web, raising health and environmental concerns. To study this transformation process at the basic level, specifically the reduction of mercuric mercury (HgII) to elemental mercury (Hg⁰), we examined the green algal species *Chlamydomonas reinhardtii*, which is key in Hg uptake and known to play a role in Hg cycling through its production of thiols. **Methods:** We prepared gas-tight vial cultures to observe the kinetics of gaseous Hg concentrations measured over 18 days in comparison to the controls under light and dark conditions. To compare conditions, a Lumex analyzer device measured the time to reach the maximal Hg⁰ concentration and the area under the curve. An ANOVA test has been performed to statistically test the significant differences (p-value < 0.05). **Results:** Our results showed that algae inoculated with Hg can effectively reduce Hg through volatilization. In all cultures inoculated with algae (50 μ L), peak gaseous Hg⁰ emissions were observed at a faster rate under light conditions in comparison to our controls. However, peak Hg⁰ concentrations in dark conditions were not as quick, which may point to other reduction processes performed. **Conclusions:** For future studies, we will measure the HgII concentrations in the liquid to quantify the remaining Hg in our samples. This will tell us how much Hg is transformed by the algae and how much Hg remains inside the algae, which will assist in potential mercury bioremediation efforts.

PS 4439 Ozone-Mediated Oxidation of 4-Alkoxy-2(3)-nitroacetanilides: Rapid Mineralization and Implications for Wastewater Treatment

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Background and Purpose: 4-Alkoxyacetanilides (4-AAs), such as phenacetin, were widely used analgesics from their introduction before World War I until concerns over kidney toxicity led to their withdrawal in the 1980s. Despite discontinued medical use, they have occasionally appeared as adulterants in illicit drugs and been linked to toxicity. Our laboratory has shown that nitroderivatives of 4-AAs can form through reactions with cellular oxidants such as peroxynitrite-CO₂. These nitrated analogs, particularly N-(4-alkoxy-3-nitrophenyl)acetamides, are environmentally concerning due to their persistence and potential toxicity. In contrast to halogenation by hypochlorite/hypochlorous acid, which produces chlorinated intermediates [e.g., N-(4-alkoxy-3-chlorophenyl)acetamides], ozonation may offer a rapid and cleaner pathway for degradation. This study examined the efficacy of ozone (O₃) in oxidizing nitroacetanilides under aqueous conditions relevant to water treatment. **Methods:** Ozone was generated from O₂ by corona discharge, and the O₃ in O₂ mixtures (~1% O₃) were bubbled through 100 mL of 0.1 M phosphate buffer (pH 7.0) containing 0.4 mM N-(4-methoxy-2-nitrophenyl)acetamide, N-(4-methoxy-3-nitrophenyl)acetamide, N-(4-ethoxy-3-nitrophenyl)acetamide, or N-(4-methoxy-2-methyl-5-nitrophenyl)acetamide at 25 $\pm$ 1 °C. Reaction progress was monitored by UV-Vis (220-540 nm) and

RP-HPLC with UV detection at 254 nm at intervals 0.5-2.0 min for a total of 10 min. RP-HPLC analysis was carried out using an Agilent ODS column (4.6x250 mm; particle size: 5 μ) and a mobile phase consisting of 50% acetonitrile in water at a flow of 1.2 mL.min⁻¹ (injection volume: 20 μ L). **Results:** Ozone treatment caused rapid loss of UV-Vis absorbance and RP-HPLC peaks of the parent *N*-(4-alkoxy-2[3 or 5]-nitrophenyl)acetamides, with complete disappearance in 4-8 min. Transient, uncharacterized intermediates appeared briefly but were fully degraded by the end of ozonation. HPLC-UV showed >99% reduction within 8 min, consistent with near-complete mineralization. Compared with hypochlorite-hypochlorous acid and peroxydinitrite-CO₂ oxidations, O₃-mediated oxidations were more efficient and produced no persistent byproducts with significant absorption at 254 nm (indicative of aromatic rings). The UV-Vis results were fully corroborated and confirmed by RP-HPLC analysis. **Conclusions:** This study demonstrates that O₃ is a highly effective oxidant for rapid degradation and mineralization of nitroacetanilides under aqueous conditions. The findings highlight the potential of ozonation as a viable advanced oxidation process for removing pharmaceutical residues and their nitro-derivatives from wastewater sources. These results also underscore the mechanistic differences between O₃ and other oxidants, supporting its role as a clean, sustainable technology in environmental toxicology and water treatment.

PS 4440 Overlooked role of sulfur in the production of microcystin.

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Background and Purpose: Cyanobacterial harmful algal blooms (HABs) represent a growing global toxicological concern because of their ability to produce microcystin (MC), a family of cyclic heptapeptide hepatotoxins that inhibit protein phosphatases PP1 and PP2A, disrupt phosphorylation-dependent signaling, and contribute to liver injury, including non-alcoholic liver disease. Forecasting MC occurrence and potency remains a major challenge in environmental toxicology. Despite decades of research, sulfur availability has never been systematically evaluated as a determinant of MC biosynthesis, largely because sulfur is absent from the final MC structure. However, sulfur metabolism is deeply embedded in the enzymology of MC biosynthesis: sulfur atoms at catalytic centers of NRPS/PKS enzymes support chain elongation and cyclization, while cysteine-derived metabolites such as S-adenosylmethionine (SAM) and malonyl-CoA facilitate biosynthesis. Beyond biosynthesis, sulfur also underpins thiol metabolism and detoxification pathways, including glutathione conjugation, highlighting its relevance to both toxin production and organismal susceptibility. We hypothesized that environmental sulfur availability regulates growth and MC quotas in *Microcystis aeruginosa*. **Methods:** To test this hypothesis, *M. aeruginosa* was cultured in batch systems across a sulfate gradient (0-300 μ M) under identical light and nutrient conditions aside from sulfur. Biomass, cell counts, and MC quotas were measured using ADDA-ELISA at defined intervals, with three independent replicates per treatment. The experimental design simulated environmentally relevant sulfate concentrations to assess how sulfur availability influences cyanobacterial growth and toxin biosynthesis. **Results:** Sulfur availability and MC production were positively related. Biomass and cell density increased with sulfur concentration, and MC quotas expressed on a per-cell and per-biomass basis rose strongly with sulfur supply, culminating in an eight-fold increase across the sulfur gradient. These results provide one of the first empirical demonstrations that sulfur is a critical driver of MC production in cyanobacteria. **Conclusions:** This study reveals that an element historically overlooked in bloom toxicology, because it is absent from the final toxin structure, is nonetheless indispensable to the biochemical machinery of toxin synthesis. In natural ecosystems, sulfur availability varies over 100-fold due to watershed geology, redox cycling, and anthropogenic inputs, suggesting a potential mechanistic link between sulfur dynamics and spatial or temporal heterogeneity in MC concentrations. By identifying sulfur as a key environmental regulator in cyanotoxin biosynthesis, this work introduces a new dimension in environmental toxicology, ecological monitoring, and the management of eutrophication and its toxic symptoms in Oklahoma and globally.

PS 4442 Germination to Pollination: Effects of Heavy Metals on *Phacelia parryi*

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Background and Purpose: Pollination is a vital ecological process that sustains biodiversity and food production, yet it is increasingly threatened by environmental pollution. Heavy metal contamination, particularly copper (Cu) and zinc (Zn) pollution in cities, originates from automotive wear and runoff, accumulating in roadside soils during rainfall. Areas with increased traffic and urbanization have elevated concentrations of Cu and Zn pollution in roadside soil. This study examines how Cu and Zn pollution affect the germination rate and reproductive traits of *Phacelia parryi*, a native southern California wildflower. We hypothesized that elevated Cu and Zn exposure would cause higher levels of metal sequestration, negatively affecting germination rate, floral development, and decreasing pollinator visitation rate. **Methods:** Four soil treatments were applied to *P. parryi* seeds and plants: 60 parts per million (ppm) Cu, 85 ppm Zn, and 60 ppm Cu + 85 ppm Zn in addition to a control group. All of the seedlings were grown in an incubator at 28° C with 12 hours of light, then 24° C with no light. Germination was tracked during the first two weeks,

then 35 seedlings were transplanted into their corresponding soil treatments. For example, seedlings grown in 60 ppm Cu were transplanted to 60 ppm Cu soil. The control group was split into an additional delayed 60 ppm Cu group, representing post-germination pollution. This provides insight into how the timing of contamination influences plant development and reproductive success. All plants were grown in pollinator exclusion cages outside during the summer of 2025. Morphological traits such as flower petal length, flower petal width, anther length, and leaf size were measured in mm using a caliper. Additional phenological markers, such as first bud, first bloom, and total flower count, were collected, and pollinator visitation data were collected once flowering began. **Results:** Preliminary analyses revealed no significant differences in germination rate among treatments ($\chi^2 = 3.50$, $df = 4$, $p > 0.05$) and no significant effect of heavy metal exposure on petal length (ANOVA: $F = 1.30$, $df = 4$, 153 , $p > 0.05$). These results suggest that moderate levels of Cu and Zn may not impair early developmental or petal length in *P. parryi*. Additional traits such as petal width, anther length, leaf morphology, and pollinator visitation are being analyzed to determine whether there are any differences in reproductive or ecological responses. X-ray fluorescence (XRF) analysis verified that soil Cu and Zn concentrations matched intended treatment levels, confirming that non-significant results were not caused by inaccurate metal applications. Future data analysis will look at metal sequestration. **Conclusions:** These findings suggest that *P. parryi* shows early-stage tolerance to Cu and Zn exposure, as neither germination nor petal length was significantly affected. Future analyses will clarify whether other morphological or time-dependent effects occur. Understanding how heavy metal contamination influences native wildflowers is key to predicting ecological responses to urban pollution. This research contributes to the field of toxicology by demonstrating how soil contaminants can alter biological systems, linking environmental toxicology to plant reproduction and pollination ecology.

PS 4443 Unique Toxic Mechanism of *Chlamydomonas reinhardtii* Exposed to Gold Nanoparticles with Different Shapes

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Background and Purpose: Nanoparticles (NPs) are widely utilized in a variety of commercial and research applications. In the process of NPs production, usage, transportation, and disposal, they inevitably enter the environments through migration and transformation. The aquatic environment is the primary medium through which NPs enter and diffuse into other environmental resources. Microalgae play a crucial role in mitigating eco-environmental changes. The ecotoxicological impact of nanoparticles with different shapes on aquatic systems, particularly their cellular and molecular interactions with algae, remain insufficiently characterized. **Methods:** In this study, 100 nm star (AuNS) and spherical (AuNP) silica-coated gold nanospheres were used as the representative shapes of NPs, and exposed *C. reinhardtii* to varying concentrations (1-50 mg/L) NPs with different shapes to investigate their differential effects on algal growth and primary photosynthetic processes. Confocal and transmission electron microscope were employed to detect the interaction and accumulation of different shapes of AuNPs and *C. reinhardtii*. RNA-seq was employed to elucidate the unique toxicity mechanisms of these differently shaped AuNPs in microalgae. **Results:** The results demonstrate that exposure to 10 mg/L AuNS for 72 hr results in more pronounced inhibition of cell growth and photosynthesis compared to the same concentration of AuNP. Morphometric profiling reveals that AuNS induce greater cellular damage in *C. reinhardtii* than AuNP at equivalent exposure concentrations, including cell enlargement, debris production, and subcellular structure disruption. Confocal imaging suggests this heightened toxicity may stem from distinct internalization patterns: AuNP primarily adhere to chloroplast surfaces, while AuNS penetrate more deeply into intracellular compartments. RNA sequencing identifies 9 upregulated and 38 downregulated differentially expressed genes (DEGs) in the 10 mg/L AuNP group, impairing photosynthesis and energy storage via the photosystem II subunit S1 (PSBS1)/ early light-inducible protein (ELI3) pathway. In contrast, the AuNS group exhibits 246 upregulated and 145 downregulated DEGs, affecting membrane integrity and nitrogen metabolism through the nitrate reductase (NIT1)/ aminomethyl transferase (AMT1)/ protein kinase domain-containing protein (AOA2K3CRU5) pathway. **Conclusions:** This study elucidates the unique toxic mechanisms by which different nanoparticle shapes interact with microalgae, underscoring the importance of nanoparticle shape in assessing ecotoxicity. Our findings suggest that in the development and production of nanoparticles, consideration should extend beyond application performance stability and functional enhancements to include potential ecological and human health impacts. Emphasizing nanoparticle shape optimization to maintain safety and stability while preserving functionality is essential for minimizing adverse environmental effects.

PS 4444 Embryonic exposure to Bisphenol F disrupts early neurodevelopment and induces anxiety-like behaviour in zebrafish (*Danio rerio*)

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Background and Purpose: Bisphenol F (BPF), a structural analogue of bisphenol A, is increasingly used in industrial and consumer applications and has been detected in aquatic systems worldwide. Although promoted as a safer alternative, limited information exists regarding its developmental neurotoxicity. This study aimed to evaluate whether embryonic exposure to environmentally relevant concentrations of BPF disrupts early neurodevelopment and behaviour in zebrafish (*Danio rerio*) and to identify potential molecular mechanisms underlying these effects. **Methods:** Zebrafish embryos were exposed to 0, 5, 20, and 50 µg/L BPF from 4-120 hours post-fertilization (hpf). Spontaneous tail-coiling was assessed at 20 and 30 hpf to detect early neuro-excitation. At 120 hpf, larvae were tested for thigmotaxis, dark/light preference, and locomotor activity using automated video tracking system. Whole larvae were analyzed for BDNF protein and the relative expression of neuroplasticity- and stress-related genes. **Results:** Larval zebrafish exposed to BPF at 5, 20, and 50 µg/L from 4 to 120 hpf exhibited distinct behavioural and molecular alterations, despite showing no significant change in spontaneous tail-coiling activity at 20 or 30 hpf. In the thigmotaxis assay, BPF-exposed larvae demonstrated heightened anxiety-like behaviour, as reflected by a significant reduction in total distance traveled within the outer (peripheral) zone at 5 and 50 µg/L. Moreover, the proportion of time spent in the outer zone was consistently reduced across all treatment groups (5-50 µg/L), further indicating increased thigmotactic preference for the inner zone and a greater tendency toward anxiety. The reflexive movement test showed a significant increase in activity per minute at 5, 20 and 50 µg/L, with the most pronounced response at 20 µg/L, indicating transient hyper-reactivity or heightened neural excitability. In the dark/light preference assay, locomotor activity during the dark phase was markedly suppressed in all BPF-exposed groups, suggesting hypoactive or anxiogenic behaviour under reduced illumination, whereas total locomotor distance increased at 20 and 50 µg/L, indicative of mild hyperactivity and altered exploratory behaviour. At the molecular level, significant upregulation of *bdnf* mRNA and BDNF protein was observed at 50 µg/L, implying activation of neurotrophic signaling. Similarly, *grin2a* expression increased at 50 µg/L, suggesting enhanced N-methyl-D-aspartate receptor (NMDAR) activity and excitatory neurotransmission. Conversely, *ntrk2a* was downregulated across all concentrations, indicating suppression of TrkB-mediated feedback regulation. Elevated *akt1* expression at 50 µg/L supported activation of the PI3K-Akt survival pathway, whereas increased *inhibab* transcripts at the same concentration pointed to activin/inhibin A-mediated stress modulation. In contrast, *sypa* expression was significantly reduced at 50 µg/L, suggesting potential impairment of synaptic vesicle cycling and neurotransmission. **Conclusions:** Embryonic exposure to BPF at concentrations between 5 and 50 µg/L perturbs neurotrophic and excitatory-inhibitory signaling in developing zebrafish, resulting in anxiety-like and hyperactive behavioural phenotypes. These findings identify BPF as a potential developmental neurotoxicant, demonstrating that even short-term exposure during embryogenesis can disrupt early neuronal excitability and behaviour. The results contribute to understanding bisphenol analog-induced neurotoxicity and emphasize the need for mechanistic and generational studies to evaluate long-term risks of BPF contamination in aquatic environments.

PS 4445 Mixture Interactions of Haloacetonitriles Disinfection By-Products in Zebrafish

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Background and Purpose: Haloacetonitriles (HANs) are nitrogenous disinfection by-products (DBPs) commonly formed during chlorination or bromination processes. Previous studies have shown that HANs exhibit higher cytotoxicity than regulated DBPs such as trihalomethanes, haloacetic acids, and bromate. However, the interactions and mechanisms among different HAN species during combined exposure remain unclear. In this study, we used the zebrafish model to investigate the effects of three common HANs (BCAN, DBAN, and DCAN) under single and combined exposures on embryonic development, locomotor behavior, and oxidative stress responses. **Methods:** Four hours post-fertilization zebrafish embryos were exposed to various concentrations of BCAN, DBAN, and DCAN for five days, with exposure levels ranging from 0 to 200 ppb. At 120 hpf, mortality, unhatched rate, morphological abnormalities, and locomotor activity were assessed to characterize dose response relationships. Interactive effects were evaluated by three binary combinations (BCAN+DBAN, BCAN+DCAN, and DBAN+DCAN) following the same exposure scheme, with DBAN fixed at its teratogenic EC₂₀ (70 ppb). Transcriptomic and differential gene expression analyses will be conducted to identify oxidative stress responses and associated functional and disease pathways. **Results:** Following single-HAN exposures, DBAN caused a significant increase in mortality and unhatched embryos at concentrations above 150 ppb, whereas BCAN and DCAN induced only minimal lethality at the same concentrations. DBAN exposure also showed a marked increase in morphological abnormalities beginning at 80 ppb, while BCAN and DCAN did not elicit noticeable malformations. Locomotor behavior test showed that DBAN exposure induced hyperactivity at 60-80 ppb but

reduced movement at 100-120 ppb, whereas BCAN and DCAN did not cause significant effects on locomotor activity. These results indicate that DBAN exhibited the highest toxicity among the three HANs. In mixture exposure experiments, no significant differences in mortality or unhatched rate were observed compared with the control group. However, when DBAN was fixed at 70 ppb, co-exposure with 10 ppb of either BCAN or DCAN induced significant developmental abnormalities, whereas co-exposure with 50 ppb reduced these developmental effects. Behavioral patterns in the mixture groups were consistent with those observed under DBAN single exposure. Notably, these behavioral effects occurred at concentrations lower than the teratogenic (80 ppb) and lethal (180 ppb) thresholds, suggesting that the nervous system may be affected at relatively low exposure levels. **Conclusions:** Our preliminary findings indicate DBAN induces both lethal and teratogenic effects at 80 ppb and causes biphasic neurobehavioral disturbances, potentially relevant with oxidative stress responses. Mixture interactions between DBAN and other HANs suggest a possible antagonistic effect. Future work will identify the molecular mechanisms underlying these responses through transcriptomic analysis and differential gene expression profiling.

PS 4446 A Proteomic Study Assessing the Impact of Brevetoxin on Zebrafish (*Danio rerio*) Embryo Development

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Background and Purpose: Brevetoxin (PbTx) is a neurotoxin produced by the marine dinoflagellate, *Karenia brevis* which causes harmful algal blooms (HABs) known as the Florida red tide. Brevetoxins pose risks to both human and animal health. Red-tide blooms are known to cause massive fish kills linked to the brevetoxins which activate the voltage-gated sodium channels present in neuronal and skeletal muscle cells. Ingestion of filter-feeding shellfish that are contaminated with brevetoxins can also cause neurotoxic shellfish poisoning (NSP). Inhalation of aerosolized toxins can lead to respiratory problems. Given these health and ecological risks, understanding how brevetoxins impact aquatic organisms at the molecular level are crucial. While previous studies have shown the developmental impacts of environmental toxins in zebrafish and other fish species, such as medaka or mahi-mahi, the molecular mechanisms involved in brevetoxins' effects on early-stage zebrafish remain poorly understood. We aim to understand how marine biotoxins can impact early vertebrate development by comparing protein profiles. Red tide is an ongoing issue in Southwest Florida; the findings from this study will help contribute to our understanding of red-tide effects on ecosystem health by way of understanding its impacts on early fish development. **Methods:** To investigate these effects, zebrafish will be exposed to three concentrations of PbTx-2 (0.5, 1 and 5 ppm) and liquid-chromatography tandem mass spectrometry (LC-MS/MS) based proteomic methods will be applied to identify and quantify proteins across treatment groups. In this study, we sought to determine the effect of PbTx-2 on zebrafish embryos during early development. For this purpose, zebrafish were exposed to PbTx-2 at 72 hours post-fertilization with concentrations of 0.5, 1, and 5 ppb for 24 hours. **Results:** Distinct pathway alterations were observed across treatment comparisons. In the 1 ppb vs. 5 ppb group, *synaptic vesicle trafficking* was uniquely enriched. The 0.5 ppb vs. 5 ppb comparison revealed exclusive enrichment of *axon guidance*, *mannose metabolism*, and *acetate utilization* pathways. In the 5 ppb vs. 0 ppb group, *thiamine metabolism* and *adenine/hypoxanthine salvage* pathways were identified. In comparison, the 1 ppb vs. 0.5 ppb group showed exclusive enrichment of *asparagine and aspartate biosynthesis*, *JAK/STAT signaling*, *heterotrimeric G-protein signaling*, *plasminogen activation cascade*, *glutamine-glutamate conversion*, and *aminobutyrate degradation* pathways. **Conclusions:** Brevetoxin exposure disrupts key proteostasis mechanisms—including protein folding, degradation, and antioxidant defense—leading to protein accumulation, oxidative stress, and heightened vulnerability of embryos to cellular toxicity and apoptosis.

PS 4447 Drivers of Contaminant Load in Elasmobranchs: Coastal Development and Feeding Ecology

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Background and Purpose: Pollution is a major driver of biodiversity loss in marine ecosystems and poses risks to sharks, a subgroup of elasmobranchs that serve as apex/meso- predators and keystone species vulnerable to the bioaccumulation of persistent contaminants. Coastal estuaries function as critical feeding and nursery habitats for many shark species but often overlap with urban development and

pollutant inputs. While contaminant bioaccumulation varies by species, age class, and trophic position, few studies have examined how coastal urbanization and feeding ecology interact to influence contaminant burdens, and even fewer have addressed the co-occurrence and potential interactive effects of multiple contaminant classes. This research aims to (1) evaluate how residence in urbanized versus non-urbanized estuaries influences contaminant concentrations in broadnose sevengill sharks (*Notorynchus cepedianus*), and (2) assess how specialist versus generalist feeding strategies affect contaminant levels in finetooth (*Carcharhinus isodon*) and sandbar (*Carcharhinus plumbeus*) sharks. By integrating measurements of mercury (Hg) and per- and polyfluoroalkyl substances (PFAS), this study investigates mixture exposure dynamics in estuarine predators. I hypothesize that sharks from urbanized estuaries will have higher Hg and PFAS concentrations than those from less-impacted areas, and that generalist feeders will accumulate more of these contaminants through consumption of higher-trophic-level prey. **Methods:** Environmental samples of water and sediment were collected along with shark muscle and blood samples using catch-and-release methods. Project 1 focused on sevengill sharks from San Francisco Bay (SFB), CA, and Willapa Bay (WB), WA, urbanized versus non-urbanized estuaries. Project 2 targeted finetooth and sandbar sharks from Bulls Bay (BB) and St. Helena Sound (SHS), SC, with prey muscle samples for comparative analysis. Mercury was quantified via thermal decomposition, amalgamation, and atomic absorption spectrophotometry, and 21 PFAS were analyzed using LC-MS. Stable isotope ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) and prey Hg data support trophic positioning and contaminant transfer assessment. **Results:** Preliminary Project 1 results show significant differences in muscle total mercury (tHg) levels by age class in SFB broadnose sevengill sharks, with subadults having the highest concentrations versus juveniles and neonates/YOY (Young of the Year). Blood tHg from WB broadnose sevengill sharks show significant differences in tHg between adults and subadults. Across sites, subadult broadnose sevengill shark blood show significantly higher tHg levels in SFB samples versus WB. In Project 2, blood tHg levels in sandbar sharks are significantly higher than in finetooth sharks by age class, and both finetooth and sandbar tHg concentrations trend upward with size/age class. Parallel analysis of PFAS profiles will help determine whether co-exposure trends mirror or diverge from mercury accumulation patterns, revealing whether these contaminants share exposure pathways or partition differently across tissues. PFAS were detected in water and sediment samples across all sample collection locations. Prey, blood, tissue, water and sediment tHg, PFAS and Stable Isotope analyses are ongoing. **Conclusions:** These studies provide new insight into how habitat use and ecological traits shape multi-contaminant exposure in elasmobranchs. Results may inform conservation strategies, improve understanding of combined contaminant effects and trophic transfer, and support more integrative Hg and PFAS risk assessments for vulnerable marine predators.

PS 4448 Multiorgan integrative toxicity of emerging contaminants thallium and gallium in zebrafish

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Background and Purpose: Thallium (Tl) and gallium (Ga) are emerging metal contaminants that have recently attracted increasing attention. Both are widely used in the semiconductor and optoelectronic industries, such as in optical fibers, high-performance chips, light-emitting devices, and thin-film solar cells (CIGS). Although they possess significant economic value, their emissions have led to environmental accumulation risks. Tl concentrations in surface waters can reach up to 1520 µg/L, while Ga has been detected at 40 ppb in water and 40 ppm in sediments. Tl is highly stable and bioaccumulative, readily enriched in vegetables and fruits, whereas Ga can accumulate in the roots of crops such as wheat and rice and be translocated to grains, becoming a potential dietary exposure source. Both metals pose threats to food safety and ecological health. Therefore, it is essential to conduct a systematic toxicological evaluation of Tl and Ga, elucidate their molecular mechanisms involving apoptosis, cellular stress, and endoplasmic reticulum stress, and further explore potential protective strategies. **Methods:** At 6 hours post-fertilization (hpf), healthy zebrafish embryos were selected and placed in 24-well plates for exposure to different concentrations of thallium (Tl) and gallium (Ga) solutions, with embryo medium as the control. Some groups were co-treated with IXA4 or TUDCA from 6 to 48, 72, or 144 hpf. Evaluations included embryo survival, hatching rate, morphological assessment, target gene expression (qPCR), RNA sequencing (RNA-seq), and bioinformatic analysis. **Results:** This study employed zebrafish embryos to investigate the developmental toxicity induced by Tl and Ga, as well as the protective effects mediated by endoplasmic reticulum (ER) stress modulation. Exposure to Tl and Ga significantly increased embryo mortality and malformation rates. In particular, Ga exposure not only caused swim bladder shrinkage and cardiac abnormalities but also induced systemic iron metabolism imbalance and neurotoxicity, indicating strong interference with energy homeostasis and neural development. Tl exposure markedly upregulated multiple genes associated with the unfolded protein response (UPR). Treatment with the selective XBP1 activator IXA4 or the chemical chaperone TUDCA significantly improved embryo survival, hatching rates, and organ morphology. Transcriptomic analysis revealed that IXA4 reversed Tl-suppressed pathways

related to neurocardiac development and enhanced DNA repair mechanisms, particularly nucleotide excision repair (NER). Collectively, IXA4 mitigates Tl-induced multi-organ toxicity by reinforcing UPR adaptation and DNA repair, providing new insights into the toxicological mechanisms and potential protective strategies against emerging metal contaminants. **Conclusions:** Both Tl and Ga induce systemic developmental toxicity in zebrafish, while UPR modulation by IXA4 or TUDCA effectively alleviates Tl-induced toxicity and provides cellular protection.

PS 4449 Skin irritation caused by drilling fluid

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Background and Purpose: The process of extracting hydrocarbon raw materials involves the widespread application of a wide variety of drilling fluids. Drilling fluids are multicomponent mixtures containing a wide variety of organic and inorganic substances. The toxicological properties of drilling fluids have been basically studied in species of fish fauna and protozoa. At the same time the impact of the toxicological properties of drilling fluid on mammals have hardly been studied. Our previous studies have shown that drilling fluid, under acute experimental conditions, when administered directly into the stomach of adult male rats, leads to changes in behavioral responses and feeding behavior. The median lethal doses of drilling fluid were determined using the probit analysis method. The aim of the study was to investigate the skin irritant effect of drilling fluid in acute and subacute experiments on sexually mature male rats. **Methods:** The experiment was conducted on sexually mature male rats under conditions of skin irritation and skin resorption. All procedures involving animals were performed in accordance with ARRIVE guidelines and in compliance with the 3Rs ethical standards. In the course of the acute experiment the local irritant effect of drilling fluid on the skin was studied by applying epy drilling fluid at a rate of 20 ml/cm². The skin resorptive effect of drilling fluid was evaluated in the course of the subacute experiment lasting 30 days. The general state of the animals was assessed, signs of toxicity were identified, the number and time of death of animals, feed and water intake were recorded. The general condition of the animals, the condition of their coat and skin, the intensity and nature of spontaneous motor activity were assessed. A biochemical blood count was performed. Dermis samples were taken for histological examination. The research materials were prepared as part of IRN AP19676915 scientific project funded by the Science Committee of the Ministry of Science and Higher Education of the Republic of Kazakhstan. **Results:** Under the conditions of acute single exposure of drilling fluid to the skin of rats, moderately pronounced (pink-red tone) erythema was observed, which corresponded to 2 points. When assessing the intensity of skin edema some weak intensity of skin edema was identified, which corresponded to 1 point. The skin fold thickness gain was 0.3 mm. The assessment of the degree of erythema and edema was summarized and the average total score was calculated, which equaled 3 points. The effects of the skin-resorptive effect of the drilling fluid in the subacute experiment were assessed based on a number of indicators: feed and water intake, changes in body weight dynamics, behavioral reactions as well as external signs of intoxication. It is noteworthy that by the end of the experiment the test group of animals showed stiffness and impaired coordination of movement. Feed intake declined, which led to a change in the body weight of the test animals. It should be noted that the duration of posture in the experimental animals showed a pronounced tendency to decrease on day 15, with a gradual increase towards the end of the experiment (by day 30). A similar tendency was observed in the number of postures, which decreased by the middle of the experiment with a gradual recovery of behavioral responses. One of the important indicators characterizing the functional features of the animals' condition is grooming. It was found that in the experimental animals this type of behavior also tended to deteriorate compared to that in the reference animals. It should be noted that there were pronounced changes in the leukocyte formula and the activity of detoxification enzymes in the experimental groups compared to those in the reference group. Moderately pronounced focal inflammatory infiltrates were observed in the papillary layer of the dermis, and varying degrees of edema of fibrous structures and intercellular substance, swelling of collagen fibers and an increase in their thickness were noted in the reticular layer. **Conclusions:** Under conditions of skin irritation and skin resorption the drilling fluid leads to adverse changes in behavioral responses, clinical and biochemical blood parameters, and histomorphological alterations in the dermis.

PS 4450 Metabolic activities in Rainbow trout (*Oncorhynchus mykiss*) S9 fractions from liver and extrahepatic organs as an alternative *in vitro* ecotoxicity assessment approach

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Background and Purpose: Whole body biotransformation rate constants can be calculated by using *in vitro* to *in vivo* extrapolation (IVIVE) models. These models use *in vitro* intrinsic clearance $CL_{in\ vitro, int}$ rates derived with OECD Test Guidelines (TG) 319A and/or 319B to estimate liver clearance rates in Rainbow trout, which are then extrapolated to a whole-body (*in vivo*) biotransformation rate constant. Primacyt supports this approach and therefore contributes to alternative methods to animal

experiments. **Methods:** Rainbow trout were kept under environmental controlled housing conditions according to OECD TG 319A/B. Organs were obtained from sexually immature non-infectious, non-contagious and healthy animals. After removal, the organs were cut, immediately flash frozen in liquid nitrogen and stored at -80 °C. The tissues from 8 individual animals (per lot) were homogenized for preparation of S9 fractions in phosphate buffer, in total 2 lots. S9 fractions of liver, intestine, gill, brain, heart and spleen were produced and stored at -150 °C. S9 fractions were prepared to determine the Phase I and Phase II Cytochrome P450 enzyme activities by using liquid chromatography-mass spectrometry analysis. Phase I enzymatic activities of Cytochrome P450 in Rainbow trout organs were determined by using 1 mg protein extract incubated with 7 reference compounds, including Bupropion, Chlorzoxazone, Coumarin, Diclofenac, Midazolam, Phenacetin and S-Mephenytoin at 14 °C in 0.1 M phosphate buffer. Time dependence of enzyme activity were determined in intestine S9 fractions for 10 to 60 min and in liver for 5 to 120 min. The distribution of Phase I/II enzyme activities in Rainbow trout organs were determined by using 1 mg protein extract incubated with reference compounds at 14 °C for 60 min (Phase I) and 30 min (Phase II) in 0.1 M phosphate buffer. Glucuronidation and sulfation of 7-OH Coumarin were determined for Cytochrome P450 Phase II enzyme activities in all prepared organs. **Results:** The Phase I formation of Acetaminophen, OH-Bupropion, 4-OH-Diclofenac and 6-OH-Chlorzoxazone was linear up to 60 min in intestine and up to 120 min in liver. In both intestine and liver, a linear increase of protein concentration from 1 to 2 mg/ml led to a linear increase of enzyme activities in Phenacetin O-Deethylase, Diclofenac 4'-Hydroxylase and a minor increase of Chlorzoxazone 6'-Hydroxylase. Additionally, in liver Midazolam 1'-Hydroxylase activities led also to a linear increase. Bupropion-Hydroxylase and Coumarin 7'-Hydroxylase enzyme activities indicated no effect by doubling the protein concentration in both organs. Phase I Cytochrome P450 activities were found in all organs after 60 min of incubation. Liver accounted for the highest enzyme activities of all organs tested. Intestine accounted for the highest extrahepatic enzyme activities of Phenacetin O-Deethylase (57 % of total activity (OTA), Midazolam 1'-Hydroxylase (27 % OTA), Chlorzoxazone 6'-Hydroxylase (24 % OTA), Diclofenac 4'-Hydroxylase (22 % OTA). Bupropion-Hydroxylase activities were almost equally distributed (15-21 %) among all organs. Minor enzyme activities were also found in gill, heart, brain and spleen. Phase II activities were detected in liver, intestine, gill and heart. Liver accounted the highest activities of 7-OH-Coumarin glucuronidation (59 % OTA) and its sulfation (83 % OTA). 34 % of total 7-OH-Coumarin glucuronidation activity was detected in intestine and less in gill (4 % OTA) and heart (2 % OTA). Besides liver, 7-OH-Coumarin sulfation activity was only detectable in gill (17 % OTA). **Conclusions:** In summary, the liver is the major organ for detoxification of compounds in Rainbow trout. Liver and intestine have shown a linear increase of enzyme activity for at least 60 min of incubation time. However, extrahepatic organs, mainly intestine, but also brain, gill, heart and spleen exhibit certain Phase I Cytochrome P450 activities with different specification to the reference compounds. Phase II enzyme activities were besides liver also detectable in intestine and gill. Our results suggest that extrahepatic organs, mainly intestine, should also be taken into account when bioaccumulation and *in vitro* clearance rates are determined for IVIVE modeling in Rainbow trout.

PS 4451 Effect of tire wear particle 6PPD and 6PPDQ on HepG2 liver cancer cell metabolism

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Background and Purpose: In the United States alone, it is estimated that 1.12 million tons/year of tire wear particles are produced when automobile tires come in contact with the road. Tire wear particles, 6PPD and its quinine version, 6PPDQ, have been tested in many *in vivo* studies, revealing impaired swimming capabilities in zebrafish, and were found to be accumulated in animal livers. Nevertheless, little is known how 6PPD and 6PPDQ are impacting organisms on a cellular level, specifically how mitochondrial processes are interrupted in mammalian cells by 6PPD and 6PPDQ. **Methods:** In this study, we sought to understand if 6PPD and 6PPDQ cause changes in mammalian mitochondrial morphology and function. We incubated five different concentrations of 6PPD and 6PPDQ (200uM, 100uM, 50uM, 25uM, and 12.5uM) with a HepG2 liver cell line for 24 hours. XTT cell viability was performed following 24 hour treatment of 6PPD and 6PPDQ. Furthermore, mitochondrial staining, and ATP quantification experiments were performed following 24 hour treatment of 6PPD. **Results:** Dose dependent reduction in cell viability, mitochondrial mean branch length, and ATP levels were observed after 6PPD treatment. 6PPDQ showed an increase in cell viability across all concentrations, and was further studied morphologically. Qualitative results suggest linear polymerization of 6PPDQ and therefore reduced capacity to enter the cell. **Conclusions:** Ongoing studies, such as RNA sequencing, are being conducted to explore the specific genes 6PPD acts on to impact a cell's mitochondrial metabolism to better understand the specific pathways leading to mitochondrial dysfunction after 6PPD treatment.

PS 4452 Assessing PFAS Contamination and PFOS Biodegradation Potential in South Carolina Estuarine Sediments

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are synthetic chemicals widely used in industrial and consumer applications, including firefighting foams, non-stick cookware, and food packaging. Their exceptionally strong carbon-fluorine bonds contribute to their environmental persistence and tendency to bioaccumulate. Among them, perfluorooctanesulfonic acid (PFOS) remains a pervasive contaminant in marine ecosystems despite its phaseout from U.S. production, largely because it is readily transported to and retained in estuarine sediments, where it can persist for decades. Estuaries, which provide critical habitat and essential ecosystem services, are increasingly threatened by coastal urbanization and associated contaminant inputs. Charleston Harbor (CH), a highly urbanized estuary in South Carolina, is known to contain elevated PFAS levels, whereas nearby St. Helena Sound (SHS), a comparatively undeveloped system, has not yet been analyzed for PFAS contamination. Because microbial communities in estuarine sediments regulate organic matter cycling and pollutant transformation under both aerobic and anaerobic conditions, they may also initiate PFOS degradation and therefore serve as potential bioindicators of PFAS exposure and ecosystem health. In this study, we quantify PFAS concentrations and characterize sediment microbial community composition in CH and SHS. We test three hypotheses: (1) PFAS concentrations will be higher in CH than in SHS; (2) sites with elevated PFAS will harbor microbial communities enriched in taxa resistant to or capable of degrading these compounds; and (3) microbial inoculum from those sediments, when exposed to elevated PFOS, will transform it into less bioaccumulative metabolites. **Methods:** Sediment was collected with a Van Veen grab and split for PFAS and microbial analyses, and surface water for PFAS was collected in 1-L HDPE bottles. PFAS concentrations in sediment water were quantified by HPLC-MS/MS. Microbial community composition in sediment was characterized by NextGen Illumina 16S rRNA gene sequencing and processed in QIIME 2. Alpha diversity (Shannon index, Pielou's evenness) and beta diversity (Bray-Curtis dissimilarity, unweighted UniFrac) were used to compare microbial communities in sites and estuaries. To evaluate PFOS biodegradation potential, sediment inocula from selected sites were incubated at elevated PFOS concentrations under aerobic and anaerobic conditions. PFOS loss and metabolite formation were measured by IM-MS and fluoride ion release was monitored using a fluoride ion selective electrode, and TOPO cloning was used to identify candidate taxa potentially responsible for PFOS degradation. **Results:** Preliminary results show some differences in PFAS between estuaries, with PFOS emerging as the dominant PFAS in both sediment and surface water and generally higher in CH than in SHS. Shannon diversity decreased from Fall to Spring with October sediments showing higher diversity than March. Unweighted UniFrac indicated that microbial communities were more similar within the same site and season, while Bray-Curtis shows distinct, site-specific clustering across months. These patterns, together with the persistence and dominance of PFOS, motivated the targeted culture experiments to test PFOS degradation and link it to specific microbial taxa. **Conclusions:** Overall, we find PFAS contamination in both estuaries, with PFOS consistently the dominant compound across each site. Additionally, there are clear differences in sediment microbial diversity and community composition between the more urbanized and less urbanized sites. Because these field patterns are correlative and multiple stressors can influence sediment microbiomes, they highlight the need for controlled culturing studies. Ongoing incubations with sediment inocula and elevated PFOS exposure will directly test whether estuarine microbes can transform PFOS and help identify the taxa that may contribute to the natural attenuation of PFAS in these systems.

PS 4453 Mechanism of Liver Injury Induced by Cr⁶⁺ in Zebrafish and Protective Effect of Organic Selenium

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Background and Purpose: Hexavalent chromium (Cr⁶⁺) is a form of chromium with extremely strong oxidizability, high mobility, and the highest toxicity, and it is classified as a Group A human carcinogen. It is mainly released into the environment through leather and textile production processes and metallurgical applications, leading to widespread contamination of soil and groundwater. After transmission through the food chain, Cr⁶⁺ accumulates in target organs (such as the liver) in humans and animals, posing a potential threat to human health. In addition to promoting hepatocyte death and secondary liver fibrosis, Cr⁶⁺ can also induce hepatic steatosis. However, the mechanism underlying the association between Cr⁶⁺ and these liver pathological changes has not been fully elucidated. **Methods:** In this study, liver transgenic zebrafish were used as an experimental model to explore the mechanism of liver steatosis and hepatocyte death induced by Cr⁶⁺. Meanwhile, we evaluated the protective effect of low-dose selenomethionine (Se-Met) on Cr⁶⁺.

mediated hepatotoxicity. **Results:** Exposure to Cr⁶⁺ caused hepatic steatosis and hepatocyte mitochondrial damage in zebrafish. At the biochemical and molecular levels, Cr⁶⁺ exposure upregulated the expression of genes related to lipid metabolism and ferroptosis, while reducing the protein expression level of glutathione peroxidase 4 (GPX4) and the content of glutathione (GSH). Notably, both ferroptosis inhibitor Fer-1 and low-dose Se-Met could alleviate Cr⁶⁺-induced hepatic steatosis, mitochondrial dysfunction, and GPX4/GSH regulatory imbalance. Cr⁶⁺ significantly affected fatty acid metabolism, fatty acid biosynthesis, linoleic acid metabolism, and oxidoreductase activity. **Conclusions:** The results of this study indicate that the mechanism of liver injury induced by Cr⁶⁺ is related to the ferroptosis pathway, and the ferroptosis inhibitor Fer-1 and low-dose Se-Met can prevent this injury. The results also suggest that low-dose Se-Met can be used as a feed additive to prevent the harm caused by chromium.

PS 4454 Ecotoxicological Assessment of Effluents from a Combined Anaerobic-Anoxic-Aerobic Wastewater Treatment System Reveals Unexpected Oxidative Stress in *Chironomus sancticarioli*

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Background and Purpose: Traditional wastewater treatment plants are usually based on anaerobic or aerobic processes and often fail to efficiently remove organic micropollutants, posing risks to both environmental and human health. Besides low removal efficiencies, biotransformation products are usually derived from biological processes, which may have potentially higher toxicity. To overcome these drawbacks, combined biological processes have been proposed as a hidden solution. Notwithstanding research examining the ecotoxicity of effluents from treatment units, investigation into the ecotoxicological effects of effluents from combined alternative systems, such as anaerobic-anoxic-aerobic systems, remains lacking, especially involving biomarkers assessments. It was hypothesized that effluents from the aerobic stage would exhibit lower toxic potential than those from preceding units, as enhanced oxidation could favor micropollutant degradation and reduce the formation of reactive intermediates. **Methods:** This study assessed the ecotoxicological effects of an innovative treatment system integrating anaerobic, anoxic, and aerobic fixed-film reactors, producing effluents with low organic matter and nitrogen. The system comprised an anaerobic reactor fed with raw domestic wastewater (RDW), whose effluent supplied an anoxic-aerobic stage. The anoxic reactor received the anaerobic effluent with sodium bicarbonate addition and a recirculated stream from the aerobic reactor, which finalized treatment. Four samples were tested at 25%, 50%, and 100% dilutions: RDW, anaerobically treated wastewater (ATDW), ATDW with sodium bicarbonate (ATDW+NaHCO₃), and anoxic-aerobically treated wastewater (AATDW). *Chironomus sancticarioli* was used in chronic exposures (7 days) assessing survival, dry weight, and biomarkers as catalase (CAT), glutathione (GSH), glutathione S-transferase (GST), and lipid peroxidation (LPO). **Results:** No significant changes occurred in survival or dry weight. However, distinct oxidative stress patterns emerged. RDW and ATDW showed no significant CAT variation, but GSH was significantly upregulated at 50% and 100% RDW and all ATDW dilutions, especially at 100% ATDW. This increase coincided with a remarkable GST downregulation in 100% RDW and all ATDW dilutions. LPO decreased in 100% RDW and 50-100% ATDW, suggesting effective antioxidant compensation. The stable CAT levels may reflect compensation via GSH-Px activity or oxidative imbalance driven by organic micropollutants rather than H₂O₂. The rise in GSH indicates adaptive redox regulation against stress, while reduced GST suggests enzyme inhibition or structural damage. Despite redox disturbance, oxidative damage (LPO) remained controlled in anaerobic treatments. Conversely, the anoxic-aerobic effluent (AATDW) produced unexpected effects. CAT was consistently downregulated across all dilutions, while ATDW+NaHCO₃ showed no changes. GSH was upregulated in both ATDW+NaHCO₃ and AATDW, whereas GST was reduced. Notably, LPO levels doubled in 50% and 100% AATDW, indicating enhanced lipid damage despite higher organic matter and nitrogen removal. It is worth noting that all the exposures were carried out under aeration, which rules out the hypothesis of oxygen levels present in the AATDW samples. Thus, the anoxic-aerobic stage induced greater oxidative stress than the anaerobic stage, challenging the notion that higher oxidative levels from aerobic systems necessarily produce effluents with less alteration. **Conclusions:** The combined anaerobic-anoxic-aerobic system effectively reduced conventional pollutants but induced distinct oxidative stress responses at the cellular level. Anaerobic treatment maintained redox balance without oxidative damage but affected the antioxidant defense system. On the other hand, the anoxic-aerobic effluent triggered lipid peroxidation and enzyme inhibition in *C. sancticarioli*. These findings highlight that improved conventional removal does not necessarily correlate with reduced ecotoxicity, reinforcing the importance of biomarker-based assessments in evaluating advanced treatment systems.

PS 4455 Hair analysis as a non-invasive method for assessing the exposure of mammals with different dietary habits to poly- and perfluoroalkyl substances

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Background and Purpose: Poly- and perfluoroalkyl substances (PFASs) are persistent environmental pollutants found across various ecosystems. While human and domestic animal exposure to PFASs is well documented, data on wild terrestrial mammals remain scarce, particularly when using hair as a biomonitoring matrix. Hair appears particularly valuable for PFAS biomonitoring in wildlife, as it accumulates chemicals over time and reflects long-term exposure remaining intact after death. Despite this potential, very few studies have investigated PFAS accumulation in the hair of wild terrestrial mammals. This study aimed to assess the levels of selected PFASs in the hair of different wild mammal species with varying diets, using liquid chromatography-tandem mass spectrometry (LC-MS/MS). **Methods:** Samples were collected between January and June 2023 in the Abruzzo region, located in central Italy. The study encompassed 58 adult individuals, belonging to three groups in term of feeding behaviour: 1) herbivores (n=23) including roe deer and red deer; 2) predators (n=25) including red fox, European badger and wolf; 3) omnivores (n=10) including wild boar. The PFASs analysed included perfluorobutanesulfonic acid (PFBS), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorohexanesulfonic acid (PFHxS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), linear and branched perfluorooctane sulfonic acid (L-PFOS and B-PFOS), perfluoropentanesulfonic acid (PFPeS), perfluorheptanesulfonic acid (PFHpS), perfluoroundecanoic acid (PFUnDA) and perfluorododecanoic acid (PFDoDA). Quantification of analytes was conducted using LC-MS/MS, employing a Sciex Triple Quad™ 7500 system (Sciex, Framingham, MA, USA) operated in negative electrospray ionization (ESI-) mode. Descriptive statistics were applied to summarize the data. To compare PFAS concentrations among animals with different feeding behaviours, the non-parametric Kruskal-Wallis test was employed. **Results:** The levels of PFASs detected in this study varied significantly depending on both the specific compound and the mammalian species analysed. When considering all 58 hair samples collectively, the highest mean concentrations were observed for PFNA and PFOA. PFNA exhibited a mean ± SD of 0.309 ± 0.33 ng/g (median: 0.37 ng/g) and was detected above LOQ in 93.1% of samples. PFOA was present in 96.6% of samples (mean ± SD: 0.272 ± 146.051 ng/g; median: 0.15 ng/g). Significant inter-group differences were also observed for individual compounds. The clearest differences between predators and herbivores were noted in the case of L-PFOS and PFDoDA. Mean level of L-PFOS in predators was 0.291±0.046 ng/g (median 0.143 ng/g) and was statistically significantly higher (p<0.0001) than value noted in herbivores (mean 0.033±0.09 ng/g, median<LOQ). Mean level of L-PFOS in generalist omnivores was 0.093±0.08 ng/g (median 0.068 ng/g) and was also statistically significantly higher (p=0.0269) than that noticed in herbivores. Similar situation was found in the case of PFDoDA. Moreover, mean level of PFUnDA in predators amounted to 0.091±0.11 ng/g (median 0.054 ng/g) and was statistically significantly higher (p=0.031) than the level in herbivores (mean 0.038±0.03ng/g and median 0.033 ng/g). **Conclusions:** This study contributes valuable insights into the environmental exposure of wild terrestrial mammals to PFASs, reinforcing the utility of hair as a biomonitoring matrix. Trophic position appears to play a central role in PFAS bioaccumulation, with higher concentrations observed in carnivores. Species-specific behaviours (such as opportunistic feeding near human settlements) may also influence exposure levels. The differences in PFAS compound profiles across studies underscore the complex environmental dynamics and highlight the need to monitor both legacy and emerging PFAS. Despite generally low concentrations, the toxic potential of PFASs at environmentally relevant levels, combined with possible mixture effects, raises concerns for wildlife health and conservation.

PS 4456 Developing a Protocol for Measuring Environmentally Sourced Microplastics through Flow Cytometry

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Background and Purpose: Microplastics are ubiquitous in our environment, being found in every medium. A microplastic, as defined by the EPA, is derived from any different form of plastic in the size range of 5 millimeters to as small as 1 nanometer or referred to as nanoplastic. Evidence suggests that microplastics are present in nearly every environment, regardless of location. However, existing methods for measuring and identifying these microplastics have typically relied on lab-milled plastics and microplastics under ideal conditions, with minimal consideration of natural variability. The goal of this project was to develop a protocol to not only detect the presence of microplastics, but also efficiently quantitate environmentally sourced microplastics. **Methods:** For this study, water samples were taken from two sources around Missoula, MT, Frenchtown Pond State Park and the Clark Fork River through Missoula. Both being highly recreated bodies of water with lots of anthropogenic activity in their vicinities. The samples were treated with hydrogen peroxide and left to sit for 24 hours before each measurement was taken. Milli-Q water was used as a control to establish a baseline for microplastics. Fluorescent Nile

Red Dye was used to stain for microplastics in the samples, as it has a high affinity towards plastic particles, allowing quantitation of microplastic particles based on fluorescence intensity. Each sample was stained with Nile Red for 30 minutes before measuring, to give it ample time to adhere to the plastic within the water samples. Flow Cytometry was used as the main method for identifying microplastic presence using excitation at 488 nm and emission at 530 nm to separate microplastics from the background signal of the sample. As Flow Cytometry proved to be more efficient than other methods established, another method tried was measuring the fluorescent intensity, with a plate reader, as an indicator for microplastic concentration. **Results:** Flow Cytometry allowed us to separate the microplastic content from fluorescent intensity to get an accurate concentration. By developing a detailed and reproducible protocol we were able to quantify different microplastic concentrations from multiple environmental samples from Frenchtown Pond and the Clark Fork. For each, Frenchtown Pond had concentrations of microplastics ranging from 87-222 𝜇g per 100 𝜇l and the Clark Fork had concentrations ranging from 126-254 𝜇g per 100 𝜇l. **Conclusions:** Flow Cytometry proved to be an effective and efficient quantitation of microplastics in environmental sampling.

PS 4457 Crested porcupines (*Hystrix cristata*) as sentinels of environmental metal contamination in Central Italy

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Background and Purpose: Contamination by potentially toxic metals represents one of the main environmental issues threatening biodiversity and the proper functioning of ecosystems, with direct repercussions on human health. In this context, toxicological biomonitoring plays a fundamental role in assessing the presence and spread of such substances. The selection of representative "sentinel" species is crucial to ensure effective monitoring. In Italy, one such species is the crested porcupine (*Hystrix cristata*), a large semi-fossorial rodent of African origin. Its broad ecological tolerance, generalist diet, sedentary and territorial habits, and close contact with soil and vegetation make *H. cristata* an excellent indicator of local contamination. Furthermore, its long lifespan allows for the progressive accumulation of contaminants over time, making it a suitable species for evaluating chronic exposure to pollutants in mixed agricultural and forested environments. This study aimed, for the first time on a global scale, to assess the concentrations of barium (Ba), cadmium (Cd) and lead (Pb) in liver and kidney of crested porcupine. The investigation sought to determine the presence and quantitative levels of these elements within a selected area, the Abruzzo region, to evaluate potential differences in concentrations between the two tissues, sex and age and to explore possible correlations between contamination levels and land use. **Methods:** Between 2022 and 2025, liver and kidney were collected from 30 carcasses of *Hystrix cristata*. Local authorities granted permission for the necropsy of the carcasses. Anamnestic information including age and sex were desumed from the necropsy and sampled animals were categorized based on the latitude and longitude coordinates in the WGS84 (EPSG: 4326) reference system of their deaths for inclusion in the CORINE Land Cover (CLC) classes. The instrumental analysis of Ba, Cd, and Pb was performed using an inductively coupled plasma mass spectrometer (Q-ICP-MS iCAP RQ) in standard mode using matrix-matching calibration to minimize the effect of the matrix. Shapiro-Wilk normality tests showed that the distribution of elements' concentrations was not normal. Non-parametric Mann-Whitney and Kruskal-Wallis tests were applied to compare concentrations of inorganic elements among matrixes, animal sex, age and CLC distribution. Data were shown as median with 1st (Q1) and 3rd (Q3) quartiles and as mean and standard deviation (SD). **Results:** In liver and kidney metal concentrations were distributed as follows: Cd>Ba>Pb. The mean and median value of metals in liver were: Ba (138.7±118.7 µg/kg; 112 µg/kg Q1=46.8, Q3=171.8); Cd (140.3±199.6 µg/kg; 40 µg/kg Q1 22, Q3 220); Pb (61.5±87.4; 33 µg/kg Q1=24.5, Q3=54.5). In kidney mean and median concentrations were as follows: Ba (140.2±302; 60.5 Q1=42.3, Q3=114.5); Cd (950.4±1453.3, 326.5 Q1 121, Q3 1191.8); Pb (75.8±71.7, 53.5 Q1=35.5, Q3=88). The differences between matrixes showed as Cd were statistically significant higher in kidney compared to liver (p<0.05). Differences in concentrations of metals were not significant according to sex, age and CLC distribution. However, according to CLC dated animals were distributed as follows: 17% urban fabric, 27% arable land, 13% permanent crops, 27% heterogeneous agricultural areas, and 17% forests. **Conclusions:** The ecological traits of *Hystrix cristata* support its use as an effective sentinel species for terrestrial ecosystem monitoring. The results presented in this study provide the first global baseline values for potentially toxic metal concentrations in this species, contributing to a better understanding of contaminant bioaccumulation processes in Mediterranean terrestrial mammals. Cadmium concentrations in the kidney matrix were found to be significantly higher than those of other metals. According to the CLC dataset, the species' distribution is consistent with its ecological characteristics. Considering its ongoing northward expansion and increasing interaction with anthropogenic environments, *H. cristata* may represent a highly valuable biological model for integrated studies on wildlife toxicology, environmental change, conservation management, and human health protection.

PS 4458 Ecological Risk Assessment and CLP Classification of Beryllium and Beryllium Compounds in Aquatic Environments

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Background and Purpose: Beryllium (Be) and its compounds are used in various industries and may be released into aquatic environments. Despite their widespread use, ecological risks to aquatic species are not well characterized, and regulatory classifications under the Globally Harmonized System (GHS) and European Union (EU) Classification, Labeling, and Packaging (CLP) regulations are inconsistent and lack a clear scientific basis. **Methods:** Aquatic toxicity thresholds for Be and Be compounds were compiled from peer-reviewed literature and regulatory databases. Environmental Be concentrations in surface waters were obtained from the National Water Quality Monitoring Council (NWQMC) Water Quality Portal (WQP). Acute and chronic risk quotients (RQs) were calculated by comparing environmental concentrations to toxicity thresholds. CLP hazard classifications were assigned according to EU guidance. **Results:** Aquatic toxicity thresholds for Be compounds ranged from 'practically nontoxic' to 'moderately toxic,' with most values orders of magnitude higher than measured environmental Be concentrations. Acute and chronic RQs were generally below 1.0, indicating low risk to aquatic species. CLP classification varied by compound: insoluble Be metal and Be(OH)₂ were assigned to Chronic 2 and 4, respectively, while soluble compounds such as BeCl₂, BeNO₃, and BeSO₄·4H₂O were classified as Chronic 1-3 and Acute 1. Only one chronic RQ exceedance was observed for BeSO₄·4H₂O in crustaceans at the 95th percentile Be water concentration. **Conclusions:** Be and its compounds are not anticipated to pose significant acute or chronic risks to aquatic life under current environmental conditions, but compound-specific hazard classification is warranted. Data gaps remain for chronic toxicity and the influence of water chemistry, highlighting the need for further research.

PS 4459 Microplastics in Motion: Comparative Uptake and Immune Responses in Coastal Shark Species

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Background and Purpose: Microplastics (MPs) are an emerging class of environmental pollutants that are now considered a global ecotoxicological concern due to their persistence, ubiquity, and potential for biological interaction. MPs are introduced into marine systems through urban runoff, wastewater effluent, and degradation of larger plastic debris, where they absorb hydrophobic contaminants and concentrate in sediments and biota. Mounting evidence from laboratory models, including zebrafish, bivalves, and marine worms, demonstrate that MPs can induce oxidative stress, inflammation, reproductive impairment, as well as altered behavior through physical obstruction and endocrine disruption. However, data on the biological effects of MPs in large marine vertebrates remain sparse, particularly in long-lived predators such as sharks. Coastal South Carolina estuaries, including Charleston Harbor and Winyah Bay represent MP hotspots, receiving high inputs from urban, industrial, and riverine sources. MPs have been documented in local sediments, plankton, fishes, and even dolphins, but shark exposure remains uncharacterized. Because sharks occupy high trophic positions, exhibit species-specific feeding strategies (pelagic, demersal, omnivorous), and possess a conserved vertebrate immune system, they represent ideal sentinels for evaluating MP bioaccumulation and associated physiological effects. **We hypothesize that demersal feeders will exhibit higher MP burdens than pelagic species due to greater benthic contact and ingestion of sediment-associated prey, and that increasing MP load will correspond with elevated oxidative stress and innate immune activation in liver and blood tissues.** **Methods:** Sharks are collected exclusively from incidental bycatch mortalities during ongoing South Carolina Department of Natural Resources (SCDNR) longline, drumline, and gillnet surveys to ensure ethical sample acquisition. Three ecologically distinct species are targeted: finetooth (*C. isodon*; pelagic mesopredator), blacktip (*C. limbatus*; generalist carnivore), and bonnethead (*S. tiburo*; demersal omnivore) for cross trophic comparisons of MP uptake. Dissected tissues (gut, liver, brain, gonad, kidney, intestine) are digested in 10% potassium hydroxide (KOH) at 40°C, sieved through a graded cascade, and filtered on 10 µm membranes. Retained particles are stained with Nile Red and visualized under 254 nm UV light for fluorescent quantification, while polymer identity is confirmed using Fourier Transform Infrared Spectroscopy (FT-IR). To evaluate physiological responses, liver homogenates are analyzed for key antioxidant and oxidative stress biomarkers, including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), total glutathione (GSH), and lipid peroxidation (MDA). Blood samples are assessed for leukocyte differentials, plasma lysozyme activity, and total protein as indicators of immune activation. **Results:** Pilot analyses demonstrate efficient tissue digestion and recovery of synthetic polymers. MPs were visualized in liver, gastrointestinal, and muscle tissues, confirming bioavailability and retention in higher-trophic vertebrates. Quantitative assessments show tissue-specific variation, with the highest MP concentrations in intestine, followed by liver, muscle, and gonads. Particle morphologies, fragments, fibers, and films, suggest multiple exposure routes through both waterborne and dietary pathways. Ongoing analyses will use FTIR to confirm polymer composition and integrate MP load with oxidative stress and immune biomarkers to assess potential dose-dependent physiological responses. Continued sampling through 2025-2026 will expand

species coverage and enable modeling of exposure, accumulation, and biological impact. **Conclusions:** This work represents one of the first integrative studies to quantify MP exposure risk factors and associated biological effects in estuarine sharks. By linking trophic ecology, contaminant characterization, and biomarkers of oxidative and immune function, the project will establish a mechanistic foundation for understanding plastic-associated toxicity in marine predators. These findings will inform ecotoxicological risk assessment frameworks, contribute to the development of biomonitoring indicators for apex species, and support conservation strategies that address plastic pollution impacts in South Carolina's coastal ecosystems.

PS 4460 Neurotoxicity of β -N-methylamino-L-alanine (BMAA) in larval zebrafish: behavioural and transcriptomic insights

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Background and Purpose: β -N-Methylamino-L-alanine (BMAA), a neurotoxic non-protein amino acid produced by cyanobacteria, diatoms and dinoflagellates, has become a global concern due to its potent neurotoxicity and its possible link to neurodegenerative diseases. BMAA bioaccumulates and biomagnifies through aquatic food webs, posing ecological risks to aquatic organisms and raising concerns for human exposure. Despite this, investigation of BMAA neurotoxicity to aquatic species is still in its early stages. **Methods:** To address this gap, zebrafish (*Danio rerio*) embryos were exposed to three environmentally relevant concentrations of BMAA (10, 20, and 40 μ g/L) and a control for 28 days. Behavioural assay was performed to evaluate social interactions, and transcriptomic profiling was conducted to identify dose-dependent alterations in molecular, cellular, and biological pathways. **Results:** Exposure to higher concentrations of BMAA (20 and 40 μ g/L) significantly reduced the time larvae spent with conspecifics, indicating altered social behaviour. Transcriptomic analysis revealed that BMAA disrupts multiple molecular pathways in a dose-dependent manner, including excitatory signalling, mitochondrial function, oxidative stress regulation, lipid, protein, and energy metabolism, proteostasis, and neuroinflammatory responses. These disruptions resulted in apoptosis, neuronal dysfunction, and developmental impairments. **Conclusions:** This study provides important insights into the neurotoxic effects of BMAA on zebrafish, establishing a clear link between exposure to environmentally relevant concentrations of BMAA and adverse behavioural and molecular outcomes. Advancing mechanistic insights into BMAA effects in aquatic organisms is essential for effective ecological risk assessments and the development of regulatory standards to safeguard aquatic ecosystems and public health.

PS 4461 Parent vs. Metabolite: Transcriptional Responses to the Phthalate DEHP and its Alternative Plasticizers in *Danio rerio* embryo-larvae

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Background and Purpose: Non-phthalate plasticizers (NPPs) are increasingly being used as replacements for traditional phthalates such as di(2-ethylhexyl) phthalate (DEHP), due to growing evidence of adverse human health effects associated with phthalate exposure. Among these alternatives, di(2-ethylhexyl) adipate (DEHA) and tris(2-ethylhexyl) trimellitate (TOTM) have been introduced as potential substitutes for DEHP. However, these NPPs share key structural similarities with DEHP, including the primary ester-linked 2-ethylhexyl side chains (TOTM and DEHA) and the phthalic acid head (TOTM). Emerging evidence indicates that these compounds can produce metabolites identical to those of their DEHP counterparts. This raises important questions about whether such replacements represent a meaningful improvement in safety. The objective of this study was to compare the induction of Phase I-III biotransformation transcripts in zebrafish (*Danio rerio*) larvae following exposure to DEHP, DEHA, TOTM, and their shared metabolite, 2-ethylhexanol (2EH), to determine whether DEHP-induced effects are mediated by 2EH. **Methods:** Zebrafish embryos were exposed under matched dose conditions equimolar concentrations of DEHP, 2EH, DEHA, and TOTM. Following five-day exposures, gene expression of Phase I-III transcripts were measured, followed by measurement of endocrine-associated transcripts that have been identified to be altered during DEHP exposure. Fold changes relative to controls were calculated, and treatment effects were assessed. **Results:** Principle component analysis revealed that TOTM, DEHA, DEHP, and 2EH were associated with the same Phase II-III transcripts, PXR, UGTb5, SULT1st3, SULT2st3, and Steroid sulfatase. Analysis of endocrine-associated transcripts revealed no difference among treatments. **Conclusions:** These results suggest that TOTM and DEHA may differentially modulate genes associated with Phase II-III metabolism in a similar manner to DEHP.

PS 4462 Estrogenic activity of PFAS with variable length and structure using human cell bioassays and rainbow trout (*Oncorhynchus mykiss*) liver slice assays

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are a diverse class of synthetic fluorinated chemicals that are ubiquitous in the environment due to their diverse usage in both consumer and industrial products. The majority of PFAS have little or no toxicity information. Previous high throughput screening identified three PFAS - 1H,1H,8H,8H-perfluorooctane-1,8-diol (FC8-diol), 1H,1H,10H,10H-perfluoro-decane-1,10-diol (FC10-diol), and 1H,1H,8H,8H-perfluoro-3,6-dioxaoctane-1,8-diol (FC8-DOD) as the most estrogenic PFAS among 140 structures tested. *In vivo*, these three PFAS caused concentration-dependent increases in the expression of estrogenic genes with their rank order of potency *in vivo* matching the *in vitro* results. The aim of this study was to further evaluate the potential of PFAS with structural similarity to FC8-DOD, FC8-diol, or FC10-diol, to cause estrogenic effects. **Methods:** Five PFAS, four diols with chain lengths varying from five to 12 carbons, and PFAS with different structure but a high similarity score, were selected using tools in the U.S. EPA's CompTox Chemicals Dashboard v2.5.3. The five selected PFAS were tested using Indigo Bioscience human estrogen receptor bioassay kits. To further test potential estrogenic effects and verify the activity previously seen in FC10-diol and FC8-DOD, liver slices derived from immature male rainbow trout were exposed to selected PFAS. Slices were incubated with six doses of each PFAS for 48 h, then RNA extracted, and expression of estrogen receptor regulated genes was investigated by RT-qPCR. **Results:** Among the five tested PFAS, FC9-diol and FC12-diol elicited significant estrogenic activity in the human-based system. Exposures to FC9-diol, FC10-diol, FC12-diol and FC8-DOD induced vitellogenin mRNA expression in exposed liver slices, but exposure to diols of other chain lengths did not. Hepatic transcription of insulin-like growth factor 1 and apolipoprotein b mRNA was significantly upregulated in FC9-diol and FC8-DOD at their highest concentrations, but significant modulation was not observed in FC10-diol. **Conclusions:** The present study demonstrates that some PFAS exhibited strong estrogenic potential in both a human estrogen receptor (ER) reporter assay and *ex vivo* rainbow trout liver slices. Active PFAS were found to be those with 10.3 to 13.39 angstroms distance between hydroxyl groups suggesting diol chain lengths confer estrogenic activity which relates to their affinity for the ligand binding domain. These data provide insights into the narrow range of PFAS structures capable of eliciting estrogenic activity in humans and fish. The contents of this abstract neither constitute nor necessarily reflect US EPA policy.

PS 4463 Xenometabolome Profiling of Structurally Diverse PFAS in Rainbow Trout (*Oncorhynchus mykiss*) Liver Slices

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Background and Purpose: Per- and polyfluoroalkyl substances, or PFAS, are manmade chemicals that are now widely distributed throughout the environment from human usage. Many PFAS are resistant to biodegradation leading to concerns over sublethal toxicity. Fish are known to bioaccumulate PFAS and the ability of the fish to detoxify potential reactive species via Phase I and II metabolism is an ongoing research area. **Methods:** This study investigated the xenometabolome of liver slice media exposed to three PFAS chemicals differing in chain length and structure including FC8-DOD (1H,1H,8H,8H-Perfluoro-3,6-dioxaoctane-1,8-diol), FC9-Diol (1H,1H,9H,9H-perfluorodecane-1,9-diol), and FC10-Diol (1H,1H,10H,10H-perfluorodecane-1,10-diol), with the study PFAS compounds shown to have estrogenic effects. Liver slices derived from immature male rainbow trout were incubated with six doses of PFAS treated exposure media for 48 h before the liver slice media was collected and non-targeted mass spectrometry analysis was performed to profile the biological transformation products (bioTPs) from PFAS metabolism (xenometabolome). **Results:** The main enzymatic pathways detected to be involved in biotransformation of the three PFAS were alcohol oxidation and glucuronidation. At comparable nominal concentrations, FC9-Diol and FC10-Diol had greater summed peak area of bioTPs compared to the precursor analyte reflecting a potential greater capability to detoxify and differences in estrogenicity relative to FC8-DOD. **Conclusions:** Differences in chain length and structure could have implications regarding the ability to detoxify PFAS chemicals and the respective toxicological response. Future research efforts will use molecular docking of the PFAS diols and their bioTPs to understand binding affinity to estrogen receptors.

PS 4464 Evaluation of Aquatic Toxicity of Phototransformed Hexanitrohexaazaisowurtzitane

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Background and Purpose: Energetics and munitions are used in nearly every aspect of military operations and have potential to be released both directly and indirectly into the environment through production, training, use, and disposal; poten-

tially affecting warfighters and civilians by drinking water, and crop contamination. Hexanitrohexaazaisowurtzitane commonly known as CL-20, is known as one of the most powerful non-nuclear compounds created. However, assessing the toxicity risk to the warfighter and the environment prior to fielding is imperative prior to its adoption as a commonly used energetic material at home and abroad. While degradation of CL-20 with environmental exposure was confirmed in a previous study using *Ceriodaphnia dubia* exposed to CL-20, using aged and weathered Sassafras Sandy Loam soil; it resulted in limited data and variable findings. Therefore, we investigated the hypothesis that CL-20 toxicity to *D. magna* increased after radiant sunlight exposure. **Methods:** Daphnia neonates <24 hours old, were obtained by EA Engineering in Hunt Valley, MD. The experimental design was validated by using potassium chloride (KCl) according to OECD TG202. The published KCl EC₅₀ is 340.0 mg/L and our observed KCl EC₅₀ = 324.0 mg/L. Using a stock concentration of 1mg/L, CL-20 was irradiated using the Atlas Suntest CPS+ photoreactor to simulate the natural UV/VIS irradiation at 16.203 MJ/m² representing sun exposure during an average 24H period in Bagdad, Iraq. Eight nominal concentrations were made following the exposure ranging from 1.0mg/L to 0.001mg/L. Daphnia were exposed, kept in the dark at room temperature, and immobilized daphnia were recorded at 24 and 48 hours. **Results:** 24H EC₅₀ of UV(-) CL-20 was determined to be 0.001 mg/L and UV(+) is 0.12 mg/L, 48H exposure data demonstrated increased variability in mortality rates. Water quality before and after UV exposure was also monitored; however, little change was observed in salinity, pH, and total nitrates, nitrites, ammonia, and formic acid; though, we noted some control death in a previous round with irradiated water. **Conclusions:** A decrease in toxicity of CL-20 after UV radiation was observed, demonstrating that CL-20 does breakdown in the presence of UV exposure. A repeated study should be conducted on daphnia cultures cultured in house, instead of obtaining them from external sources. This will allow us to troubleshoot the control mortality we observed in previous exposures, and allow us to increase our sample size and modify our concentration range. This study was run in conjunction with other *in vivo* tests consisting of acute (one dose), 14-day repeated dose, and 90-day repeated dose exposure studies to assess toxicity.

PS 4465 The drivers of chemosensitization: naturally-occurring marine microbial organohalogenes are substrates of the drug transporter ABCB1

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Background and Purpose: The drug transporter ABCB1, highly expressed at barrier tissues and conserved across species, is well studied for its role in limiting the accumulation and toxicity of xenobiotics. Although ABCB1 effluxes many toxic compounds, it is ineffective against certain persistent organic pollutants (POPs) such as PBDE-100 and DDT, which instead bind to and inhibit the transporter. These are termed Transporter Interfering Compounds (TICs), and the inhibition the cause results in chemosensitization: the heightened toxicity of additional compounds that are normally effluxed. Importantly, inhibition alone is not toxic; toxicity emerges only when ABCB1 substrates are present concurrently with TICs. However, the natural substrates of ABCB1 in marine environments—the compounds that may drive toxicity during chemosensitization—remain unknown. We propose that naturally occurring marine microbial organohalogenes (MOHs) are substrates of ABCB1, based on: (1) high drug transporter expression in marine invertebrates inhabiting microbially rich sediments; (2) heightened immune response in ABCB1-knockout (KO) sea urchin larvae challenged with bacteria; and (3) structural similarity between microbial MOHs and synthetic organohalogenes known to bind ABCB1. Using the sea urchin as a model organism, we sought to determine if ABCB1 protects marine embryos from MOH toxicity by limiting their intracellular accumulation. **Methods:** We developed a high-content imaging pipeline using sea urchin embryos, enabling large-scale dosing, imaging, and phenotyping of thousands of individuals. Using a recently established ABCB1-KO line, which exhibits reduced efflux and heightened sensitivity to transporter substrates, we screened 68 purified MOHs produced by Pacific marine bacteria (*Streptomyces*, *Pseudoalteromonas*, *Hormosilla*, and others). A point screen at 2 μ M identified compounds causing developmental toxicity; hits showing >45% abnormal development were subjected to full dose-response assays in both wildtype (WT) and KO backgrounds. EC₅₀ values were compared to determine ABCB1-dependent protection. Embryos were also harvested for LC-MS analysis of intracellular compound accumulation. **Results:** Sixteen of the 68 compounds tested induced >45% abnormal development. These included three pyrroles, one bipyrrrole, nine phenyl pyrroles, one indole, and two hydroxylated PBDEs. Five have known bioactivities (cytotoxicity, antimicrobial activity, thyroid disruption, and mutagenesis). All 16 hits were more potent to ABCB1-KO embryos than WTs. WT EC₅₀ values ranged from 0.08-6.4 μ M, whereas KO EC₅₀ values ranged from 0.02-4.1 μ M. On average, KO embryos were 3.3X more sensitive, with maximum potency increase of 5.3X. Compounds showing the greatest potency increase had log Kow values of 3-4, consistent with other strong ABCB1 binders. Preliminary LC-MS data indicate KO embryos accumulate roughly twice as much of certain compounds as WT embryos. **Conclusions:** We identified 16 naturally occurring MOHs as toxic substrates of ABCB1 in a marine organism, the sea urchin. This fills a long-standing knowledge gap in chemosensitization: as TICs and their binding interactions with ABCB1 are increasingly characterized, the environmental substrates whose toxicity is amplified by transporter inhibition have remained unclear. For instance, both synthetic

and naturally-occurring organohalogenes bioaccumulate in marine organisms and are transferred to humans via consumption of contaminated seafood. While drug transporters may be effective at limiting accumulation of microbial MOHs in low-pollutant conditions, co-exposure with TICs could increase their tissue burden by reducing efflux activity. The consequences of such mixture interactions on human health and development are unknown. Identifying which compounds may be of concern, as we have done here, is a crucial step forward for predicting, understanding, and mitigating the toxic effects of chemosensitization.

PS 4466 Integrated Spatial Transcriptomic, Behavioral, and Immunophenotypic Analyses Reveal Heat Wave-Driven Neurocognitive and Neuroinflammatory Vulnerabilities in Aging

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Background and Purpose: Climate change-associated heat waves are an escalating environmental stressor and represent an under-recognized form of environmental neurotoxicity with disproportionate impact on the elderly. While both aging and acute heat stress impair brain function, their combined effects on cognition and neuroimmune processes remain poorly understood. Young brains may mount adaptive stress programs, whereas aged brains are hypothesized to exhibit maladaptive inflammatory cascades and accelerated decline. We applied spatial transcriptomics, behavioral profiling, and immunophenotyping to map how heat waves differentially affect young and aged brains. **Methods:** Young (3 mo) and aged (18 mo) C57BL/6J mice (2x2 design) were exposed to periodic heat stress (40 °C, 3 h) for 14 consecutive days or maintained at thermoneutrality. Spatial transcriptomics (10x Xenium) was performed on prefrontal cortex, hippocampus, hypothalamus, and brainstem to resolve cell-type-specific transcriptional programs within intact tissue architecture. Cognitive and motor performance were assessed using Morris Water Maze (MWM; spatial memory), Novel Object Recognition (NOR; recognition memory), Rotarod (motor coordination), and Open Field (locomotion/anxiety). Multi-parameter flow cytometry quantified resident microglia (CD11b⁺CD45^{low}) and infiltrating myeloid cells (CD11b⁺CD45^{high}) to validate transcriptomic findings at the cellular level. **Results:** Spatial transcriptomics uniquely revealed hippocampal and hypothalamic microdomains in aged heat-stressed mice where proinflammatory transcripts co-localized with downregulation of synaptic plasticity markers (Bdnf, Camk2a), forming maladaptive inflammatory “hotspots.” These signatures were not that elevated in young cohorts, which instead showed transient upregulation of heat-shock genes without sustained inflammatory activation. Behaviorally, aged heat-stressed mice exhibited impaired spatial learning (MWM escape latency $\approx$ 52 $\pm$ 3 s vs. 38 $\pm$ 2 s in aged controls, p < 0.05), $\sim$ 20% lower NOR discrimination index (p < 0.05), and reduced center time in the Open Field ($\sim$ 15%, p < 0.05), whereas young mice demonstrated resilience across tasks but MWM. Flow cytometry confirmed $\sim$ 2-fold expansion of activated microglia and CD45^{high} infiltrating myeloid cells in aged heat-stressed brains (p < 0.05), with only modest activation in young mice. **Conclusions:** Preliminary findings indicate that acute heat stress elicits fundamentally different responses across the aging spectrum. Spatial transcriptomics provided exclusive resolution of region- and cell-type-specific vulnerabilities linking synaptic dysfunction with localized inflammatory hotspots that corresponded with behavioral decline in aged mice. Young brains mounted adaptive, reversible stress programs, while aged brains exhibited maladaptive immune activation and measurable cognitive impairments. By integrating spatial transcriptomics, behavioral outcomes, and immunophenotyping, this study establishes a mechanistic brain-behavior-immunity framework for environmental neurotoxicity. These results highlight aging as a critical modifier of climate-linked neurotoxicity and demonstrate the value of advanced multimodal approaches for predicting population vulnerability to heat waves. This study was supported by NIH/NIEHS Grant 1P01ES028942-01#Project 4 Toxicology awarded to Dr. Saurabh Chatterjee.

PS 4467 Air pollution and heat co-exposure induced cardiac dysfunction: Role of Nod-like Receptor X-1

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Background and Purpose: Extreme heat events are increasing in frequency, duration and severity. These increases are associated with raised air pollutant levels. Epidemiological data associate cardiovascular events as major drivers of increased mortality due to air pollution. While individually air pollution and heat stress are extensively studied, the mechanism behind the cardiac impact of their combined exposure are still largely unknown. NLRX1 is a mitochondrial protein involved in the cellular stress pathway. However, its involvement in air pollution and heat exposure co-exposure-induced pulmonary and cardiac dysfunction is still unknown. **Methods:** *Nlr1^{+/-}* and *Nlr1^{-/-}* mice were exposed to either Air (CTR), 2.5 mg/m³ ultrafine carbon

black (CB) with 1 ppm ozone (O₃), at room temperature or 102°F high temperature (HT). Exposure was performed by whole body inhalation for 3 hours and animals were euthanized 24 hours after exposure and lung inflammation was assessed by bronchoalveolar lavage cytology and inflammation-related gene and protein expression. Cardiac function was assessed immediately after exposure using ultrasound Doppler Echocardiography (Vevo F2 High Frequency Ultrasound Echocardiography). LV M-mode images were used to assess conventional echocardiographic assessment of short and long axis, while strain analysis was applied to B-mode videos captured in the parasternal short and long axes. Heart tissue mitochondrial function was assessed by mitochondrial complex activity assays and isolated mitochondria coupling assay. **Results:** Aerosol characterization by scanning and transmission electron microscopy demonstrated ultrafine size agglomerates. Stable aerosol concentration, temperature, and humidity were achieved and monitored by real-time software-assisted feedback loops. Combined heat and air pollution exposure induced greater lung inflammation in *Nlr1^{-/-}* mice compared to *Nlr1^{+/+}* mice. In contrast to *Nlr1^{+/+}* mice, no significant changes in stroke volume and cardiac output were observed in *Nlr1^{-/-}* mice after air pollution exposure and heat stress. In addition, *Nlr1^{-/-}* mice demonstrated a significant decrease in longitudinal time to the peak (TTP% %) strain which indicates a potential cardiac contractile issue after co-exposure to heat and air pollution. Moreover, *Nlr1^{-/-}* mice demonstrated a significant decrease in activity of mitochondrial complexes (III, IV, V) after HT+CB+O₃ co-exposure. The coupling assay further demonstrated a significantly impaired state 3 respiration in *Nlr1^{-/-}* mice. **Conclusions:** In conclusion, we demonstrate that air pollution exposure induces impaired cardiac functional physiological adaptation to heat in an NLRX1-dependent manner. **Funding:** Supported by NIH grants R01ES031253 (SH), R01ES03125304S1 (SH).

PS 4468 Effect of Heat Stress on Vascular Functions

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Background and Purpose: Thermal stress is an important environmental stressor which affects cardiovascular disease. Both hypothermia and hyperthermia have been suggested to increase the risk of atherosclerosis, the underlying cause of most cardiovascular disease. While the effect of hypothermia has been examined in experimental animals, no animal models have been established to rigorously test the plausibility that hyperthermia is sufficient to exacerbate cardiovascular disease and delineate the underlying cellular and molecular mechanisms. In this study, we established murine model of thermal stress-induced vascular function - the primary drivers of vascular inflammation and atherosclerosis. **Methods:** Young adult (12-20-week-old) male C57BL/6J mice were exposed to heat stress (37 °C, 3h/day, 4 days/week) for up to 4 weeks in temperature and humidity (40%) controlled Sable Systems cabinets. Mice maintained at 22 °C (room temperature) and 30 °C (thermoneutral conditions) in identical cabinets served as controls. Core body temperature was measured using SoHo implants and the data were analyzed by Ponemah-DSI software. For metabolic profiling, the mice were housed singly for 4days/week, 3h/day and the data were analyzed by microinterpreter & calR software. To examine the effect of heat stress on vascular functions we measured vascular permeability (Evans blue dye) (n=10/group), measured arterial stiffness (by ECHO cardiography) (n=10/group) and leukocyte trafficking (by multi-photon intravital microscopy) (n=10/group). **Results:** Core body temperature of mice maintained at thermoneutral conditions was comparable with the core body temperature of mice maintained at 22 °C (36 °C-37 °C). However, exposure to heat stress (37 °C) increased the core body temperature to 40 °C. The core body temperature in heat stress-exposed mice returned to normal within an hour of withdrawal of heat stress. Metabolic profiling showed significant increase in total food (32.24 kcal) & total water (6.8 mL) intake of heat stressed mice than the control group (18.95 kcal & 4.8 mL) (P < 0.01). But no significant change in body weight was observed. The heat-stressed mice also showed decrease in oxygen consumption (78.96 mL/h) and carbon dioxide expulsion (70.92 mL/h) than the control group (P < 0.01). Mice subjected to 37 °C also showed significant decrease in locomotor activity (beam breaks/h) and pedestrian locomotion (m). Vascular permeability was also compromised with heat stress, and we observed a decrease in permeability by 42% (P < 0.01). Heat stress also increased leukocyte adhesion. We observed a significant increase in adhering cells (3.07 cells/2 min) and rolling cells (2.5 cells/2 min) (P < 0.0001) than the mice subjected to 22 °C (room temperature) (0.27 cells/2 min) & (0.5 cells/2 min) respectively. There was no significant change in arterial stiffness. **Conclusions:** These studies established a well characterized murine model of heat stress-induced vascular dysfunctions.

PS 4469 Heat Stress and Perfluorooctanoic Acid impacts Endometrial Function in post-pubertal gilts: Implications for Reproductive Toxicology

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Background and Purpose: Heat stress (HS) and chemical exposure alter physiological homeostasis and can negatively impact reproductive performance, impair chemical biotransformation, and increase health risks. Heat stress occurs when

the environmental temperature surpasses the core body temperature of the organism, resulting in physiological and metabolic disturbances. Perfluorooctanoic acid (PFOA) impairs fertility and induces endocrine disruption. This study aimed to understand the individual and combined effects of PFOA exposure and HS on endometrial health and functioning in post-pubertal gilts. **Methods:** Pigs (n = 48) were housed in thermal neutral (TN; 20 °C) or heat stress (HS; 26.6 to 32.2 °C) conditions, synchronized in estrus with MATRIX® and fed either unadulterated cookie dough or cookie dough containing 70 mg/Kg of PFOA. Thus, there were four treatment groups: TN control (TC); TN PFOA (TP); HS control (HC), and HS PFOA (HP), with treatments lasting for 19 d. The uterus was weighed at euthanasia and endometrial samples collected. Isolated protein was analyzed by LC-MS/MS coupled with bioinformatic analysis. Fold-changes were identified between pre-planned contrasts with significance considered at P < 0.05. **Results:** Neither PFOA nor HS altered uterine weight. Compared to TC, PFOA exposure altered 69 proteins, with 36 proteins increased and 33 decreased. HS alone increased 59 proteins and decreased 90 compared to TC gilts, resulting in a total of 149 proteins altered. In the HS group, PFOA exposure increased the abundance of 78 proteins and decreased 33 proteins, resulting in a total of 111 proteins altered. In the groups exposed to PFOA, HS impacted 114 proteins, with 54 increased and 60 decreased. Exposure to PFOA during HS decreased the expression of 37 proteins and increased the expression of 40 proteins, compared to TC. PFOA altered six identical proteins in varying directions and extent between TC vs TP and HC vs HP gilts. Similarly, HS altered fourteen identical proteins between TC vs HC and TP vs HP. Metascape analysis identified biological pathways affected, which included reproduction and homeostatic processes. **Conclusions:** This study reveals that the endometrial proteome of post-pubertal gilts is altered by PFOA exposure, both basally state and in combination with HS, potentially contributing to endometrial dysfunction.

PS 4470 Perfluorooctanoic acid exposure differentially affects the follicular fluid proteome in post-pubertal gilts during thermal neutral and heat stress conditions

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Background and Purpose: Environmental stressors, such as heat and chemical exposures, can adversely affect female reproduction. Perfluorooctanoic acid (PFOA), a long-lasting pollutant, is linked to reproductive toxicity, including reduced fertility and endocrine disruption. Heat stress (HS), which occurs when environmental temperature exceeds core body temperature, impairs reproductive function in mammals by disrupting gamete development, oocyte maturation, and early embryonic survival. To explore the individual and combined effects of HS and PFOA exposure, this study investigated the hypothesis that PFOA would alter the follicular fluid proteome of post-pubertal gilts, with differential impacts observed under thermal neutral (TN) and HS conditions. **Methods:** Pigs (n = 48) were estrus synchronized and experienced TN (20 °C) or HS (26.6 to 32.2 °C) conditions during which they were exposed to cookie dough ± 70 ng/kg PFOA daily for 19 d. Thus, there were four treatment groups: TN control (TC); TN PFOA (TP); HS control (HC), and HS PFOA (HP). Follicular fluid was collected on d 20 post-euthanasia and analyzed by LC-MS/MS with bioinformatic analysis. **Results:** In the follicular fluid, 221 proteins were identified. When compared to TC pigs, PFOA exposure altered the abundance of one protein (P < 0.1). In HC gilts, 22 proteins differed in abundance (P < 0.1) when compared to TC gilts. Of these, two were increased and 11 were decreased at P < 0.05, and three were increased and six were decreased at P < 0.1. The follicular fluid of HP gilts had two proteins increased and six proteins decreased at P < 0.05, while an additional two proteins were increased and six proteins were decreased at P < 0.1. String gene ontology analysis of TC vs HC identified 17 biological pathways associated with immune and inflammatory response, stress response, and metabolic processes, with a protein-protein interaction network comprising 15 nodes and 17 edges. In the TC vs HP gilt follicular fluid proteome, 16 biological pathways associated with translation and gene expression were identified, comprising 15 nodes and 18 edges. **Conclusions:** This study identified the follicular fluid proteome of post-pubertal gilts and examined how PFOA affects protein levels, both with and without HS. The results indicated that PFOA exposure could lead to or contribute to metabolic disorders and disrupt the ovarian steroidogenic pathway. HS may impair immune and inflammatory responses, alter structural and cellular components, and contribute to metabolic disorders. Additionally, the data suggest that PFOA exposure in combination with HS might compromise the immune and inflammatory response, alter cellular and structural components, and interfere with the coagulation and transport systems.

PS 4471 Fresh water salinization exposure heightens risk of poor Vibriosis outcomes and systemic inflammation

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Background and Purpose: Freshwater salinization and subsequent human exposure via drinking water is a global health concern, yet its role in modulating susceptibility to bacterial infections is unknown. *Vibrio vulnificus* is a halophilic pathogen that

causes severe septicemia, especially in individuals with underlying hepatic dysfunction. This study investigated whether chronic saltwater exposure alters gut microbiota composition, systemic inflammation, and liver injury in mice challenged with *V. vulnificus*. **Methods:** Male C57BL/6 mice were divided into four groups: control (regular water), salt-water treated (1.2 g NaCl/L), *V. vulnificus* infection (10^8 CFU, oral gavage), and combined salt-water plus *V. vulnificus* exposure. Salt-water treatment continued for 4 weeks prior to bacterial challenge. Mice were euthanized 24 hours post-infection. Serum samples were analyzed for endotoxin, proinflammatory cytokines (IL-1 β , IL-6, TNF- α), C-reactive protein (CRP), and hepatic enzymes (ALT, AST). Metagenomic sequencing of fecal DNA was performed to evaluate microbial diversity, taxa abundance, and antimicrobial resistance gene profiles. **Results:** Chronic salt-water exposure alone did not elevate serum endotoxin or cytokine levels compared to controls. In contrast, *V. vulnificus* infection markedly increased serum endotoxin ($p < 0.001$), IL-1 β , IL-6, and TNF- α (all $p < 0.001$), and CRP ($p < 0.001$), confirming systemic inflammation. The combination of salt-water and *V. vulnificus* induced synergistic cytokine elevation (IL-1 β and TNF- α , $p < 0.001$) and a modest but reproducible increase in CRP ($p = 0.041$), suggesting a heightened inflammatory response. Liver enzyme analysis revealed significant AST elevation in infected and salt-water + infection groups ($p < 0.001$), whereas ALT remained unchanged, indicating hepatic stress without overt necrosis. Metagenomic profiling showed reduced α -diversity in salt-water + infection mice, with notable enrichment of *Clostridioides difficile* and *Staphylococcus xylosus*, taxa associated with dysbiosis and intestinal inflammation. Antimicrobial resistance gene abundance did not differ significantly among groups. **Conclusions:** Prolonged salt-water exposure sensitizes the host to enhanced inflammatory and microbial responses following *V. vulnificus* infection, accompanied by altered gut microbiome composition. These findings highlight a potential mechanistic link between high-salt consumption, gut-liver axis perturbation, and increased susceptibility to enteric pathogens. Understanding how environmental and dietary salt interact with pathogenic bacteria could inform risk mitigation strategies for coastal populations and individuals with compromised hepatic or gut barrier function.

PS 4472 Metabolic Stress Potentiates Renal Maladaptive Repair Following *Vibrio vulnificus* Sepsis: Implications for a Warming Climate

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Background and Purpose: In the United States, cases of non-cholera *Vibrio* infections are projected to increase by 50 % by 2090 relative to 1995 due to rising sea surface temperatures—underscoring how climate change is intensifying exposure to pathogens such as *Vibrio vulnificus* (VV). Sepsis caused by VV is a growing public health concern, particularly for individuals with underlying metabolic disease. Acute kidney injury (AKI) caused by sepsis is a major driver of chronic kidney disease (CKD) when maladaptive repair leads to persistent inflammation and fibrosis. High-fat diet (HFD) consumption induces metabolic dysfunction, mitochondrial stress, and inflammation, all of which heighten renal vulnerability. This study investigated how HFD-driven metabolic stress influences tubular epithelial maladaptive repair and fibrotic progression following sepsis-induced AKI using a clinically relevant VV infection model. **Methods:** Male C57BL/6J mice ($n=60$) were assigned to chow or HFD (60% kcal fat). At 18 weeks, mice received either VV intragastric infection or vehicle, generating four groups: CHOW, HFD, VV, and HFD+VV. Mice were sacrificed at 24 hours, 30 days, and 90 days post-infection. Serum creatinine was measured, and renal structural injury, tubular degeneration, inflammation, and fibrosis were quantified using Masson's Trichrome staining. **Results:** At 24 hours, serum creatinine was significantly elevated in HFD+VV versus HFD and VV, demonstrating parallel metabolic and infectious stress. Tubular injury increased across groups, with HFD+VV showing extensive tubular dilation, epithelial vacuolization, dense inflammatory infiltrates, and early peritubular fibrosis. Glomerular injury peaked in HFD+VV. At 30 days, HFD+VV mice retained the highest structural injury and fibrosis, without differences in serum creatinine values. By 90 days, creatinine levels again differed significantly between VV and HFD+VV, indicating recurrent or persistent injury. Cortical fibrosis remained highest in HFD+VV, with sustained glomerular damage in VV and HFD+VV groups. **Conclusions:** HFD-induced metabolic dysfunction markedly exacerbates renal injury, prolongs maladaptive tubular repair, and accelerates fibrotic progression following VV sepsis. These findings highlight a critical interaction between metabolic stress and a climate change-driven emerging infectious threat. Understanding these mechanisms provides crucial insight for identifying vulnerable populations and designing interventions to mitigate long-term renal consequences in a changing climate.

PS 4473 Enhancing Disaster Preparedness Through Inclusive Science Communication and Early Warning Systems

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Background and Purpose: Effective science communication is crucial for bridging the gap between research and its societal impact, particularly in disaster preparedness and risk management. This study explores risk perception, protective actions, and the role of early warning systems during the July 18-19, 2023, flash floods in Mayfield, Kentucky. **Methods:** Using a mixed-methods approach, which included

interviews and surveys, we analyzed how residents responded to warnings and the factors that influenced their actions. **Results:** We found that traditional media sources, such as local television and the Weather Channel, were the most relied upon for information during the event. However, many residents received warnings only shortly before the flood, limiting their preparedness. Risk perception and access to diverse information channels significantly influenced protective actions. At the same time, demographic factors, such as ethnicity, revealed disparities in response behaviors, with Hispanic or Latino residents less likely to take protective measures. **Conclusions:** These findings underscore the urgent need for inclusive, timely, and targeted communication strategies to enhance disaster preparedness across diverse communities. We propose integrating localized social media alerts and culturally tailored messaging into existing frameworks to improve communication effectiveness. This research informs disaster preparedness strategies and provides a scalable framework for science communication across various fields, including public health and environmental risk management. By engaging diverse stakeholders and leveraging modern digital tools, this study highlights the critical role of tailored communication in fostering societal resilience and driving equitable policy change.

PS 4474 ECHA Writes Back: The Reasons for Rejection of Read-Across in Compliance Check Decisions by the European Chemicals Agency

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Background and Purpose: As part of the European Union's REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) regulation, the European Chemicals Agency (ECHA) is required to assess the completeness of data submitted by registrants to demonstrate the safety of their substances. By law, ECHA must examine at least 20% of registration dossiers through a process known as compliance checks (CCHs). If a dossier is found to be non-compliant with REACH requirements, a formal decision is published. Common reasons for non-compliance include missing required information or insufficient justification for adaptations to standard information requirements (e.g., read-across, weight-of-evidence approaches). **Methods:** Our prior review of CCH decisions published between 2010 and 2023 identified 2,313 issued decisions. Of these, 48% ($n = 1,111$) included read-across hypotheses for at least one endpoint, and among those, 93% contained read-across adaptations deemed inadequate. In this study, we systematically evaluated and extracted information on all read-across hypotheses that ECHA found unacceptable for fulfilling standard information requirements, including extension of the dataset to the decisions published in 2024 and 2025. For each case, we recorded the adapted endpoint, source substance(s), read-across type, and the scientific rationale supporting the proposed adaptation. Information related to the proposed read-across rationales was categorized using 17 assessment elements that represent key components of read-across hypotheses, including structural, toxicokinetic, and toxicodynamic similarities. **Results:** These 17 elements were used to identify the reasons for ECHA's rejection of the proposed read-across and to analyze associations between hypothesis characteristics and CCH outcomes. Our analysis revealed that inadequate read-across adaptations often lacked supporting data demonstrating toxicokinetic and toxicodynamic similarity. Furthermore, we identified specific elements within the read-across hypotheses that could be strengthened by incorporating data from New Approach Methods (NAMs) to reduce uncertainty. **Conclusions:** Overall, this systematic evaluation provides critical insights into the reasons why ECHA deems certain read-across adaptations inadequate. The findings highlight opportunities to enhance confidence in read-across approaches and to design scientifically robust adaptations that can better withstand regulatory scrutiny.

PS 4475 New EU Endocrine Disruptor Hazard Classes: Classification Criteria, Testing Requirements, and Industry Implications

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Background and Purpose: The European Union has introduced new hazard classes for endocrine disruptors in the Classification, Labelling and Packaging (CLP) Regulation, establishing the first dedicated classification system for these substances. The European Commission's Delegated Regulation (EU) 2023/707 mandates compliance from May 1, 2025, for substances and May 1, 2026, for mixtures. This framework introduces two distinct hazard classes: endocrine disruption for human health (ED HH) and endocrine disruption for the environment (ED ENV), each subdivided into Category 1 (known or presumed endocrine disruptors) and Category 2 (suspected endocrine disruptors). Previously, endocrine disruptors were regulated indirectly through other hazard categories, such as reproductive toxicity or aquatic toxicity, creating regulatory gaps. This presentation describes the classification system, assessment criteria, critical testing endpoints, and implications for industry and regulators. **Methods:** This presentation analyses the new ED classification framework based on EU guidance, CLP amendments, and ECHA criteria guidance published in late 2024. The classification system operates on a weight-of-evidence approach requiring demonstration of three key criteria: an adverse effect in an intact organism or its progeny, an endocrine mode of action, and

a biologically plausible link between the two. The presentation outlines Category 1 versus Category 2 decision logic and testing strategies for estrogenic, androgenic, thyroid, and steroidogenic (EAST) modalities, including *in vitro* screening assays and *in vivo* confirmatory studies. The integration of New Approach Methodologies, including high-throughput screening, adverse outcome pathways, and computational predictions alongside traditional studies, is examined. Case studies demonstrate how data packages are evaluated and classification decisions evolve based on evidence quality and completeness. **Results:** ED classification requires specific criteria based on evidence strength, mechanistic understanding, and EAST modality. Category 1 demands convincing evidence from human or robust animal data demonstrating adverse effects linked to endocrine mechanisms; Category 2 applies when evidence suggests but doesn't confirm endocrine disruption. Classification under the new CLP ED hazard classes triggers regulatory consequences under REACH, including potential inclusion in the Candidate List of Substances of Very High Concern (SVHC), inclusion on the Candidate List for authorisation, Annex XIV (requiring authorisation for use), or Annex XVII restrictions (imposing use bans or limitations), and enhanced risk management requirements in chemical safety assessments and Safety Data Sheets. Critical endpoints include reproductive organ weights and histopathology, hormone measurements, developmental effects, thyroid parameters, sexual maturation effects, and transgenerational effects. Data gap analysis reveals that many REACH registrations lack endocrine-specific endpoints, requiring additional information to conclude the ED classification. Industry implications include mandatory portfolio screening, potential triggering of REACH authorisation or restriction procedures, reformulation requirements for classified substances in consumer products, and supply chain communication obligations through updated Safety Data Sheets. **Conclusions:** The new ED hazard classes establish a science-based framework for identifying endocrine-disrupting substances, closing critical regulatory gaps. Implementation requires industry engagement through systematic evaluation, high-quality data generation, transparent reporting, and participation in harmonised classification proposals. These classifications drive prioritisation across EU regulations, including REACH, cosmetic and food contact materials regulations to enhance chemical safety protection.

PS 4476 Enhancing Chemical Hazard Assessment in California's Safer Consumer Products Program: Translating Toxicological Data into Hazard Scores and Addressing Challenges with Alternatives Analysis

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Background and Purpose: California's Safer Consumer Products (SCP) Program uses a hazard-based approach to evaluate chemicals, rather than traditional risk assessment. Our science-driven process identifies specific consumer products that contain potentially harmful chemicals. Manufacturers who produce these specific product-chemical combinations, or Priority Products, perform a comprehensive Alternatives Analysis (AA) to evaluate potential alternatives to avoid regrettable substitutions. An integral part of the AA process is the chemical hazard assessment (CHA), which involves evaluating chemicals to determine their potential to cause harm to human health and the environment. However, challenges have emerged in these manufacturer-led CHAs, prompting the exploration of new approaches to improve the quality and consistency of CHAs for the benefit of product manufacturers, regulators, and consumers. **Methods:** We reviewed AAs submitted by manufacturers and evaluated their comparison of alternatives, with particular focus on: (1) CHA methodologies and scoring systems, (2) approaches to translate diverse toxicological data into hazard benchmarks, and (3) strategies for addressing incomplete data. **Results:** While manufacturers may employ a range of different CHA methods and criteria for benchmarking, many fail to address all the hazard endpoints required by SCP regulations. Manufacturers use the results of their CHA to weigh trade-offs and identify an alternative to replace the harmful chemical in their product. However, we have encountered several challenges, including variability in the quality of submitted CHAs, limited industry expertise in alternatives assessment and hazard evaluation, inconsistent translation of toxicological data into comparable hazard scores, lack of tools to consider transformation products, different approaches to handling data gaps, and limited methodologies to assess the hazards of mixtures and formulations. Collectively, these challenges can increase the potential for regrettable substitutions, because the quality of the CHA may not sufficiently support a manufacturer in weighing trade-offs. **Conclusions:** SCP is exploring several initiatives designed to strengthen CHA quality, reduce resource disparities across manufacturers, and facilitate informed substitution. These include updating our AA guide to provide clearer guidance to manufacturers performing CHAs, increasing earlier engagement with manufacturers to better understand performance needs and increase awareness of SCP's AA process and requirements, and the creation of an AA database that publicly lists potential alternatives with hazard assessments completed by product manufacturers. SCP is also exploring a collaborative AA approach, in which the program would lead elements such as the CHA, but meaningfully engage with and receive targeted information from industry for a given Priority Product. These steps could help reduce disparities between manufacturers while improving consistency and scientific rigor. We welcome input to continue to refine these concepts and ensure they are scientifically sound and implementable.

PS 4477 California Cannabis Consumption Survey - a Foundation for Health-Based Pesticide Limits for Edible Cannabis

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Background and Purpose: Cannabis remains a federally illegal crop in the United States, and pesticide use on cannabis and pesticide residue on cannabis products cannot be regulated in the same way as other agricultural crops. This has left US states that have legalized cannabis to develop their own regulatory frameworks. In California, the Department of Pesticide Regulation (DPR) advises the Department of Cannabis Control on health-protective pesticide residue limits known as "action levels" for cannabis products, including edibles, beverages, and tinctures. A major challenge in establishing health-based action levels from pesticide residues in cannabis is the lack of both cannabis pesticide concentration data and cannabis product use and consumption data, both of which are essential for estimating pesticide exposure from cannabis use. When DPR initially developed its pesticide action level methodology, the department selected watermelon as a surrogate agricultural commodity because of its high consumption rates. This was the most health-protective strategy to account for the wide variety and consumption range of cannabis-infused edibles. However, this model lacked real-world relevance. To refine its methodology, DPR partnered with California State University, Sacramento (CSUS) to conduct the California Cannabis Consumption (C3) Survey in 2020 and 2023. The C3 Survey provides the first comprehensive dataset on cannabis consumption patterns in the California marketplace and supports the development of more accurate, health-based exposure scenarios. **Methods:** DPR and CSUS's Public Health Survey Research Program jointly developed the C3 Survey with the main objective of determining the quantity of commercial and legal cannabis products consumers use in the California marketplace for each type of manufactured cannabis product. The 31-page survey was administered in both pilot and full cohort phases via in-person interviews. The survey was conducted in 2020 and 2023 throughout California, including in the Los Angeles/San Diego area, Central Valley, greater Sacramento area, and the North Coast. The survey tool was designed to ensure representative sampling of California legal cannabis consumers over 18 years old. **Results:** The C3 survey included 381 participants who averaged 38.8 years old (range 18-95 years old), with 54% male and 45% female respondents. Most participants self-identified as White, non-Hispanic (46%) or Hispanic/Latino (32%) and they reported using an average of 2.7 cannabis product types in the past 28 days. Flower was the most commonly used product (324 respondents), followed by vapes (220), edibles (148), and resin (121). Fewer participants reported using topicals, beverages, or liquid concentrates. Among non-inhalable products, significant differences in consumption rates were observed. Notably, cannabis-infused beverages (e.g., teas, sodas) were consumed at rates approximately 14 times higher than other edible products (e.g., gummies, cookies, chocolates, mints, and liquid concentrates). This consumption rate reflects total product volume or mass, not delta-9-tetrahydrocannabinol (THC) content. **Conclusions:** California is the first state to develop a comprehensive methodology for the health-based evaluation of pesticide residues in cannabis products. DPR's methodology initially used an agricultural surrogate for cannabis consumption data. However, with the completion of the C3 Survey, DPR has now refined its methodology for deriving health-based action levels of pesticides in cannabis based on upper-bound (95-99th percentile) consumption data for various cannabis products. This framework is grounded in toxicology, real-world consumption data, and the best regulatory practices, and could be applied by regulatory agencies seeking to assess the health impact of pesticide residues on hemp products.

PS 4478 Developing Human Health Reference Levels for Pesticide Residues in Groundwater

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Background and Purpose: In accordance with California's Pesticide Contamination Prevention Act (PCPA), the Department of Pesticide Regulation (DPR) established pesticide detection and response processes to prevent potential contamination of groundwater from legal agricultural use of pesticides. Detection of pesticides in groundwater following routine monitoring may trigger a request to DPR's Human Health Assessment (HHA) Branch for a human health risk evaluation. These evaluations are fit-for-purpose formal reviews that determine if the pesticide was detected after legal agricultural use and, if so, whether uses can continue with or without added restrictions. HHA evaluations establish one or more Groundwater Human Health Reference Levels (HHRLs) for screening pesticides and any relevant pesticide degradates in groundwater. **Methods:** An HHRL is the threshold pesticide and/or pesticide degrade concentration for a maximum water intake that results in the maximum safe oral exposure. A pesticide and its degradate(s) may be covered by the same HHRL when they are considered to be toxicologically equivalent and their residue concentrations summed for evaluation. DPR uses established regulatory reference doses (RfDs) and high-end drinking water intakes (DWIs) to calculate HHRLs. Pesticide concentrations in groundwater at or below the HHRL are considered to have no significant health concern. Detections above the HHRL may indicate potential pollution of groundwater as defined by PCPA. **Results:** Unique HHRLs have been used to screen residues of the parent pesticide as well as chemically

related pesticides and degradates with equivalent toxicity. As of November 2025, 19 Groundwater HHRLs have been established for pesticides and related degrade species. **Conclusions:** DPR HHRLs are based on health-protective and scientifically supportable assumptions and are specific for evaluations of pesticides in groundwater. They provide context for interpreting the potential human health impact of groundwater pesticide monitoring data.

PS 4479 Setting Maximum Levels for PFAS in Farm Products

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Background and Purpose: The State of Maine has been testing soil, water, and farm products for per- and polyfluoroalkyl substances (PFAS) at properties with records of past sewage sludge spreading. This testing has identified PFAS contamination of a number of products, including milk, beef, eggs, and certain vegetables. The Maine Center for Disease Control and Prevention (Maine CDC) developed perfluorooctane sulfonic acid (PFOS) action levels for milk in 2017, beef in 2020, and eggs in 2025 for the state's Department of Agriculture, Conservation and Forestry (DACF) to identify when a product would be considered adulterated and no longer fit to be sold by a producer. In 2025, the state legislature formally established the DACF PFAS Response Program and authorized the state to set maximum levels for PFAS in farm products. As part of expected DACF rulemaking, Maine CDC is updating existing maximum levels for PFOS in milk, beef, and eggs, and developing additional maximum levels for PFOS in other farm products produced in Maine. **Methods:** We calculated product consumption rates in g/kg-day using 2015-2023 National Health and Nutrition Examination Survey (NHANES) dietary intake data. Survey foods were mapped to individual farm products using the Food and Drug Administration's (FDA) Food Disaggregation Database. Consumption rates were calculated as two-day means for individuals who completed dietary surveys on two non-consecutive days and reported product consumption on at least one day. As-consumed product quantities were converted to raw, edible forms using adjustment factors calculated from composition data reported on the United States Department of Agriculture's Food Data Central website. We estimated mean and 90th percentile consumption rates for child subpopulations recommended by the FDA using the "survey" package in R to account for the complex NHANES survey design. We calculated maximum levels for PFOS as the toxicity value divided by the consumption rates for 14 farm products. We used PFOS toxicity values published by the Agency for Toxic Substances and Disease Registry (ATSDR) and the United States Environmental Protection Agency (USEPA) and compared the results to maximum limits set by the European Commission in 2023 and to current laboratory reporting limits. **Results:** Of the 14 farm products, 90th percentile consumption rates calculated from NHANES for children aged 0 to 6 years were highest for fluid milk (42.52g/kg-day) and lowest for lettuce (0.99g/kg-day). Using ATSDR's 2021 minimum risk level (MRL) of 2ng/kg-day for PFOS, the maximum level for fluid milk would be 0.05ng/g (48ng/L) and for other products would range from 0.2ng/g to 2.0ng/g. The maximum levels for PFOS in beef and eggs (both 0.5ng/g) would be similar to the maximum levels set by the European Commission for combined beef, pork, and poultry meats (0.3ng/g) and for eggs (1.0ng/g). Maximum levels calculated using USEPA's 2024 PFOS reference dose (RfD) of 0.1ng/kg-day would be 20-fold lower and well below current commercial lab reporting limits for PFOS. **Conclusions:** Maximum levels for PFAS in farm products have not been established at the federal level in the US. Maximum levels for PFOS in beef and eggs calculated using ATSDR's MRL and consumption rates from NHANES would be similar to levels set for these products by the European Commission. Using either ATSDR's MRL or USEPA's RfD as the toxicity value would result in PFOS maximum levels near or below current laboratory detection limits for many products.

PS 4480 Tracking the Persistence of Replaceable Animal Tests: The Replace Animal Tests (RAT) List as a Tool for Advancing Non-Animal Toxicology

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Background and Purpose: It is often assumed that animal tests for which non-animal replacements exist are no longer conducted, or at least rarely used. However, evidence shows that these tests continue—and in some cases even increase—long after scientifically valid alternatives have been established. The *Replace Animal Tests (RAT) List*, developed by Cruelty Free International, is a data-driven tool designed to systematically track regulatory animal tests that remain in use despite the availability of scientifically validated non-animal approaches. The tool currently monitors ten key tests across regions including Europe, North America, South America, and Asia. **Methods:** For each test, the list includes the estimated number of animals used annually, based on EU data, to show the scale of impact. It also tracks trends over time to see whether animal use is going up, down, or staying the same. A detailed description of the animal tests and the available non-animal methods is included. It sets out which sectors are driving the use of these tests, what guidelines or legislation require them, and in which regions. It tracks the acceptance status of the non-animal replacements across different regions. And finally, it highlights the key barriers to replacement — and some possible solutions. **Results:** The RAT list tests include rabbit pyrogen, skin and eye irritation, skin sensitisation, antibody production, and

batch potency and safety tests. Collectively, these ten tests result in an estimated 1.2 million animals being used annually in Europe, according to publicly available data from 2022. This persists even though the EU has legal requirements for the adoption of non-animal methods where feasible. In regions without such legal mandates, the extent of animal use is likely even higher. While most other regions do not yet have equivalent legal obligations, global momentum is building. Governments, regulators, and international harmonization bodies are developing strategies and roadmaps to reduce animal testing. The U.S. Food and Drug Administration's roadmap, for example, identifies several RAT List tests—such as shellfish toxin, leptospirosis potency, pyrogen, and skin irritation tests—as priority areas for reduction. **Conclusions:** By integrating regulatory data and expert input, the RAT List quantifies the persistence of outdated tests and identifies scientific, regulatory, and operational barriers impeding adoption of modern toxicological methods. The RAT List provides an evidence-based framework to help scientists, regulators, and policymakers prioritize areas for transition and advance global efforts toward predictive, human-relevant toxicology.

PS 4481 An Evaluation of the Science and Recent Regulatory Initiatives Regarding Formaldehyde

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Background and Purpose: For more than five decades, formaldehyde has been exhaustively studied and been the focus of numerous recent regulatory initiatives. Few chemicals have been examined with greater experimental rigor or generated more policy disagreement. The extensive toxicological and mechanistic data present a remarkably consistent picture, e.g., a concentration-dependent continuum of portal-of-entry effects with clear biological thresholds. Nonetheless, regulatory agencies have recently reached markedly different conclusions about acceptable exposure limits. This divergence reflects not a deficiency of data, but rather fundamental differences in the interpretation of evidence and the application of the precautionary principle. **Methods:** Relevant literature was identified through systematic searches of PubMed, Scopus, and Google Scholar (1960-2025) and supplemented by key technical assessments (CIIT, 1999; WHO, 2010; ECHA, 2012). Only peer-reviewed studies or authoritative reports with quantitative exposure-response, histopathologic, or mechanistic data were considered. Human, animal, and computational findings were integrated to characterize the relationships among concentration, exposure duration, and tissue response. The weight of evidence was used to describe the biological processes linking irritation, cytotoxicity, and tumorigenesis. **Results:** Controlled human chamber studies, chronic rodent bioassays, and computational dosimetry collectively demonstrate that formaldehyde's toxicity is confined to tissues of initial contact. Mild sensory irritation occurs at 0.3-0.5 ppm, reversible epithelial adaptation near 1 ppm, and cytotoxic injury with regenerative proliferation occurring above 6 ppm. Chronic inhalation studies in rats and mice confirmed that nasal tumors developed only under conditions of sustained epithelial injury and compensatory hyperplasia, findings consistent with a threshold-driven, local mode-of-action. Biologically based dose-response (BBDR) modeling utilizes the experimental DPX kinetics which continues to support that formaldehyde's effects arise from the local saturation of metabolic and binding capacities in the nasal epithelium, resulting in prolonged cytotoxicity and regenerative proliferation: a mechanism not influenced by cumulative systemic dose. Despite this mechanistic clarity, regulatory decisions have often emphasized epidemiologic correlations and default linear low-dose extrapolations. Today, occupational and environmental limits among different countries vary by more than an order of magnitude. The EPA's reliance on linearized cancer models for carcinogenesis contrasts sharply with threshold-based approaches adopted by ACGIH, ECHA, and several international agencies. **Conclusions:** The history of formaldehyde regulation highlights the persistent gap between the science of dose-response and the policy frameworks that govern its interpretation. Integrating mechanistic toxicology into risk assessments allows for an approach that 1) links inhaled concentration to local tissue dose, 2) differentiates adaptive from adverse responses, 3) appropriately weights controlled human and animal data, and 4) promotes regulatory consistency grounded in empirical evidence. Formaldehyde, therefore, stands as both a case study in inhalation toxicology and a lens through which to examine how abundant, well-characterized science can yield divergent regulatory outcomes when viewed through differing philosophical and statutory frameworks.

PS 4482 Impact of single-use plastics at intensive care units: Costs, toxicity, and environmental sustainability

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Background and Purpose: The extensive use of single-use plastics in Intensive Care Units (ICUs) presents a growing challenge in environmental, health, and economic terms. Although their use ensures asepsis and clinical efficiency, it generates large volumes of waste and high disposal costs, which can be up to ten times higher than those of household waste. In hospitals, plastic waste represents between 20% and 30% of total waste generated, with averages of up to 2 kg per bed per day. In 2023,

healthcare systems in Europe and North America faced costs exceeding 56 billion USD, and by 2040 these figures could surpass 76 billion if no mitigation strategies are implemented. In ICUs, the most commonly used plastic devices include catheters, IV bags, ventilator components, endotracheal tubes, dialysis systems, cannulas, connectors, and sterile packaging. Most are made of polyvinyl chloride (PVC), polyethylene (PE), polypropylene (PP), polycarbonate (PC), and polyurethane (PU). Among these, PVC and PC remain critical materials due to the potential presence of plasticizers such as di(2-ethylhexyl) phthalate (DEHP) and Bisphenol A (BPA), compounds with recognized endocrine-disrupting effects that may pose health risks to critically ill patients. In Colombia, ICU waste generation has been documented at up to 3.87 kg/bed/day, and circular-economy models have been proposed for the recovery of these materials; however, research on BPA or DEHP exposure in clinical settings remains limited. **Methods:** This review was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. Based on the PICO method (Population, Intervention, Comparison, Outcome), the following scientific question was formulated: Can circular-economy strategies generate successful models for the recovery and reuse of materials derived from single-use plastics in ICUs, in order to reduce environmental impact, decrease the consumption of high-value raw materials, improve energy recovery, and enhance sustainability? Databases such as Medline, Latin America & Iberia, and platforms including PubMed, ScienceDirect, Scopus, and Scielo were used, applying inclusion and exclusion criteria to publications from 2020 to 2024, reviewing hospitals worldwide. Critical appraisal and quality assessment of the included studies were performed using the Cochrane risk-of-bias tool. **Results:** This review will provide information to support circular-economy models that reintegrate single-use ICU plastics into the value chain. **Conclusions:** The information generated will allow the development of alternative strategies for circular-economy models.

PS 4483 EPA's Implementation of the Endocrine Disruptor Screening Program (EDSP) Near-Term Strategies

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Background and Purpose: The Federal Food, Drug, and Cosmetic Act (FFDCA) 408(p) required EPA to develop a screening program to determine whether certain substances (including pesticides) may have an effect in humans similar to an effect produced by "naturally occurring estrogen, or other such endocrine effects". As a result, the Agency established the EDSP in 1998, which includes human and wildlife testing for estrogen, androgen, and thyroid pathways using a two-tiered testing approach. Since then, EPA has faced several challenges with program implementation. In order to begin addressing these challenges and rebuilding the EDSP over the next several years, EPA is prioritizing screening of conventional pesticides in order to fulfill the Agency's obligations and commitments under FFDCA, which requires the EPA to screen for and protect against endocrine disrupting effects in humans. **Methods:** In 2023, EPA issued a Federal Register Notice describing the development of initial near-term strategies, which includes how existing data submitted to EPA can be used to evaluate endocrine systems. Under the EDSP near-term strategies, EPA is evaluating existing data obtained through Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) registration and registration review and other scientifically relevant information (OSRI) to determine if additional human health data are needed to inform FFDCA section 408(p) decisions. For example, endocrine-related data for numerous conventional pesticides are available through either two-generation reproduction toxicity or extended one-generation reproductive toxicity (EOGRT) studies that would eliminate the need for any further estrogen or androgen data. Additionally, EPA developed a framework to organize and prioritize the large number of conventional pesticides in registration review. "Group 1" cases demonstrated potential activity in ToxCast Pathway Models and were identified as highest priority for needing additional data. EPA committed to issuing data call-ins (DCIs) to obtain additional data for "Group 1" cases. For the remaining pesticides in "Group 2" or "Group 3", EPA committed to reevaluating the available data to determine what additional studies, if any, might be needed to confirm EPA's assessment of the potential for impacts to endocrine pathways in humans. Since the release of the EDSP near-term strategies, EPA has updated these groups for conventional pesticides based on public comments and additional information. **Results:** EPA has made progress in EDSP implementation since the release of the near-term strategies. Over 100 conventional pesticide cases were identified with existing reproductive toxicity studies performed under current guidelines that provide adequate estrogen and androgen data for humans. Additionally, EPA has completed FFDCA 408(p)(6)-related commitments and obligations for several new active ingredients and chemicals undergoing registration review. EPA has created a website to track the status of EDSP DCI Notices for "Group 1" chemicals. To date, out of the DCIs issued for approximately 30 "Group 1" chemicals, EPA has received responses for at least 21. EPA has begun evaluating these responses, which include waiver requests and/or data submissions, with a small number planning to generate new data. For "Group 2" and "Group 3" chemicals, EPA's Hazard and Science Policy Council (HASPOC) continues to consider the need for additional thyroid data when there is evidence of thyroid toxicity. Additionally, the Endocrine Disruptor Screening Policy Council (EDSPOC) was formed in 2022 to review hazard and exposure data and recommend whether additional endocrine-related data are needed. In fiscal year 2025, EDSPOC considered endocrine-related data needs or exemptions for over 10 chemicals and recommendations will be

incorporated into public documents to inform 408(p) EDSP decisions in the future. **Conclusions:** The near-term strategies that have been implemented thus far are the initial steps EPA is taking to generate momentum toward the longer-term goal of addressing its FFDCA 408(p) obligations and commitments. Use of the EDSP near-term strategies thus far has highlighted the utility and sufficiency of existing data to address data needs and decisions under FFDCA 408(p). EPA will continue to provide status updates through its tracking website and consider further improvements for implementation.

PS 4484 Network toxicology applied to titanium dioxide nanoparticles: implications for human health

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Background and Purpose: Titanium dioxide nanoparticles (TiO₂-NPs), widely used in consumer products, are emerging environmental contaminants. Although their ability to induce oxidative stress is known, the molecular mechanisms underlying their toxicity remain unclear. This study explored the immunotoxic mechanisms of TiO₂-NPs by analyzing transcriptomic changes in human macrophages. **Methods:** The study was based on a network toxicology approach that integrated a dual strategy for gene identification, construction of molecular interaction networks, functional characterization, and projection to pathological contexts. The analysis included transcriptomic data from human CD14⁺ macrophages exposed to TiO₂-NPs (GSE179543), processed using the limma package ($|\log_2FC| > 0.58$, FDR < 0.05), complemented by exhaustive mining of toxicogenomic databases (CTD, GeneCards, DisGeNET) using "titanium dioxide nanoparticles" as the search term. This integration enabled the selection of 200 genes with altered expression for subsequent analysis. Protein-protein interaction networks were constructed in STRING (confidence > 0.900), and hub genes were identified through topological analysis with CytoHubba in Cytoscape. Functional characterization was performed through enrichment analysis in Reactome (FDR < 0.05), with validation via multi-platform triangulation (Metascape, g:Profiler, DAVID), exclusively retaining significant functional terms consistently reproduced in at least two analytical tools. **Results:** Network topology analysis identified four central hub genes: CCL2, CXCL10, CXCL9, and CD4, functioning as key regulators of the TiO₂-NP response. These demonstrated significant enrichment in critical biological pathways including Interleukin-10 signaling (FDR = 6.24×10^{-7}), interleukin signaling cascades (FDR = 1.51×10^{-5}), chemokine receptor binding (FDR = 1.51×10^{-5}), and the NFE2L2 oxidative stress pathway (FDR = 3.42×10^{-5}). Functional analysis confirmed sustained activation of both inflammatory responses and oxidative stress mechanisms. Disease ontology assessment revealed that titanium dioxide nanoparticles exhibit significant associations (FDR < 0.05) with systemic lupus erythematosus, atherosclerosis, rheumatoid arthritis, neurodegenerative disorders, and neoplastic processes, highlighting their broad pathogenic potential. **Conclusions:** Titanium dioxide nanoparticles possess the potential to induce transcriptional reprogramming in CD14⁺ human macrophages through coordinated activation of immunoinflammatory and oxidative stress pathways governed by key molecular hubs. The widespread presence of these nanoparticles in consumer products positions them as significant components of the global human exposome, calling for greater attention regarding their toxicological implications. The identified molecular and pathological outcomes support the critical need to prioritize safety assessments and implement specific protective measures against this emerging public health concern worldwide. [UniCartagena. Support to Research Groups and Doctoral Programs. 2024-2025].

PS 4485 Cross-Platform Translation of Toxicogenomic Profiles Using a Transformer Model to Enable Data Harmonization

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Background and Purpose: Toxicogenomic studies rely on transcriptomic profiling platforms that vary in dynamic range, probe chemistry, and transcript coverage. These differences introduce systematic technical variation that limits cross-study analysis and re-use of historical data. There are at least three generations of profiling platforms with many legacy data samples. For example, two main toxicogenomics databases, open-TGGATs and DrugMatrix, contain measurement data from older platforms including CodeLink and Affymetrix. There remains a gap for toxicologists to utilize the broadest possible range of existing data. We developed a transformer-based model to translate treatment-derived gene expression profiles across platforms, enabling harmonization and integrated interpretation of toxicology datasets. **Methods:** Paired gene expression datasets were curated from the DrugMatrix and used to train a customized profile-to-profile attention model that learns a shared latent representation of treatment-induced transcriptomic responses and reconstructs them in a target platform space. Different from Seq2Seq transformers, our model does not enforce artificial ordering on the gene profiles, and is able to concurrently decode for multiple regions in the target profiles. In our evaluation we focused on translating CodeLink and Affymetrix microarray profiles to BioSpyder Whole Transcriptome (BioSpyderWT) TempO-Seq profiles. Model performance was evaluated by gene-wise correlation, concordance of differential expression analyses,

and pathway enrichment similarity. We also compare prediction accuracy against two baselines, a multi-layer perception (MLP) and a seq2seq transformer. **Results:** Dependent on the amount of available data samples per tissue in the database, we explore three training modes (mixed-tissue, single-tissue, and cross-tissue). In all three modes, our model beats the two baselines in prediction performance. In mixed-tissue mode, our model reduces mean absolute error by more than 50%, that is, 0.043 vs. 0.09, and doubles the Pearson correlation, ≈ 0.71 vs. 0.37, relative to baselines. Critically, it preserves rare but biologically important over- and under-expressed signals with MAE < 0.22 . Single-tissue models yield further gains for well-represented organs, for example, liver mean absolute error $> 10\%$, while highlighting the need for data-augmentation strategies in low-sample tissues. The model accurately reconstructed target platform expression signatures. Across chemicals and tissues, gene-level **Pearson Correlation Coefficients are up to 0.75**, with foldchange data for tissues with plenty of training samples. Differential expression analyses performed on translated profiles exhibited **$\sim 60\text{--}80\%$ overlap in top perturbed genes**, and downstream biological interpretation was preserved, with **pathway-level Jaccard similarity of up to 0.85**, particularly for pathways associated with xenobiotic metabolism, oxidative stress, immune activation, and mitochondrial function. These results indicate that the model captures conserved biological signal despite technical platform differences. To further demonstrate the utility of our model for modern AI applications, we harmonize liver data from two platforms in DrugMatrix, CodeLink and BioSynderWT, for bio-marker identification in liver necrosis analysis. Our experiment shows that harmonized data improves the predictive performance of a simple MLP model over data on the BioSynderWT platform alone. **The F1 score increases from 0.64 to 0.72. Conclusions:** By enabling reliable cross-platform harmonization, our model unlocks decades of legacy toxicogenomic data for contemporary analyses, bolsters biomarker discovery, and promises more reproducible predictive toxicology and regulatory assessments. This study demonstrates that transformer-based platform translation can harmonize toxicogenomic datasets across measurement technologies while preserving biologically meaningful response patterns. This approach supports greater reuse of existing *in vivo* transcriptomic data and aligns with New Approach Methodologies aimed at improving mechanistic inference and reducing reliance on new animal testing.

PS 4486 Mapping the landscape of pharmacological target interactions in RPTEC/TERT1 cells using transcriptomics and cell painting

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Background and Purpose: New approach methods (NAMs) could revolutionize current safety assessment through mechanistic understanding of chemical-biological interactions, but their regulatory uptake remains limited. A perceived limitation is reproducibility across methods and cell systems. Does pharmacological modulation of a target by different compounds always induce identical downstream effects? Do transcriptional or phenotypic signatures predict upstream target interactions? And what is the complementarity of transcriptional and phenotypic changes in describing downstream effects of target modulation? We evaluated transcriptional and morphological profiles upon exposure to a large pharmacologically active reference chemical set with overlapping target interactions. **Methods:** The reference chemical set was derived from RefChemDB, which compiles chemical-target-mode-activity relationships from multiple public sources. This set includes > 300 compounds that interact with some 150 distinct drug targets, with each target associated with 2-4 compounds designed to modulate a specific cellular function. First, we collected affinity measurements from ChEMBL of compound-target activity calls to evaluate the call-combination in RefChemDB and identify further possible targets and performed MS experiments to evaluate the stability of reference chemicals. To determine transcriptional and phenotypic profiles, we exposed RPTEC/TERT1 cells to 8 non-cytotoxic concentrations and conducted TempO-Seq high-throughput transcriptomic profiling and Cell Painting PLUS (CPP) after 24 h. We projected transcriptomic profiling to *in vitro* human RPTEC/TERT1 TXG-MAPr, a weighted gene co-expression network built on toxicogenomic responses upon exposure to nephrotoxics. Finally, BMD modeling of morphological features and TXG-MAPr module responses was performed to determine PODs and evaluate if both methods captured biological activity of reference chemicals. **Results:** Quantitative affinity data collected from ChEMBL confirmed compound-target activity calls defined in RefChemDB as most sensitive but further identified additional significant interactions. Transcriptomics identified 75 targets for which at least one associated compound gave transcriptional changes. When pairs were both able to trigger a transcriptomic response, differential gene expression correlation between pairs showed high similarity. When using the *in vitro* human RPTEC/TERT1 TXG-MAPr, clusters of co-expressed genes for pairs of reference compounds could be related to their target. CPP showed lysosome features to be often perturbed by the selected compound set. Compounds showing high transcriptomic correlation often had similar CPP profiles, e.g., EGFR antagonists Lapatinib and Canertinib had similar transcriptomic profiles marked by a downregulation in the activity of RPTEC/TERT1 TXG-MAPr modules 88 and 95, associated with MAPK signaling. CPP profiles for the ER, mitochondria, lysosome, and actin displayed similar activity, indicating that both platforms capture a shared mechanism. Many compounds that showed positive results in the transcriptome exhibited minimal activity in the CPP assay. Surprisingly, MAP2K1 inhibitors Selumetinib and

PD0325901 showed high correlation with EGFR antagonists at gene and module level, yet the CPP profile of the MAP2K1 inhibitors was not perturbed. Also, TOP1 antagonists Topotecan and Irinotecan exhibited transcriptomic activity but only Topotecan gave changes in CPP features related to ER and mitochondrial activity. This indicates that the different assays may offer different biological interpretations. **Conclusions:** We present a definition on the target space leading to transcriptomic and morphological responses in RPTEC/TERT1 and an interpretative framework based on co-expressed network analysis to obtain mechanistic information for safety assessment. Our work aims to define the chemical-target transcriptomic landscape to assess which biological events can be captured by which cell lines and tissues, thus contributing to define the relevance for chemical risk assessment.

PS 4487 Single cell RNA-sequencing reveals divergent genes in cynomolgus macaque versus human in CD3-CD28 mediated immune response

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Background and Purpose: The binding specificity achieved by many new immunomodulating therapeutics necessitates the use of non-human primates to achieve thorough safety assessment. Cynomolgus macaques (cyno) are the most widely used non-human primate for drug safety assessment due to their small size, non-seasonal reproductive cycle, and high homology with humans. However, differences in the adaptive immune response between species is not fully characterized, slowing development of immunomodulating therapeutics. **Methods:** To elucidate differences in this response between species, we utilized single cell RNA-sequencing (scRNA-seq) to perform a cross-species integrative analysis of gene expression in immune cells across a 3-day time course after *in vitro* anti-CD3/anti-CD28 stimulation. **Results:** We observed that T cell activation markers upregulated at different time points after stimulation: human CD8+ T cells reaching max CD25 expression 48 hours after stimulation ($\sim 80\%$ of cells expressing) and cyno 72 hours after stimulation ($\sim 85\%$ of cells expressing). However, there were limited statistically significant differences in the expression of T cell signaling related genes between species. To more thoroughly assess temporal differences in gene expression between species, we assessed the variance explained by the interaction of time and species. We identified 270 genes with over 55% of the variation observed in gene expression explained by the interaction between time and species. **Conclusions:** An in-depth understanding of this response between species will accelerate decision-making in the safety assessment of immunomodulating therapeutics.

PS 4488 Evaluation of Anti-Arthritic Effects of Acupen a Polyherbal Formulation Using Adjuvant-Induced Arthritis Model in Wistar Rats

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Background and Purpose: Arthritis is a chronic inflammatory disorder of the joints characterised by pain, swelling, stiffness, and progressive structural damage, ultimately impairing musculoskeletal function. Despite available treatments, long-term management remains challenging due to the adverse effects of conventional drugs. Acupen (300 mg tablet) is a polyherbal formulation containing extracts of *Apium*, *Leptophyllum*, *Aloe vera*, *Commiphora mukul*, *Ricinus communis*, *Myristica fragrans*, *Boerhavia diffusa*, and *Triphala*. The diverse bioactive compounds in these herbs are reported to possess anti-inflammatory and antioxidant properties, offering a potential synergistic effect against arthritis. The objective of this research is to evaluate the potential antiarthritic properties of the Acupen using adjuvant-induced arthritis model in Wistar rats. **Methods:** Arthritis was induced by Subplantar injection of 0.1 mL complete Freund's adjuvant (CFA) in the left hind limb in all rats except the negative control group. Negative control (G1) received vehicle only (0.5% CMC), Disease Control (G2) was kept untreated, Positive Control (G3) received methotrexate (1 mg/kg b. wt./day), and the Acupen-treated groups were treated at 60 (G4) and 120 (G5) mg/kg b. wt./day. Vehicle, methotrexate, and acupen treatment were given orally for 28 consecutive days. Endpoints evaluated included clinical signs, mortality, arthritis scoring, paw volume measurement, cytokine estimation, and histopathology. **Results:** The disease control group showed progressive inflammation and increased paw volume throughout the study period. Methotrexate treatment significantly reduced arthritis scores and paw edema compared with the disease control. Acupen treatment reduced arthritis severity, with a higher dose, which was marginal compared to the positive control. **Conclusions:** Based on the results obtained in the current study, it is concluded that Acupen polyherbal formulation exhibits promising anti-inflammatory and anti-arthritic activity in the CFA-induced arthritis model in Wistar rats, supporting its potential as a complementary therapeutic approach for arthritis management.

PS 4489 Differential Gene Expression in 3D-printer exposed BEAS-2B Human Bronchial Cells Varies with Filament Material and Exposure Time

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Background and Purpose: The use of 3D printers, particularly fused filament fabrication (FFF) printers, has increased in residential, educational, and occupational spaces. These printers emit aerosols, including particulate matter and volatile organic compounds, which may cause toxic effects in humans when inhaled. The health effects after exposure have not been adequately characterized. **Methods:** This study analyzes RNA-sequenced data from an FFF printer exposed to a BEAS-2B lung cell model for five different filament types: black-colored acrylonitrile butadiene styrene (ABS), transparent ABS, blue-colored polylactic acid (PLA), transparent PLA, and a mixed steel-and-polymer. Differential gene expression (DEGs) and protein-protein interactions (PPIs) were compared at 1 and 4 hours of exposure. **Results:** There was significant variability in the numbers and identities of DEGs and PPIs among the different exposure groups. The highest number of DEGs and PPIs was observed in the 4-hour exposure to the transparent PLA (401 DEGs and 474 PPIs), and the smallest number of DEGs was observed in the 1-hour exposure to the steel filament (12 DEGs and 0 PPIs). Only a few perturbed genes were common across exposure groups, possibly indicating distinct mechanisms of action induced by each aerosol exposure. **Conclusions:** This research emphasizes the need for further investigation into the underlying mechanisms of 3D-printer emission-induced toxicity.

PS 4490 A similarity-based transcriptomics analysis workflow enables rapid investigation of potential mechanisms of compound toxicity during nonclinical development

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Background and Purpose: Investigating the mechanisms causing adverse effects during nonclinical toxicity testing directly impacts drug development by derisking potential human health liabilities. While inferences of a compound's safety profile can be made using toxicity data from structurally similar compounds, shared structural features alone may not always be indicative of adverse or undesirable effects. Toxicogenomics provides an additional means of assessing compound safety prior to initiating further testing, as historical compounds with similar profiles of differentially expressed genes could be examined for mechanisms of toxicity. Here, we developed a computational workflow to (1) identify compounds from historical nonclinical studies with similar gene expression to compounds of interest, and (2) compile and contextualize multimodal safety data for rapid interpretation and use in mechanistic investigation. **Methods:** Bulk RNA-seq and mid-density array data from internal *in vivo* and *in vitro* studies comprising over 11000 gene expression profiles were harmonized into a reference database. Cosine similarities (a measure of similarity between two vectors) were then computed from log-transformed fold-change values for all pairs of profiles. A downstream workflow and web application was developed to assemble internal safety data for profiles exceeding a user-defined threshold of cosine similarity versus a user-supplied compound or list of gene fold change values. Data collected for each query included chemical structures, ancillary pharmacology, clinical chemistry, pathway enrichment via overrepresentation analysis and transcriptomic signature scores, and histopathology phenotypes. The application was developed to enable flexible querying starting with any data modality (e.g. any phenotype, biomarker, etc.) to support a variety of mechanistic investigations. **Results:** To evaluate whether cosine similarity accurately prioritizes compounds with similar transcriptional effects, cosine similarity values were compared across 560 compounds that were profiled in multiple mid-density array studies. Cosine similarity values for matching treatment groups (i.e. same compound and dose) and matching compounds only (i.e. same compound at different doses) were both significantly higher than values for mismatching compounds ($p=1.4E-79$ and $p=4.7E-44$ via Mann-Whitney U tests). Cosine similarity values between 1673 compounds profiled via mid-density array were also compared to Tanimoto similarity values (a measure of 2D chemical similarity) computed from Morgan fingerprints to evaluate whether chemical structure-based similarity was associated with cosine similarity values. Tanimoto similarity values were not predictive of cosine similarity values via ordinary least squares regression ($R^2=0.015$), indicating that compounds eliciting similar gene expression may not necessarily be structurally similar. A case study querying compounds known to modulate xenobiotic nuclear receptor CAR yielded analogous compounds with enriched activity towards CAR via transcriptional biomarker scores. Compounds with high incidences of liver hypertrophy in rats were also queried for differentially expressed genes in rat liver samples, where hits were defined as an absolute $\log_{10}$ (fold-change) ≥ 0.5 . These compounds were found to have high hit rates of CAR-regulated genes CYP1B1, CYP1B2 and CYP2C6 (48%, 65% and 49% respectively) whereas compounds that did not cause hypertrophy had lower hit rates (29%, 40% and 18% respectively), in agreement with the known association of CAR activation with liver hypertrophy. **Conclusions:** This workflow underscores the value of large-scale multimodal datasets for providing rapid support during nonclinical toxicology investigations. These data can also support new initiatives to predict safety liabilities earlier in drug development by serving as a basis for machine learn-

ing models and AI-enabled hypothesis generation. Integration of external data could augment predictive capabilities by expanding the diversity of compounds and model systems tested. Altogether, this workflow accelerates current safety investigations and supports future drug development by providing real-time mechanistic insights into potential safety liabilities.

PS 4491 Visualizing Structural Relationships with Clustering in Mass Spectrometry Datasets

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Background and Purpose: We have developed an R Shiny application to visualize molecule clustering using dendrograms based on mass spectrometry features. Feature-based clustering can identify structural relationships between molecules without requiring prior knowledge of molecular formulas. This work expands upon the SCOPE (Structural-based Connectivity and Omic Phenotype Evaluations) cheminformatics toolbox, which generates dendrograms and heatmaps to help assess links between lipid class and changes in lipid abundance. SCOPE uses SMILES (Simplified Molecular Input Line Entry System) to represent molecular structure and it can effectively cluster lipids in a dataset that belong to the same class. The use of SMILES limits the clustering to molecules that have known annotations. The feature-based clustering used in the Shiny app may be applied to datasets that contain unknown compounds. **Methods:** Features that can be included in the clustering are the LC retention times (RT), IMS collision cross sections (CCS), and mass-to-charge ratio (m/z) values. The features are detected in a platform that contains coupled liquid chromatography, ion mobility spectrometry and mass spectrometry separations (LC-IMS-MS). The Kendrick mass defect (KMD) may optionally be included in the clustering, where different homologous series in a dataset can have distinct values. The app displays interactive scatter plots for the individual features to help identify potential dataset outliers. The dendrograms show how molecules cluster based on structural similarities for the user-selected features. As with SCOPE, for datasets that have abundance data, the fold change values are displayed on a heatmap adjacent to the dendrogram. This combined visualization can be used to ascertain whether clustered molecules show similar changes in abundance for sample comparisons. **Results:** The functionality of the app is not restricted to a specific molecule type and we highlight two use cases from published studies for lipid and per- and polyfluoroalkyl substances (PFAS). In the first study, mice were exposed to PFAS compounds and the resulting changes to the hepatic lipid abundance were evaluated. The characterization of lipid samples is often challenging due to the variety of molecules and physicochemical properties of the lipidome. In the second study, PFAS with previously unknown structures were elucidated through nontargeted analyses. Like lipids, there are thousands of different PFAS, and having a visualization tool that provides insights into the structural properties of unknown compounds can be useful for these types of datasets. **Conclusions:** The resulting dendrograms show molecule clustering by both structural complexity and group classifications, where the contribution of each is dependent upon the composition of the dataset.

PS 4492 Using methylation profiling to improve bioinformatics pipelines for the identification of dioxin response elements

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Background and Purpose: The aryl hydrocarbon receptor (AHR) is a cytosolic ligand-activated transcription factor that binds a wide range of ligands, including families of dioxin-like substances such as polychlorobiphenyls, polychlorinated dibenzo-para-dioxins, and polycyclic aromatic hydrocarbons. AHR forms a nuclear-localized complex that recognizes and binds dioxin response elements (DREs) in gene promoters. The resulting target gene regulation causes various transcriptional, epigenetic, and metabolic responses leading to physiological and developmental effects on immune response, cell cycle control, cellular differentiation, and organ development, and reproductive outcomes. Although the canonical DRE is well-defined by a 5-bp core motif (5-GCGTG-3), studies have shown that extended flanking sequences influence AHR binding and tissue specificity. This raises an unresolved computational challenge of distinguishing functional DREs from the many instances of the core motif across the genome. **Methods:** While considerable attention has been given to the effects of AHR on gene transcription, DNA methylation has also been shown to be intertwined with the AHR gene regulation process, through direct inhibition, or indirectly through effects on chromatin accessibility. The standard approach to the identification of AHR targets has included a combination of motif searching for a DRE consensus genomic sequence, with additional refinement based on transcriptomics and ChIP-seq or other protein-DNA binding assays to better distinguish among putative DREs. We propose to further extend our understanding of active DREs by including results from methylation profiling in response to chemical exposure and activation of AHR, which can be used in combined analyses to produce refined computational models to identify DREs. **Results:** As proof of principle in an important model organism, we analyzed whole-genome bisulfite sequencing (WGBS) data from the sperm of F2-generation zebrafish for which progenitors

had been subjected to a factorial treatment design consisting of the presence or absence of an *ahr2* morpholino knockdown and exposure to benzo[a]pyrene (BaP, known to activate AHR). We quantified differential methylation and examined how these changes correspond to the DRE motifs of varying lengths (5-bp, 8-bp, and 12-bp) to investigate the relationship between AHR binding potential and epigenetic modification. We applied machine learning models to evaluate which variants best predict AHR-associated methylation responses. **Conclusions:** To identify additional AHR targets in zebrafish, we generated a position specific scoring matrix (PSSM) based on the DRE motifs identified for CYP1A to construct a genome-wide library of DRE motifs, expanding beyond the core 5-base-pair consensus motif. Generating PSSMs using these longer motifs reduces the number of genome hits and may lead to discovery of relevant DREs. Results from the machine learning models revealed a subset of longer motifs with improved predictive scores for methylation response. In addition, we describe an approach for gene-level analysis that effectively considers DRE consensus sequences as “soft” pathways suitable for gene set enrichment analyses in the analysis of differential methylation responses.

PS 4493 Comparative Toxicogenomics Database: A data integration and visualization resource to inform the exposome and disease etiologies

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Background and Purpose: Environmental exposures are critical contributors to most chronic diseases. Exposure sources include, but are not limited to, industrial contamination, dietary factors, commercial products, lifestyle choices, and psychosocial stressors. The Comparative Toxicogenomics Database (CTD; <https://ctdbase.org>) was established and publicly launched in 2004 to facilitate data integration and discovery about how environmental factors affect human disease. CTD is a highly cited, globally-used resource that continues to evolve and inform emerging environmental health frameworks such as Adverse Outcome Pathways (AOPs) and the Exposome, which aims to describe the totality of exposures across an individual's lifetime. Here, we present updates on CTD's data and functionality and demonstrate how this resource can be used to survey the environmental health landscape - from model-based mechanisms to exposed populations - to generate testable hypotheses that advance environmental health research. **Methods:** CTD biocurators read the peer-reviewed literature and use community-based data standards to manually curate relationships between chemicals, genes/proteins, phenotypes, anatomical terms, diseases, taxa, and exposure measurements. These data are integrated with select external data sets and analytical and visualization tools designed to discover novel, mechanistic connections between exposures and adverse health outcomes. CTD is accessible via its public website and provides user-friendly query pages, analytical and visualization tools, and downloadable data files. **Results:** CTD currently contains manually curated information for over 19,000 chemicals; 56,000 cross-species genes; 6,800 phenotypes; 7,200 diseases; 630 organisms; and 1,000 anatomical terms. Integrating these curated data-sets further generates more than 100 million toxicogenomic relationships. Importantly, CTD continues to expand, with ~17,000 interactions from over 300 newly curated scientific articles added every month. There are a variety of methods by which users access CTD data, including browser-based keyword searches, advanced query options, analytical tools, and embedded functionality that enables refinement of queries and analyses on demand. Here, we demonstrate CTD's breadth and utility with examples that explore connections between specific chemicals and disease outcomes; however, the same methodologies can be applied to any chemical or disease of interest in CTD. **Conclusions:** Over the last two decades, CTD has evolved into a unique, analytical tool to explore cross-species information about the biological repercussions of chemical exposures. CTD's data and functionality are positioned to inform testable hypotheses that advance understanding about the etiologies and underlying mechanisms of chronic diseases, and as such, environmental health frameworks such as AOPs and the Exposome.

PS 4494 NeTan: A Network-based Tool for Multi-Omics Integration across Cohort and Molecular levels

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Background and Purpose: Large multi-omics datasets exhibit complex structures at both cohort and molecular levels. Relationships among data points, whether representing individuals within a cohort or molecules within a biological system, provide key insights into system behavior and individual responses to environmental challenges. While several computational tools have been developed to construct cross-omics molecular networks, few account for cohort-level relationships. We developed NeTan, a user-friendly, graph-based tool that connects the exposome, molecular responses, clinical parameters, and phenotypes across both cohort and molecular level. This unified framework provides a mechanistic basis for hypothesis generation and individualized risk-relevant interpretation. **Methods:** NeTan constructs networks in two modes: (1) cohort-level, where nodes represent participants, and (2) molecular-level, where nodes represent omics features. At the cohort level, NeTan can generate either a single “stacked” consensus network graph or

a multi-layer graph preserving participant relationships within each omics layer. Edges inference is supported by multiple algorithms, including Spearman correlation, Context Likelihood of Relatedness for mutual information, Random Forest similarity for nonlinear associations, and Graphical Lasso for partial correlations. Adjacency matrices are thresholded but retain continuous edge weights, enabling users to tune edge strength and facilitate interpretation. The resulting graphs include network statistics, node attributes, and community detection for identifying hubs and sub-network modules. **Results:** NeTan builds single-layer or multi-layer networks at the cohort level and the molecular level. It is implemented as a user-friendly web application, available at <https://netan.io>, and as a Python package at <https://github.com/bm-boris/netan-lib>. The platform ensures data security by not retaining uploaded data and supports interactive network visualization, standard export formats (e.g., Cytoscape) for downstream visualization and analysis. Users can save and share their analysis configurations to reproduce results. A unique advantage of NeTan is the population network, where edges between individuals reveal similarities in molecular profiles. Using this mode, we identified network communities in a cohort of young healthy adults, showing that occupational exposures have a dominant effect on metabolomics profile in that cohort. This exemplifies the power of NeTan in prioritizing factors that drive biological responses. **Conclusions:** NeTan provides a unified, scalable framework for multi-omics integration that simultaneously captures inter-sample (cohort) structures and molecular co-variations. By combining multiple edge inference algorithms, NeTan enhances robustness and confidence in detected associations. This platform facilitates mechanistic interpretation of complex biological responses and supports risk assessment through transparent, reproducible network-based analysis. Research reported in this publication was supported by the Advanced Research Projects Agency for Health (ARPA-H) under award number D24AC00345-00.

PS 4495 In Vitro Profiling of Cosmetic Ingredients within the Tox21 10K Compound Library for Bioactivity and Potential Toxicity

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Background and Purpose: The United States Food and Drug Administration (FDA) defines cosmetics as “articles intended to be rubbed, poured, sprinkled, or sprayed on, introduced into, or otherwise applied to the human body...for cleansing, beautifying, promoting attractiveness, or altering the appearance” in the Federal Food, Drug & Cosmetic Act (FD&C Act) Section 201(i). However, the ingredients within these cosmetics are not currently monitored by the FDA. Thus, some states such as California are banning specific chemicals at the state level in bills such as AB-496 Cosmetic safety. This bill bans 26 chemicals (and their salts totaling 70 compounds) from being used in cosmetics which includes known endocrine disruptors such as dibutyl phthalate, diethylhexyl phthalate, and per- and polyfluoroalkyl substances (PFAS). **Methods:** The Tox21 10K compound library was previously screened against a panel of ~80 cell- and biochemical-based assays. The Tox21 10K compound library included 34 compounds (and their variations) that were within the California ban. In this study, banned cosmetic ingredients and non-cosmetic compounds were compared across the Tox21 assays. **Results:** We found that the banned cosmetic compounds were significantly more active in 10 antagonist assays and 7 agonist assays compared to the non-cosmetic compounds. The antagonist assays included enzymes such as CYP2C19 and aromatase (CYP19A1), and the agonist assays included the Keap1/Nrf2 antioxidant response element pathway (KS-ARE). **Conclusions:** The results of this analysis will help identify specific pathways affected by the cosmetic compounds and create a further understanding of the compounds mechanisms of action.

PS 4496 Evaluating Potential Hepatotoxic and Metabolic Disruption by Emerging HFPO Homologues in Adult Female Mice

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Background and Purpose: The phase-out of legacy per- and polyfluoroalkyl substances (PFAS) such as perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) has led to the introduction of replacement compounds based on hexafluoropropylene oxide (HFPO) chemistry. Among these, HFPO dimer acid (HFPO-DA), trimer acid (HFPO-TA), and tetramer acid (HFPO-TeA) are increasingly detected in the environment, yet their toxicological profiles remain poorly characterized. Given their structural similarity to legacy PFAS, this study aimed to evaluate the immunotoxic, hepatotoxic, and metabolic effects of HFPO homologues in adult female mice to assess potential health risks associated with PFAS substitution. **Methods:** Adult female C57BL/6J mice were exposed via drinking water to HFPO-DA, HFPO-TA, or HFPO-TeA at concentrations that were similar to human exposure levels over 3 orders of magnitude for 28 days, followed by immunization with sheep red blood cells to assess a T-cell-dependent antibody response (n = 8/group). To evaluate hepatic and metabolic outcomes, a 14-day study exposed mice

to equal concentrations of HFPO-DA, HFPO-TA, HFPO-TeA, or vehicle control without immunization ($n = 8/\text{group}$). A 7-day comparative study included PFOS at the EFSA tolerable daily intake (TDI) level or at a much lower sub-TDI concentration. Liver and serum samples were analyzed for histopathology, metabolic biomarkers, and transcriptional changes in lipid metabolism and inflammatory pathways. **Results:** Across all exposure durations, HFPO-TA consistently induced dose-dependent increases in liver-to-body weight ratios and significantly decreased circulating triglyceride concentrations, indicative of hepatic and metabolic disruption. HFPO-DA and HFPO-TeA elicited no significant hepatic or metabolic effects. Histopathology confirmed mild hepatocellular hypertrophy in HFPO-TA exposed mice. No overt immunotoxicity was detected in antibody response assays. **Conclusions:** HFPO-TA was identified as the most hepatotoxic and metabolically-disrupting of the HFPO homologues tested, producing consistent hepatic enlargement and altered lipid metabolism. HFPO-DA and HFPO-TeA showed limited biological activity at comparable concentrations. These findings suggest that not all HFPO-based PFAS are toxicologically equivalent, and that HFPO-TA may pose hepatometabolic risks similar to or exceeding those of legacy PFAS. This work underscores the need for mechanistic and dose-response studies to evaluate the safety and regulatory implications of PFAS alternatives currently detected in the environment. Ongoing analyses aim to elucidate the molecular mechanisms underlying HFPO-TA induced hepatotoxicity to inform risk assessment of emerging PFAS alternatives.

PS 4497 Innovative Co-Culture Approach to Investigate Cellular Responses to Fluorotelomer Alcohol Exposure

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Background and Purpose: Fluorotelomer alcohols (FTOHs) are neutral, volatile compounds widely used for their water-, grease-, and stain-repellent properties in consumer products such as textiles, carpets, outdoor gear, and food packaging. These compounds are primarily released from treated materials under ambient conditions and represent the most abundant polyfluorinated substances detected in indoor air. Human exposure occurs mainly through inhalation and ingestion of contaminated house dust, posing particular concern for children due to behavioral and developmental factors. In this study, we aimed to characterize the effects of exposure and identify potential protein targets of four FTOHs in relevant airway cell models. **Methods:** We first employed live-cell monitoring technology to evaluate the biological effects of varying concentrations of the selected PTOHs compounds on airway cellular models using two human airway epithelial cell lines: A549 (lung adenocarcinoma) and BBM (bronchial epithelial cell line derived from BEAS-2B cells). These two models represent distinct areas of the human airway. We exposed these cells to increasing concentrations of 4 compounds: perfluorooctylethyl acrylate, 2-Perfluoro-Octyl ethanol, 2-Perfluoro-Hexyl ethanol, and 2-Perfluoro-Decyl ethanol for up to 120 hours. Real-time assessments of cell proliferation, growth rates, and morphology were used to establish baseline toxicity profiles and characterize alterations in cellular health and function. We also performed an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay to assess cell metabolism/viability. Additionally, we employed a co-culture model of primary human bronchial epithelial cells differentiated at the air-liquid interface (ALI) over THP-1-derived macrophages, representing a physiologically relevant human lung airway model. We assessed macrophage activation (CD86) expression utilizing a flow cytometry assay. Finally, we utilized bottom-up quantitative proteomics to measure differential protein expression of human ALI cells upon exposure to 2-Perfluoro-Octyl ethanol. **Results:** Our preliminary experiments showed that perfluorooctylethyl acrylate showed increased toxicity as demonstrated by significant decreased growth and cell viability in both cell lines tested in a concentration-dependent manner. We also observed increased toxicity with the other three compounds, but only at higher concentrations in both A549 and BBM cells. Preliminary experiments involving exposure to FTOH compounds in the ALI-macrophage co-culture model demonstrated macrophage activation, particularly following exposure to perfluorooctylethyl acrylate and 2-perfluorooctyl ethanol. Additionally, we identified 176 unique proteins with significantly altered expression levels in response to FTOH exposure in this model. **Conclusions:** Our findings demonstrate that FTOHs can elicit concentration-dependent cytotoxic effects and alter cellular function in human airway models. Collectively, these results highlight the potential impact of FTOHs on human respiratory health through both cytotoxic and immunomodulatory mechanisms, warranting further investigation into their molecular targets and long-term effects on human airway physiology.

PS 4498 Cell type-specific transcriptomic effects of PFAS in human cortical organoids

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are a large class of synthetic chemicals widely used in industrial and consumer products. Due to their chemical stability, PFAS persist in the environment, accumulate in biological systems, and have been linked to cancer, immune dysfunction, endo-

crine disruption, and developmental toxicity. Although PFAS can cross both the blood-brain barrier and the placenta, their effects on human neurodevelopment remain poorly understood. **Methods:** We investigated the cell type-specific transcriptomic effects of six PFAS included in the U.S. EPA National Primary Drinking Water Regulation (Perfluorobutanesulfonic Acid, PFBS; Perfluorohexanesulfonic Acid, PFHxS; Perfluorooctanoic Acid, PFOA; Perfluorononanoic Acid, PFNA; Perfluorooctanesulfonic Acid, PFOS; and GenX) on early neurodevelopment. We generated human cortical organoids (COs) from six iPSC lines derived from healthy female and male donors, and chronically exposed them to each PFAS for up to 50 days, followed by single-cell RNA sequencing. **Results:** Single-cell clustering identified neurons, progenitors, and intermediate progenitor cells as major cell type populations. PFAS exposure did not significantly alter overall cell type proportions, although subtle non-significant reductions in radial glia-like progenitors were observed following PFOS, PFOA, and PFHxS exposure. Pseudo-bulk differential gene expression analyses revealed that PFOS induced the greatest number of differentially expressed genes, followed by PFOA and PFHxS, with progenitors exhibiting greater transcriptional vulnerability compared to neurons. Across compounds, gene expression changes were positively correlated, reflecting shared molecular responses. Most PFAS broadly perturbed non-neuronal processes (RNA metabolism, translation, cell cycle), while neuronal processes (axonogenesis, synaptogenesis) were mainly impacted by PFOS, PFOA, and GenX. Weighted gene co-expression network analysis revealed modules preserved across PFAS and cell types, suggesting disruption of convergent pathways. Notably, PFOS-affected modules were enriched for high-risk neurodevelopmental disorder genes. Preliminary multielectrode array recordings indicated that PFOA and PFHxS may reduce neuronal network activity, although effects were variable and not statistically significant. **Conclusions:** These findings provide mechanistic insight into how PFAS exposure may impact human brain development. Our findings indicate that PFAS exert both cell type-specific and convergent transcriptional effects in cortical organoids, with progenitors showing particular vulnerability, and highlighting shared molecular pathways.

PS 4499 Mechanistic Toxicity Profiling of Legacy and Emerging PFAS Using New Approach Methodologies Integrating Target-Based and Translational Models

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are persistent environmental contaminants posing significant health risks, including endocrine disruption, hepatotoxicity, immunotoxicity, and developmental effects. Following recent FDA actions—including the 2024 withdrawal of PFAS-containing grease-proofing agents and information requests on PFAS in seafood—comprehensive toxicological assessment has become essential. Current assessments predominantly focus on legacy PFAS such as PFOA, PFOS, and PFHxS, leaving critical data gaps for emerging compounds. EPA Method 1633 enables detection of 40 PFAS across multiple matrices at low detection limits. However, mechanisms of action remain poorly characterized for many compounds, including nuclear receptor modulation, metabolic enzyme interference, oxidative stress, and inflammatory pathway activation. This study systematically screened EPA 1633 PFAS using target-based toxicology panels and translational assays to inform safety assessment and regulatory decision-making. **Methods:** I Compound selection: Selected PFAS from the EPA Method 1633 list, including perfluoroalkyl carboxylic acids (PFCAs), perfluoroalkyl sulfonic acids (PFSAAs), precursors, and replacement compounds. I Primary screening: Conducted target-based assays including (1) nuclear receptor panel to assess related cellular functional activation/inhibition, and (2) cytochrome P450 panel to evaluate enzyme inhibition through fluorescence changes in specific substrates. I Testing conditions: Compounds tested at 10 μM ; hits defined as >50% inhibition or activation. I Follow-up assessment: PFAS showing significant activity are being evaluated in the BioMAP® translational platform to assess human-relevant responses across vascular, immune, and fibroblast tissue systems. **Results:** CYP inhibition was the most common PFAS activity, with 63% of compounds inhibiting at least one isoform (>50%). CYP2C9 was the most frequently affected (58%), followed by CYP2C19 (32%) and CYP2C8 (21%), while CYP1A2 and CYP2B6 showed minimal susceptibility (5%). Structure-activity patterns emerged: perfluoroalkyl sulfonates showed the strongest CYP2C9 inhibition (e.g., PFHpS 97%), sulfonamide precursors exhibited broad multi-CYP activity (e.g., PFOSA 91%, NMeFOSA 88%, NeTFOSA 78% CYP2C9 / 74% CYP2C19), and short-chain perfluorocarboxylic acids (C7-C9) displayed selective CYP2C9 inhibition (PFHpA 76%, PFNA 92%). Nuclear receptor modulation was less frequent (16% of compounds). PFDA inhibited ER β (55%) and PR (87%), NeTFOSAA showed dual inhibition (ER β 73%, PR 100%), and 9Cl-PF3ONS had the broadest profile (5 α -reductase 77%, PR 100%, TR α 70%), indicating highly selective receptor targeting. These results highlight that sulfonate head groups with longer chains preferentially inhibit CYP2C9, sulfonamide modifications broaden CYP selectivity, and structural complexity (ether linkages, halogen substitutions) may drive multi-target nuclear receptor activity. BioMAP® studies are being used to validate these mechanistic trends in human-relevant cellular phenotypic systems. **Conclusions:** This integrated approach mechanistically characterizes PFAS toxicity, identifying CYP inhibition as the predominant activity and nuclear receptor modulation as structure-selective. Structure-activity relationships, influenced by head group chemistry, chain length, and substituents, enable mechanism-informed risk assess-

ment. Target-based assays combined with BioMAP® translational systems generate human-relevant data linking molecular targets to tissue-level responses. This framework supports comprehensive toxicity profiling without animal testing, addressing data gaps for legacy and emerging PFAS, and informing regulatory decision-making aligned with FDA/EPA priorities.

PS 4500 Role of the gut microbiota in PFAS-induced changes to hepatic toxicity and cholesterol metabolism

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are a large family of man-made chemicals used in many consumer and industrial products. Due to their high stability and resistance to degradation, PFAS persist and accumulate as environmental contaminants around the world. PFAS have been measured in human blood and found associated with numerous adverse health effects, including liver disease and elevated circulating cholesterol. In previous studies, we have observed that exposing mice to a PFAS mixture led to increased hepatic toxicity, especially in female mice, as well as elevated plasma cholesterol and bile acid levels, which may be due to PFAS-induced modulation of intestinal bile acid reuptake. Additionally, it was also observed that the form and function of the gut microbiota was changed due to PFAS exposure. PFAS exposure led to alterations of gut microbiota diversity measures and female PFAS-exposed mice also had increased abundance of the genera *Clostridium* and *Bifidobacterium*, which have important roles in the production of secondary bile acids. Therefore, we wanted to test the hypothesis that PFAS exposure modulates hepatic toxicity as well as cholesterol and bile acid metabolism through its effects on the gut microbiota. **Methods:** To do this, female C57BL/6 murine pathogen-free (MPF) mice or germ-free (GF) mice were fed an atherogenic diet and exposed to a mixture of PFAS representing legacy, replacement, and emerging subtypes (i.e., PFOA, PFOS, PFHxS, GenX, and 2-(Perfluoroalkyl)ethanol). Mice were exposed to the PFAS mixture through their drinking water for 7 weeks, with each PFAS at a concentration of 2mg/L. **Results:** After the 7 weeks exposure, circulating total cholesterol was significantly lower in the GF PFAS-exposed mice vs MPF PFAS-exposed mice ($p=0.02$). In addition, total hepatic cholesterol levels were also significantly lower in the GF PFAS-exposed mice vs MPF PFAS-exposed mice ($p=0.002$). As a measure of liver injury, plasma alanine aminotransferase (ALT) levels were significantly lower in the GF PFAS-exposed mice vs MPF PFAS-exposed mice ($p=0.01$). RNA sequencing was performed for liver tissue. Hepatic expression of the inflammatory C-C motif chemokine ligand 6 (*Ccl6*) was also significantly lower in the GF PFAS-exposed mice vs MPF PFAS-exposed mice ($p<0.001$). Hepatic gene ontology (GO) enrichment analysis identified 66 pathways that had a significant interaction between PFAS exposure and mouse line (Benjamini Hochberg-adjusted p -value <0.05), with the cholesterol and sterol biosynthetic processes as the most highly significant GO terms. Individual differentially expressed genes belonging to the cholesterol biosynthetic process were further examined. Expression of *Hmgcs1* was significantly upregulated in the GF PFAS-exposed mice ($p<0.001$), while there was no significant effect in the MPF mice (interaction FDR <0.001). Expression of bile acid synthesis enzymes *Cyp7a1* and *Cyp27a1* also were significantly lower in the GF PFAS-exposed mice compared to the MPF PFAS-exposed mice. The secondary bile acid deoxycholic acid was also found to be significantly increased in the MPF PFAS-exposed mice (FDR <0.05), but not in the GF mice. Finally, PFAS levels were measured in the plasma, urine, and liver tissues collected from the mice after 7 weeks of PFAS exposure. Hepatic levels of PFOS and GenX were both significantly decreased in the GF PFAS-exposed mice compared to MPF PFAS-exposed mice with no differences observed for urine or plasma. **Conclusions:** Overall, our results suggest that GF mice are at least partially protected from PFAS-induced hepatic toxicity and hypercholesterolemia, which may in part be due to changes in the distribution of specific PFAS. Future clinical interventions that target the gut microbiome may be beneficial for individuals exposed to PFAS.

PS 4501 PPAR α -Mediated Species-Specific Hepatic Responses to PFOS Exposure in Humanized and Knockout Mouse Models of Alcohol-Associated Liver Disease

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Background and Purpose: Perfluorooctane sulfonate (PFOS) exposure is increasingly linked to metabolic dysfunction-associated steatotic liver disease (MASLD; PMID: 35475652), yet its role in alcohol-associated liver disease (ALD) remains understudied. Our previous wild-type studies demonstrated that PFOS exacerbates ALD severity via peroxisome proliferator-activated receptor α (PPAR α ; PMID: 40347464), but the mechanistic role of PPAR α and its species specificity remain elusive. Given that PFOS is a strong mouse but weak human PPAR α agonist, the relevance of murine models to human health risk is uncertain. Here, we investigated species-specific hepatic responses using humanized PPAR α (hPPAR α) and PPAR α -knockout (KO)

mice. **Methods:** Male hPPAR α and PPAR α -KO mice (C57BL/6J background) received Lieber-DeCarli liquid diet containing 5% ethanol (EtOH) or isocaloric control and were concurrently gavaged with 1 mg/kg PFOS, or Tween-80 vehicle for 10 days, followed by a 5 g/kg EtOH binge dose and euthanasia 5-6h later. Assessments included body composition, biochemistry, liver histology (H&E), gene expression analysis, and high-content imaging of isolated primary hepatocytes. **Results:** EtOH decreased body weight across genotypes, with divergent liver-to-body weight ratios (decrease in hPPAR α , increase in PPAR α -KO). Both genotypes showed ethanol-induced triglyceride and VLDL elevations, with PFOS augmenting VLDL levels. Notably, PFOS attenuated EtOH-induced ALT/AST increases in both genotypes, contrasting with wild-type studies where PFOS exacerbated hepatotoxicity (PMID:40347464). Blood alcohol concentrations decreased significantly in PPAR α -KO mice and trended lower in hPPAR α mice with PFOS co-exposure. Histological examination revealed marked genotype-dependent differences: hPPAR α mice maintained normal liver morphology in controls and developed progressive steatosis from mild (PFOS alone) to moderate (ethanol alone) to severe (PFOS+ethanol), whereas PPAR α -KO mice demonstrated significant baseline steatosis even in vehicle controls, with persistent severe steatosis across all treatments. Gene expression analyses showed that PFOS-ethanol significantly upregulated PXR and Cyp3a11 in both genotypes, with PPAR α -KO maintaining stable CAR/Cyp2b10 expression while hPPAR α exhibited decreasing trends. In pair-fed controls, PFOS alone significantly induced Cyp3a11 across all genotypes. Lipogenic genes (PPAR γ , Cd36, Fasn) showed distinct genotype-specific patterns, with PPAR α -KO displaying significant upregulation versus hPPAR α 's downregulation trend in PFOS-ethanol groups. High-content imaging confirmed cellular differences: hPPAR α hepatocytes showed 4-fold increased mitochondrial function with PFOS-ethanol, while PPAR α -KO hepatocytes exhibited 5-fold increased lipid peroxidation. **Conclusions:** These findings reveal species-specific, PPAR α -mediated responses and underscore its central role in modulating hepatic outcomes during PFOS-ethanol co-exposure. Mechanistically, PPAR α exhibits dual and context-dependent functions: it preserves lipid homeostasis yet exacerbates hepatocellular injury under combined PFOS and ethanol exposure. Humanized PPAR α mice display intermediate phenotypes, highlighting species-specific receptor activation and signaling. Notably, the alteration of PXR and CAR pathways in the absence of PPAR α suggests compensatory cross-regulatory crosstalk beyond PPAR α signaling. Collectively, this work provides mechanistic and translational insights into how environmental pollutants modulate alcohol-associated liver injury and emphasizes the need for human-relevant models in toxicological risk assessment.

PS 4502 Photodynamic Priming (PDP) Mitigates Chemoresistance and Mitochondrial Enhancements in Ovarian Cancer Cells with a History of Chronic PFAS Exposure

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Background and Purpose: Photodynamic priming (PDP), which generates reactive molecular species following light-based activation of an intracellular photosensitizer, can synergize with traditional chemotherapies to overcome chemoresistance. Such contemporary treatment combinations are needed in ovarian cancer, where ~80% of patients develop platinum-resistant disease characterized by functional mitochondrial enhancements. Importantly, several photosensitizers localize to mitochondria and enable preferential photodamage to this organelle. As a result, PDP may also be valuable for targeting mitochondrial effects known to be induced by environmental toxicants, such as perfluoroalkyl substances (PFAS). PFAS are drinking water contaminants present in the blood of nearly all Americans that have been shown to induce chemoresistance in a duration-dependent manner in ovarian cancer cells, potentially via inciting mitochondrial biogenesis. Here, we hypothesized that mitochondria-localized PDP in combination with chemotherapy would overcome PFAS-induced chemoresistance in ovarian cancer cells. **Methods:** The present study evaluated the ability of PDP $\pm$ chemotherapy to overcome chemoresistance in two OVCAR-3 cell cohorts: PFAS chronically-exposed (~35 days of 0.5 μ M perfluoroheptanoic acid exposure) and outgrown (allowed to "recover" for ~35 days following chronic PFAS exposure). Two mitochondria-localized photosensitizers, benzoporphyrin derivative (BPD) and aminolevulinic acid-induced protoporphyrin IX (ALA-PpIX), were employed for PDP. Efficacy of PDP + carboplatin (1st line) or doxorubicin (2nd line) and impacts on mitochondrial membrane potential (MMP) and content were compared. Survival post-combination therapy was measured via relative ATP content and mitochondrial parameters via high-content imaging, PCR, and/or fluorescence-based assays. All studies had at least 3 biological replicates with 2 technical replicates each. **Results:** In PFAS chronically-exposed cells, which displayed significant resistance to carboplatin and doxorubicin, heightened sensitivity to BPD-PDP + carboplatin and BPD-PDP + doxorubicin was observed compared to outgrown cells, which had renewed sensitivity to most chemotherapy only treatments. Conversely, while ALA-PpIX-PDP + carboplatin displayed similar efficacies in both cell cohorts, ALA-PpIX-PDP + doxorubicin was much more potent in PFAS chronically-exposed cells. In both cell cohorts, regardless of photosensitizer used, PDP + doxorubicin was not as effective at reducing cell survival as PDP + carboplatin. When evaluating mitochondrial parameters, significant decreases in MMP, which are suggestive of apoptosis, occurred in both cell cohorts treated with ALA-PpIX-PDP + carboplatin and ALA-PpIX-PDP + doxorubicin, but not following combination therapy using BPD-PDP. Mitochondrial content, however, decreased across all combination

therapy groups, indicative of mitochondrial photodamage. **Conclusions:** These findings demonstrate the efficacy of PDP-based combinations for overcoming chronic PFAS-induced chemoresistance and associated mitochondrial enhancements. Future studies will expand to pre-clinical models of ovarian cancer (i.e. spheroids, *in vivo*) and patient samples with the goals of 1) identifying biomarkers of PFAS exposure and/or platinum resistance, and 2) informing mechanism-targeted, PDP-based treatment regimens for patient populations at risk for chemoresistance to improve survivorship.

PS 4503 CYP2E1 Dependent Effects of PFOS in Mouse Liver and HepaRG Models

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Background and Purpose: Perfluorooctane sulfonic acid (PFOS) is a member of the perfluoroalkyl substances (PFAS), a class of environmentally persistent and bioaccumulative chemicals widely used in manufacturing. PFAS exposure has been linked to increased incidence of alcohol related liver disease, yet the mechanisms underlying PFAS interactions with ethanol remain poorly understood. CYP2E1 is a monooxygenase that metabolizes numerous xenobiotics as well as endogenous fatty acids and ketones. Although CYP2E1 is most highly expressed in the liver, it is present in multiple tissues. The interaction between PFOS and CYP2E1 and the consequences for liver function remain understudied. This study aims to define the effects of PFOS in a CYP2E1 dependent context using complementary *in vivo* and *in vitro* approaches. **Methods:** We first examined livers from wild-type (C57BL/6J), PPAR α -KO, and humanized PPAR α transgenic mice exposed to PFOS, ethanol, or both and quantified CYP2E1 expression area and intensity by immunofluorescence. We then used differentiated HepaRG hepatic cell co-culture to investigate the effects of PFOS on cellular respiration and to determine how these responses depend on CYP2E1 activity. Respiration, quantified as oxygen flux, was monitored continuously overnight before addition of PFOS, clomethiazole (a suicide inhibitor of CYP2E1), or both. Oxygen flux was measured for either 24 hours or 7 days during exposure using a Resipher continuous oxygen consumption monitoring system (Lucid Scientific). Mitochondrial membrane potential and ATP content was assessed in differentiated HepaRG cells after 7 days of exposure to PFOS, clomethiazole, or both. All experiments tested increasing doses of PFOS with and without ethanol and clomethiazole. **Results:** PFOS exposure did not alter CYP2E1 expression intensity overall, but it significantly increased the area of CYP2E1 expression surrounding the central vein, indicating a loss of normal zonation ($p < 0.05$). Ethanol increased overall CYP2E1 levels and similarly expanded the expression area ($p < 0.05$ for both effects). In HepaRG cells, PFOS alone or PFOS with ethanol had minimal effects on respiration over 24 hours at doses up to 100 micromolar. Inhibition of CYP2E1 with clomethiazole significantly exacerbated the effect of PFOS on respiration ($p < 0.001$), causing an 18 percent reduction in oxygen consumption at 50 μ M PFOS and a 29 percent reduction at 100 μ M PFOS. Over 7 days, PFOS with and without clomethiazole decreased respiration across all groups. Mitochondrial membrane potential with PFOS alone resulted in no significant change across all doses, but PFOS with CMZ resulted in a decrease of 50 percent with a dose of 0.05 μ M of PFOS ($p < 0.05$). ATP assays indicate PFOS-induced increase in total ATP content without the presence of CMZ at higher doses of PFOS (1, 5, and 10 μ M doses), but in the presence of CMZ, ATP levels are increased at lower PFOS doses (0.1 μ M to 10 μ M). Increased ATP may reflect inhibition of ATP-dependent processes, or a compensatory increase in ATP synthesis. Ongoing experiments are aimed at defining the mechanism underlying this mixture effect. **Conclusions:** These findings provide new insight into PFOS toxicity and highlight a relationship between PFOS and CYP2E1 activity that may have relevance for human exposures.

PS 4504 Impact of Albuminuria on PFAS Kinetics using Physiologically Based Toxicokinetic (PBTK) Modeling

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are persistent environmental contaminants that bioaccumulate and have been linked to diverse adverse health effects. Previous studies demonstrate that serum albumin binding governs the accumulation of PFAS with six or more perfluorinated carbons by retaining them in circulation. In advanced kidney disease such as Diabetic Kidney Disease (DKD), and Chronic Kidney Disease (CKD), glomerular damage and impaired proximal tubular reabsorption contribute to progressive albuminuria, or elevated excretion of albumin through the urine. These conditions are extremely common, especially in those with diabetes. We hypothesized that albumin-bound PFAS would be co-excreted during albuminuria, accelerating their elimination. Using a mechanistic physiologically based toxicokinetic (PBTK) model, we investigated how albuminuria influences PFAS toxicokinetics in mice, testing whether kidney disease fundamentally alters PFAS clearance pathways while accounting for parallel competing distribution and elimination processes. **Methods:** We extended an established PBTK framework

to simulate perfluorohexanoic acid (PFHxA), Perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUdA), perfluorobutanesulfonic acid (PFBS), perfluorohexanesulfonic acid (PFHxS), perfluorooctanesulfonic acid (PFOS) (npfc= 4-10). The novel model incorporates tissue blood flows, membrane permeability, albumin and phospholipid binding, and renal/hepatic transport processes. Wild-type parameters were derived from *in vitro* transporter/binding data and *in vivo* mouse studies. We modeled albuminuria by reducing serum albumin concentrations by 40% and increasing urinary albumin excretion from under 0.02 mg/day to 3 mg/day, to reflect impaired proximal tubular reabsorption. Model performance was evaluated by comparing half-lives, areas under the plasma concentration-time profile (AUC), and urine concentrations between wild-type and DKD phenotypes following single oral doses of 5mg/kg. **Results:** Simulations support the hypothesis that albuminuria accelerates PFAS elimination in a manner proportional to albumin-binding affinity. Following oral administration, the $t_{1/2}$ of PFOA decreased by 25% and AUC by 61%. PFOS showed a 20% reduction in $t_{1/2}$ and 39% decrease in AUC. The strongest effects were observed for PFUdA, with a 31% reduction in $t_{1/2}$. PFBS, which exhibits weak binding to albumin, showed only a 6.6% reduction in $t_{1/2}$ but a 52% decrease in AUC, consistent with primary renal handling of this PFAS species. Additional results to be presented at a later stage. **Conclusions:** These findings suggest albumin-mediated renal elimination as a newly appreciated and key mechanism contributing to PFAS clearance under albuminuric conditions, underscoring the need to incorporate disease-related changes in protein binding and renal function into PBTK models. Future directions include the integration of clinical data from individuals with albuminuria to evaluate model performance and quantify the impact of progressive albuminuria on PFAS exposure in clinical cohorts of patients with DKD.

PS 4505 Presence of Legacy and Emerging PFAS in Human Livers Specimens in the United States from 2000-2024

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are persistent bioaccumulative chemicals linked to liver toxicity, such as hepatocellular injury, steatosis, dysregulated lipid metabolism, and metabolic disease. The widespread use of PFAS has led to near-universal human exposure, with detectable serum levels in 98-99% of U.S. individuals. The National Academies of Sciences, Engineering, and Medicine recommends clinical monitoring for a set of 9 PFAS. However, little is known regarding tissue bioaccumulation in humans despite clear evidence that some PFAS accumulate in protein-rich organs. The liver is a target organ for PFAS-associated toxicity. Therefore, it was hypothesized that measurements of PFAS in serum may underestimate accumulation risks because legacy and some replacement PFAS concentrate in the liver. The goal of this study was to quantify PFAS concentrations in livers from individuals in the U.S. and assess whether hepatic PFAS profiles have shifted over the past 24 years due to legacy phaseouts and the emergence of replacement compounds. **Methods:** A highly sensitive solid-phase extraction liquid chromatography high-resolution mass spectrometry method using ENVI-Carb[™] sorbent was developed to measure a total of 54 PFAS - including legacy compounds, emerging alternatives, and precursors in 211 adult human livers collected between 2000 and 2024 to quantify liver PFAS profiles. Regression analyses were used to assess temporal trends in hepatic PFAS and evaluate the influence of sampling year, age, sex, and liver health status on individual PFAS and the sum of total PFAS concentrations per individual (Total PFAS). Extractable Organofluorine (EOF) was quantified in a subset of liver samples to determine the fraction of organofluorine not explained by targeted PFAS measurements. **Results:** PFAS were detected in 210 individuals, with 25 compounds, including linear and branched isomers, found in ≥ 3 livers and 15 compounds found in ≥ 30 livers. Total PFAS decreased 94% over the 24-year period, corresponding to 68% after adjusting for covariates. Since 2019, a ~950-fold variability in concentration was observed, and the PFAS profile in liver shifted from sulfonates and carboxylates to proportionally more sulfonamides and fluorotelomers. Multivariate linear regression analyses for PFAS detected in ≥ 30 ($n=15$) of the 211 human livers sampled resulted in sampling year being the strongest predictor of hepatic PFAS concentrations - associated with 13/15 compounds and total PFAS. The largest decade-scale declines were observed for 8:2 fluorotelomer sulfonic acid (8:2 FTS, -44%) and linear-perfluorooctanesulfonic acid (L-PFOS, -39%), while a 7% decade-scale increase was observed for 9-Chlorohexadecafluoro-3-oxanonane-1-sulfonic acid (9ClPF3ONS) ($p < 0.01$). Age was positively associated with several long-chain PFAS, which is consistent with years-long elimination half-lives. Males had higher PFUdA, PFDoA, and 9ClPF3ONS concentrations than females, whereas females had higher 8:2 fluorotelomer sulfonic acid concentrations than males. Metabolic dysfunction-associated steatotic liver disease (MASLD) was associated with lower concentrations of perfluorononanoic acid (PFNA, -12%), PFUdA (-18%), PFDoA (-24%), L-PFOS (-22%), branched (Br)-PFOS (-26%), 8:2 FTS (-27%), 9ClPF3ONS (-8%), and total PFAS (-23%) ($p < 0.05$). MASLD was also associated with lower concentrations of PFAS with a molecular weight range of 500-600 and/or > 600 g/mol, and an npfc of 8, 10, and 11. EOF was detected in all measured specimens, with a mean concentration of 184 ± 151 ng F/g liver (range: 50-304 ng F/g). The data illustrate that targeted PFAS comprised $< 10\%$ of

EOF, revealing that a substance-by-substance regulatory approach likely underestimates exposure. **Conclusions:** Despite two decades of regulatory action, these results highlight the human liver as a critical tissue-accumulation reservoir for both legacy and emerging PFAS and demonstrate the pressing need to expand regulatory oversight beyond currently controlled substances.

PS 4506 Oxidative Stress as a Mediator of Long-Chain PFAS-Induced Mitochondrial Impairment

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are a large group of man-made chemicals that are made of a chain of carbon (C) atoms with multiple fluorine (F) atoms. Among all the PFAS, long-chain PFAS (C>6) are more bioaccumulative, persistent, and toxic. Although some long chain PFAS production has phased out, long-chain PFAS such as perfluorooctane sulfonate (PFOS, C-8), perfluorodecanoic acid (PFDA, C-10), and perfluoroundecanoic acid (PFUnA, C-11) are of concern because they persist in the environment and bioaccumulate in fish tissues, which are consumed by humans and pose a serious threat to public health. PFAS exposure is associated with developmental toxicity, developmental neurotoxicity, carcinogenicity, endocrine disruption, and immunotoxicity. Growing evidence suggest that PFAS can disrupt oxidative balance, impair mitochondrial function and alter neurotransmitter systems. However, the molecular mechanism of long chain PFAS on mitochondrial impairment via oxidative stress such as generating reactive oxygen species (ROS) is not well documented. The hypothesis of this study is long-chain PFAS may increase production of reactive oxygen species (ROS) and oxidative stress that can cause mitochondrial impairment. Therefore, this study has been designed to evaluate the effects of long-chain PFAS induced mitochondrial impairment through oxidative stress in developmental neurotoxicity. **Methods:** This study utilized zebrafish (*Danio rerio*) embryos to investigate the developmental neurotoxicity of these compounds, with a focus on oxidative stress markers and mitochondrial distributions. Zebrafish embryos were exposed to PFOS, PFDA, or PFUnA at concentrations of 0, 0.004, 0.04, 0.4, 4, or 40 ppb (µg/L) from 1 to 72 hours post fertilization (hpf). Oxidative stress markers such as reactive oxygen species (ROS) were measured using H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate), thiobarbituric acid reactive substances (TBARS) as an indicator of lipid peroxidation, antioxidant activity such as catalase were measured using colorimetric assay at 120 hpf. Additionally, the relative mitochondrial content was assessed using MitoTracker™ Red CMXRos at 120 hpf. Larval head region was considered for measuring ROS and mitochondrial distribution. **Results:** All the three PFAS showed increased levels of ROS in all concentrations, with the exception of 0.004 ppb, where PFDA and PFUnA did not show significant changes. For all the PFAS compounds, TBARS levels also increased in almost all the concentrations compared to 0 ppb group. The results revealed a general decline in antioxidant enzyme activity across PFAS treatments compared to the 0 ppb group. The activities following PFDA exposure caused reductions at all tested concentrations, whereas the other PFAS showed a different response. Finally, relative mitochondrial content showed changes when compared to the 0 ppb group in PFOS and PFDA. **Conclusions:** These findings suggest that PFAS exposure increases oxidative stress through the induction of ROS generation resulting in lipid peroxidation and disruption of the oxidative balance, leading to mitochondrial impairment. These mitochondrial impairments can cause bioenergetics failure. However, future studies are needed to further investigate the molecular mechanisms underlying long-chain PFAS-induced oxidative stress and their role in mitochondrial impairment during developmental period.

PS 4507 Early-Life PFAS Exposure Impairs Mitochondrial Bioenergetics and Dopaminergic Development in Zebrafish

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are synthetic chemicals known for their persistence, bioaccumulation, and resistance to degradation. Long-chain PFAS such as perfluorooctanoic acid (PFOA; C8) are recognized neurotoxicants, but the safety of newer short-chain replacements, including hexafluoropropylene oxide dimer acid (GenX; C6) and perfluorobutanoic acid (PFBA; C4), remains uncertain. These compounds can cross the blood-brain barrier and may interfere with energy metabolism during development. Because mitochondria are critical for neuronal differentiation and redox balance, their disruption could impair dopamine signalling and increase susceptibility to neurodegenerative outcomes. This study examined whether exposure to PFAS of varying chain lengths during early development alters mitochondrial function and dopaminergic integrity in zebrafish (*Danio rerio*), a model organism widely used to study developmental neurotoxicity. **Methods:** Embryos were exposed to 0, 0.04, 0.4, 4, or 40 ppb of PFOA, GenX, or PFBA from 1 to 72 hours post-fertilization. Mitochondrial content and distribution were visualized with MitoTracker CMXRos fluorescence imaging, and adenosine triphosphate (ATP) levels were measured to assess mitochondrial bioenergetics. Monoamine oxidase (MAO) activity, dopamine (DA), and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were quantified using ELISA. Tyrosine hydroxylase (TH)-positive dopaminergic neurons in forebrain and midbrain regions were counted from confocal images, and cell death was evaluated using Sytox Blue staining. Data

were analyzed using one-way ANOVA with Least Significant Difference (LSD) post hoc tests ($p < 0.05$). **Results:** PFAS exposure produced chain-length-independent mitochondrial and dopaminergic impairments. Mitochondrial content and ATP levels were significantly reduced in PFOA- and GenX-treated larvae (0.4-40 ppb) and even at 0.04 ppb PFBA, indicating high sensitivity to short-chain PFAS. Elevated monoamine oxidase activity and increased DOPAC levels at higher concentrations suggested enhanced oxidative dopamine turnover, while dopamine levels declined in PFOA- and PFBA-treated larvae (0.4-40 ppb) and at 4 ppb GenX. Confocal imaging revealed fewer TH-positive neurons and smaller forebrain and midbrain regions in high-dose PFOA and GenX groups, reflecting dopaminergic developmental deficits. Increased Sytox Blue fluorescence confirmed higher neuronal death in corresponding exposure groups. **Conclusions:** These findings demonstrate that early-life exposure to both long- and short-chain PFAS disrupts mitochondrial bioenergetics and impairs dopaminergic development. Mitochondrial dysfunction appears to be a key mechanism linking PFAS exposure to developmental neurotoxicity. Notably, short-chain PFAS elicited effects at low concentrations, challenging the assumption that they are safer alternatives. This work provides mechanistic evidence relevant to developmental, neurotoxic, and regulatory toxicology, emphasizing mitochondrial disruption as a sensitive biomarker for PFAS-induced neurotoxicity. Ongoing confocal analyses of larval brain mitochondria aim to further define these mechanisms.

PS 4508 Perfluorooctanoic Acid (PFOA) exposure accelerates *Drosophila melanogaster* growth and development.

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are persistent environmental pollutants that pose a serious threat to global public health. Perfluorooctanoic acid (PFOA), the most prevalent PFAS, contaminates ecosystems across all seven continents and affects the water supplies of over 100 million U.S. residents. With PFOA exposure linked to numerous human diseases, there is an urgent need to understand the molecular mechanisms by which this molecule affects human and environmental health. **Methods:** In this study, we used the reproducible and highly predictable developmental events that occur during *Drosophila melanogaster* juvenile development to investigate how PFOA influence animal growth, development and sexual maturation. Using a combination of genetics, RNA-seq, and semi-targeted metabolomics, we examined PFOA treated animals for defects in developmentally-regulated changes in gene expression programs and metabolite abundance. In addition, we supplemented this multiomic approach with examination of mitochondrial metabolism and real-time monitoring of juvenile metabolism using high-resolution biocalorimetry. **Results:** Our phenotypic analysis revealed that PFOA exposure significantly accelerated larval development, with concentrations as low as 36 nM inducing precocious activation of the metamorphic gene expression program. Moreover, we found that these changes in gene expression were dose responsive, with the highest PFOA concentrations inducing sexual maturation nearly a day earlier than controls. Intriguingly, this accelerated growth rate had no negative effect on final body mass, with the highest PFOA concentrations inducing a significant increase in female body mass - a result that hints at an increase in the overall biosynthetic rate of PFOA exposed animals. Consistent with this finding, high-resolution biocalorimetry revealed a nearly 40% increase in heat production from PFOA exposed larvae. **Conclusions:** Together, our findings demonstrate that PFOA profoundly affects *Drosophila* development and establish the fruit fly as a powerful New Approach Methodology (NAM) for studying PFAS toxicity. Our results also suggest that PFOA contamination may have far-reaching ecological consequences, as induction of premature sexual maturation in insects would inevitably lead to altered fitness and survival in the wild.

PS 4509 Persistent Effects of PFAS on Marine Medaka (*Oryzias latipes*) Immune Competence

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are persistent environmental pollutants used in many industrial applications, including the production of non-stick cookware, water-repellent fabrics, and firefighting foams. Their carbon-fluorine bonds make them extremely resistant to degradation, making them known as "forever chemicals". The toxicity of PFAS has been extensively studied over the last years, particularly for legacy compounds such as perfluorooctanoic acid (PFOA), which have been linked to immunotoxicity, endocrine disruption, hepatotoxicity, metabolic dysfunction, and carcinogenesis. Although PFOA has been phased out in many countries due to environmental and health concerns, industry replacements like 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propanoic acid (GenX), a short-chain alternative to PFOA have emerged in production and use. While these replacements were originally considered less hazardous, growing evidence suggests they pose significant risks, particularly concerning developmental toxicity and immune function. A major gap in PFAS research is understanding the long-

term effects of early-life exposure on immune competence. The marine medaka (*Oryzias melastigma*) is a well-established model for addressing this gap due to its rapid development, well-characterized immune and endocrine systems, and relevance to aquatic toxicology. **Methods:** Marine medaka larvae were sourced from the TAMU-CC husbandry colony and subjected to PFAS exposure from 12 days post-hatch (dph) until 19 dph. Treatment groups included control, PFOA, and GenX at 0.5 ng/mL. These concentrations were selected for their relevance to human prenatal and postnatal exposure levels via maternal cord blood, breast milk, and other early-life sources. After exposure, larvae were raised until 4 months post-hatching and monitored for long-term outcomes, including immune competence through host resistance assays with *Edwardsiella piscicida* (n=15) and leukocyte profiles via flow cytometry (n=5). For transcriptomic analyses, RNA was extracted from female *O. melastigma* livers (n = 3-5). RNA-seq reads were aligned to the *O. melastigma* reference genome using HISAT2, and gene-level counts were obtained with featureCounts. Differential expression analysis and variance-stabilized visualization were performed in R using DESeq2. Pathway enrichment analysis was done using gProfiler. **Results:** Developmental exposure to PFOA and GenX resulted in reduced long-term survival and significant decreases in lymphocyte and granulocyte counts in adult females, but not in males. Preliminary RNA-seq results revealed clear, treatment-specific transcriptional responses in medaka liver following PFOA and GenX exposure. Variance-stabilized counts of the top 50 most carryable genes showed strong clustering by treatment group. Most PFOA exposed samples displayed a consistent pattern of downregulation. In contrast, GenX exposed fish showed a different pattern characterized by more frequent upregulation. These early patterns suggest exposure-dependent changes in liver gene expression and point towards potentially different effects between PFOA and GenX that may involve immune- and stress-related pathways. **Conclusions:** Collectively, these results demonstrate that short-chain replacement PFAS do not represent a biologically benign alternative; indeed, GenX evoked immunotoxic outcomes equal to or stronger than those elicited by PFOA. These findings align with the developmental origins of health and disease framework and highlight the importance of early-life exposure assessments in PFAS risk characterization. The integrated organismal, cellular, and transcriptomic evidence presented here supports the urgent need to re-evaluate regulatory assumptions regarding the safety of replacement PFAS and underscores the value of medaka as a sensitive model system for uncovering long-term immune disruption that may otherwise remain undetected. In a broader context, these data reinforce that PFAS-induced immune toxicity is not limited to legacy compounds and that continued use of structurally similar alternatives may perpetuate public health and environmental risks persistently across the life-span.

PS 4510 PFAS-mediated eicosanoid changes in a mouse model of pancreatic cancer

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Background and Purpose: Pancreatic cancer is predicted to become the second leading cause of cancer deaths in the US by 2030. Obesity, diabetes and chronic pancreatitis have been identified as potential risk factors in pancreatic cancer development. In rodents, PFOA is associated with diabetic outcomes and pancreatic cancer, while in humans, studies have linked PFOA exposure to diabetes, obesity, and pancreatic cancer. Previously, using the LSL-KRas^{G12D}; Pdx-1-Cre (KC) mouse model of pancreatic cancer, we have shown that PFOA accelerated pancreatic lesion development and altered several lipid classes in the pancreas, with thromboxane B2 (TXB2), a proinflammatory eicosanoid, being the most upregulated lipid. In humans as well as animal models, dysregulation of eicosanoid species have been associated with pancreatic cancer. However, to date, few studies have examined the link between PFAS exposure and changes in eicosanoid levels. As our previous lipidomic analysis profiled only a limited number of eicosanoids, in the present study, we evaluated additional COX and LOX-derived eicosanoids to determine if PFOA and its replacement chemical, GenX, alters their production in the pancreas of KC mice. **Methods:** Male and female KC mice were exposed to tap water (control), or water containing 1ppm PFOA or GenX beginning at 8 weeks of age through 6 months of age when tissues were collected. Approximately 10 mg of each pancreatic sample was processed for determination of eicosanoids using Ultra Performance Liquid Chromatography (UPLC) and tandem mass spectrometry (MS/MS; Agilent), using a dynamic MRM method. The COX/LOX MassSpec LCMS standard panel (Cayman Chemical) containing 9 primary eicosanoids [6-keto Prostaglandin F1α (6-keto PGF1α), TXB2, Prostaglandin F2α (PGF2α), Prostaglandin E2 (PGE2), Prostaglandin D2 (PGD2); 12(S)-HHTRE, 15(S)-HETE, 12(S)-HETE, and 5(S)-HETE] was used to quantify pancreatic eicosanoid levels. Expression of enzymes involved in eicosanoid synthesis were evaluated by qPCR, Western and IHC analyses. **Results:** All 9 eicosanoids profiled were detected in the pancreas of all experimental groups. Following exposure to 1 ppm PFOA, levels of 6-keto PGF1α, TXB2, PGE2 and PGE2 were found to be significantly elevated relative to control KC mouse pancreas. Conversely, exposure to 1 ppm GenX resulted in elevated levels of PGF2α, 12(S)-HHTRE, and 15(S)-HETE in the pancreas relative to control KC mice. Supporting an elevation in eicosanoid species by PFAS, Cox-1 levels were found to be increased in the pancreas. Further, in addition to endothelial cells, IHC analysis identified Cox-1 expression in PanIN lesions, infiltrating immune cells, and the stroma in the pancreas of KC mice. **Conclusions:** Environmental factors are postulated to play a major contributing role in pancreatic cancer development and progression as inherited genetic factors are associated with only a small percentage of pancreatic cancer cases. Functioning

as lipid signaling molecules, several eicosanoids have been implicated as drivers of pancreatic cancer by influencing cell proliferation, inflammation and immunosuppression. While GenX was introduced as a replacement chemical for PFOA, similar to PFOA, GenX also increased the incidence of pancreatic acinar adenomas and carcinomas in a 2-year rodent bioassay. Our results demonstrate that both PFOA and GenX increase levels of eicosanoids that are proinflammatory and protumorigenic. Our results suggest that further research directed at understanding the mechanistic link between PFAS-mediated changes in eicosanoids and pancreatic cancer progression is necessary.

PS 4511 Cluster of differentiation 36 (CD36) has a modest effect on perfluorooctanesulfonic acid (PFOS)-induced liver alterations and deposition

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Background and Purpose: Perfluorooctanesulfonic acid (PFOS) is a synthetic perfluoroalkyl substance widely present in the environment that is persistent and associated with adverse human health effects. PFOS exposure in humans is associated with elevation of serum liver injury markers and liver disease. In preclinical rodents, such as mice, PFOS administration markedly increases liver weight. Cluster of differentiation 36 (CD36) is a membrane glycoprotein that facilitates the import of long-chain fatty acids into cells and contributes to lipid accumulation within the liver. Because the chemical structure of PFOS closely resembles a long-chain fatty acid, it has been hypothesized that CD36 may facilitate PFOS uptake into the liver, and that CD36 is a critical mechanism for PFOS uptake and adverse liver effects. *In vitro* and modeling studies point to possible PFOS binding interactions with CD36, and CD36 has been strongly suggested in the literature as a relevant mechanism for PFOS uptake into the liver. **Methods:** To investigate *in vivo* CD36 relevance, wild-type (WT, C57BL6/J) or CD36 mice with a global deletion (CD36^{-/-}, B6.129S1-Cd36tm1Mfe/J) between the ages of 7-9 weeks were administered vehicle (0.5% Tween20 in PBS) or PFOS (10 mg/kg in 0.5% Tween20 in PBS) by oral gavage for seven days. **Results:** PFOS administration increased liver-to-body weight ratio by 66% in WT mice and 72% in CD36^{-/-} mice. It also increased total liver lipids concentration similarly in WT and CD36^{-/-} mice by ~97%. PFOS administration elevated liver triglyceride concentration by 62.3% in WT mice and by 19% in CD36^{-/-} mice. PFOS concentrations in the liver were similar between WT and CD36^{-/-} mice, suggesting that deletion of CD36 does not significantly affect PFOS uptake into the liver. Preliminary proteomic analysis identified overlapping PFOS-induced upregulation of proteins involved in lipid metabolism and oxidative processes in both genotypes within the liver. **Conclusions:** Ongoing proteomics analyses will help further clarify the extent and specificity of CD36's involvement in PFOS-associated liver responses. Overall, these preliminary findings suggest that CD36 has a modest effect on PFOS-induced liver alterations and deposition.

PS 4512 Examining sex-specific relationships between PFOS & reward responsivity

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are toxic chemicals detectable in most human blood, but its impacts on human health are unknown. Preclinical models suggest that PFAS exposure downregulates dopaminergic functioning in core reward-related brain regions, which is consistent with an anhedonic phenotype. Further evidence suggests that these effects may be sex-specific, particularly for perfluorooctanesulfonic acid (PFOS). A next step is to begin to test (a) the candidate association between PFOS exposure and reward-related brain activity in humans, and (b) the potential moderation of this association by sex. **Methods:** Forty-three young adults (28 female) had 5mL of blood extracted as part of a larger ongoing study, and blood samples were tested for a variety of PFAS compounds, including PFOS. Given prior literature suggesting sex differences in PFOS in preclinical models, only this compound was analyzed. Participants also completed an EEG recording with a battery of behavioral experiments. Of interest here was one task designed to elicit reward-related brain activity. The task is a simple guessing task where participants win or lose a nominal amount of money on each trial. The primary outcome measure is an event-related potential called the reward positivity (RewP), which is conceptualized as a neurophysiological indicator of the initial evaluation of outcomes as better or worse than expected. Larger RewP amplitude indicates greater neural sensitivity to reward outcomes. Analyses focused on characterizing relationships between sex, PFOS concentration, and RewP amplitude. **Results:** Detectable PFOS levels were available for all 43 participants included in the present analysis. Exposure levels were comparable between female (M=1.47, SD=1.26) and male participants (M=1.84, SD=2.12, t(41)= -0.24, p=0.48, d=-0.23). RewP amplitude was numerically smaller among female participants (M=3.62, SD=2.78) versus male participants (M=5.03, SD=2.80), with a medium effect size, although the group difference was not statistically significant (t(41)=1.58, p=0.12, d=0.51). Next, we calculated the correlation between PFOS level and RewP amplitude separately for female

and male participants. Although not statistically significant, opposing effects were observed, such that there was a numerically negative association among female participants ($r=-0.21$, $p=0.29$) and a numerically positive association among male participants ($r=0.43$, $p=0.11$). Fisher's r -to- z was used to compare the strength of association between male and female participants. A trend-level effect was observed ($z=1.93$, $p=.054$), offering preliminary support for a role of sex-specific processes. **Conclusions:** These preliminary findings offer initial support for clinical translation, such that higher PFOS exposure may be differentially associated with reward-related brain activity among female versus male adults. Ongoing work is being done to better understand links between PFAS compounds and reward processing in humans, with particular interest in the potential implications for anhedonic phenotype and risk for major depressive disorder. It will be of interest to revisit the present analysis within the full cohort for the parent project, which will total 300 young adults.

PS 4513 Perfluorooctane sulfonate exacerbates AHR-mediated toxicity by inhibiting CYP1A and altering its gene expression

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Background and Purpose: Per- and poly- fluoroalkyl substances (PFAS) are persistent environmental pollutants and developmental toxicants used in nonstick coatings, as surfactants, and in firefighting foams. Some PFAS (e.g. perfluorooctane sulfonate, perfluorohexane sulfonate; PFOS, PFHxS) have been shown to inhibit cytochrome p450 (CYP) proteins and (e.g. perfluorodecane sulfonate; PFDS) activate the aryl hydrocarbon receptor (AHR) which regulates many genes responsible for xenobiotic metabolism. Cytochrome p450 1A1 (*cyp1a1*, zebrafish ortholog *cyp1a*) is the most inducible AHR battery gene. Inhibition of CYP proteins can lead to reaction uncoupling and production of reactive oxygen species. Dysregulation of the AHR pathway during embryonic development can lead to "blue sac" disease characterized by pericardial edema and craniofacial malformations. **Methods:** Here we investigated how developmental exposure to PFAS interferes with CYP1A-like activity as induced by a model AHR agonist, Beta naphthoflavone (BNF) *in vivo* using a zebrafish model. We also investigated the enzyme kinetics of isolated human CYP1A1 exposed to PFAS *in vitro*. We used *in silico* molecular docking (AutoDock Vina 1.1.2, AlphaFold 3) to predict affinities of a more extensive selection of PFAS for AHR-relevant proteins as well as possible molecular interactions. **Results:** Co-exposure of 16 μ M PFOS, but not PFHxS, reduced EROD activity as induced by BNF by as much as 56%. This reduction was concomitant with increases in pericardial area and decreases in *cyp1a* gene expression. Human CYP1A1 was inhibited at tested concentrations by PFOS and to a lesser degree 6:2 fluorotelomer sulfonate (6:2 FTS). *In silico* predicted PFAS-CYP1A1 affinities aligned closely with percent inhibition *in vitro*. This suggests PFAS interferes with canonical *cyp1a* expression, CYP1A activity, or both, thus exacerbating toxicity of AHR agonists. **Conclusions:** This has important implications for environmental risk assessments that may underestimate exposure hazards of PFAS and PAH mixtures. Furthermore, this supports that *in vitro* and *in silico* experimentation can make accurate predictions about PFAS-CYP interactions.

PS 4514 PFOS and Mitochondrial Function: Unveiling an Inert Molecule's Metabolic Disruption

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Background and Purpose: Perfluorooctane sulfonate (PFOS) is a manufactured compound that has been accumulating in the soil and water sources around the globe. Despite being initially considered metabolically inert, PFOS has recently been linked to metabolic dysregulation and obesity in humans. However, the cellular mechanisms underlying potential PFOS-associated metabolic disruption are unknown. Given the central role of mitochondria in metabolic control and fuel oxidation, mitochondrial oxidative phosphorylation may represent a potential target of PFOS. Therefore, the purpose of this study is to determine whether mitochondrial respiratory function is affected by PFOS by establishing the dose-response curve across metabolically active tissues (white adipose, liver, skeletal muscle, and cardiac tissues). **Methods:** Maximal mitochondrial respiration was measured by high-resolution respirometry in permeabilized tissue samples from C57/Bl6J mice using glycolytic substrates specific to complex I (CI) or complex II (CII). Dose-response curves were analyzed to estimate 50% inhibition concentration (IC_{50}), Hill coefficient (nH), and maximum inhibition (I_{max}) of maximal CI- or CII- driven respiration by PFOS. **Results:** At physiological PFOS concentrations for humans (0.01 and 2 μ M), CI and CII specific respiration were significantly inhibited in white adipose ($p<0.01$) and cardiac ($p<0.001$) tissues. The liver tissue was significantly inhibited at physiological concentrations for CII-driven respiration only ($p<0.01$). For CI-driven respiration, I_{max} in cardiac (93 $\pm$ 4%) and skeletal muscle (91 $\pm$ 9%) tissues were significantly greater than liver (61 $\pm$ 5%, $p<0.001$) and white adipose tissues (69 $\pm$ 8%, $p<0.001$). The IC_{50} of cardiac (1461 $\pm$ 402 μ M) and skeletal muscle (1433 $\pm$ 689 μ M) tissues with CI specific substrates were lower than those of liver (2426 $\pm$ 492 μ M, $p<0.05$) and white adipose tissues (2593 $\pm$ 736 μ M, $p<0.05$). The cardiac muscle IC_{50} (1217 $\pm$ 220 μ M) was significantly lower than the skeletal muscle (1991 $\pm$ 436 μ M, $p<0.05$) with CII

specific substrates. **Conclusions:** Although IC_{50} values of PFOS on mitochondrial respiration were elevated, this study revealed that PFOS can impair mitochondrial respiratory function, with cardiac and skeletal muscle tissues demonstrating the greatest susceptibility to PFOS toxicity. In addition, physiological levels of PFOS were sufficient to substantially inhibit mitochondrial respiration in cardiac, liver, and white adipose tissues, thus providing a potential underlying mechanism for PFOS induced metabolic disruptions.

PS 4515 Evaluation of Occupational Exposure and Bioavailability of Micropowders of Polytetrafluoroethylene (PTFE): Integrated *In Vivo* and *In Silico* Approaches

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Background and Purpose: Polytetrafluoroethylene (PTFE), a high molecular weight fluoropolymer, is widely used in high performance industrial applications, yet its exposure and toxicological profile remains poorly characterized. Unlike smaller non-polymeric per- and polyfluoroalkyl substances (PFAS), fluoropolymers like PTFE are generally considered inert and non-bioavailable due to the large molecular weight. However, occupational exposure to aerosols of PTFE micropowders (ranging from tens of μ m to hundreds of μ m in size) that may be generated during manufacturing, handling and processing steps—particularly via inhalation and incidental ingestion—raises questions about potential health risks. This study was initiated to address critical data gaps related to PTFE uptake in the gut and resulting bioavailability for the ingestion exposure pathway and PTFE deposition in the lung for the inhalation pathway, with the goal of informing regulatory frameworks that currently lack substance-specific guidance for evaluating risks of potential occupational exposures to fluoropolymers. **Methods:** Two complementary approaches are employed. First, an acute *in vivo* absorption, distribution, metabolism, and excretion (ADME) study was conducted in rodents (following the OECD 417 method principles) using a composite blended PTFE sample representative of four commercially relevant forms (i.e., Suspension Fine Cut, Suspension Free Flow, Emulsion Coagulated, and Emulsion Micropowder). PTFE was administered via oral gavage, and tissue histology and serum filtration were used to assess particle absorption and distribution. Second, *in silico* inhalation dosimetry modeling of the PTFE aerosols of the four representative PTFE samples was performed using the widely used and validated Multiple-Path Particle Dosimetry (MPPD) model to simulate deposition patterns and deposited doses of PTFE aerosols in the head, tracheobronchial and alveolar regions of the human respiratory tract under relevant occupational exposure scenarios. Retained particle doses following pulmonary clearance of particles were also modeled for the tracheobronchial and pulmonary regions. Key input parameters to the MPPD model included PTFE aerosol characteristics (i.e., aerodynamic size distribution, particle density, aspect ratio) that were derived from empirical testing and published literature, as well as exposure characteristics (i.e., average airborne concentrations, exposure duration, exposure frequency) that were assessed from representative worker handling procedures, shift schedules and room conditions. **Results:** Existing literature indicates low oral toxicity and negligible systemic uptake of PTFE in rodents, with no genotoxic or carcinogenic classification. Available literature predicts that PTFE particles $\geq 10 \mu$ m are unlikely to translocate across biological barriers, providing a preliminary indication that micropowders of PTFE used in our study that range between 10 - 600 μ m in particle size are likely to show limited uptake and bioavailability. The *in vivo* study is designed to confirm presence/absence of PTFE uptake in selected tissues, while the *in silico* model provides quantitative estimates of retained PTFE dose and deposition location in the human lung. Preliminary size analysis of the four PTFE samples also indicates that there is expected to be a significant fractional deposition of these aerosolized powders in the upper human respiratory tract (i.e., the head region from nose/mouth through the larynx). These findings will help clarify whether PTFE micropowders pose a realistic risk of bioavailability or inhalation burden and toxicity in occupational settings. **Conclusions:** This integrated study provides foundational data to support differentiation of human health risks of fluoropolymers from those of other non-polymeric PFAS. The results will inform occupational safety standards and contribute to science-based risk assessments for PTFE exposures in occupational scenarios across the PTFE value chain. Given the widespread industrial use of PTFE and the evolving regulatory landscape for PFAS, these findings are critical for establishing appropriate worker health protection measures and guiding future regulatory and policy decisions.

PS 4516 Region- and sex-dependent accumulation of perfluorooctanesulfonic acid (PFOS) in the rat brain revealed by Desorption Electrospray Ionization Mass Spectrometry Imaging (DESI-MSI)

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are a group of persistent organic pollutants known to accumulate in the human body including the brain. Perfluorooctanesulfonic acid (PFOS), the most prevalent PFAS pollutant,

is estimated to have a systemic blood half-life of about 3-6 years in humans and 30 days in rodents. Although PFOS levels in the blood have decreased over time in the United States, the half-life of PFOS and other PFAS in the brain is unknown. Furthermore, how PFOS is distributed throughout the brain is also poorly understood. Because PFOS and other PFAS have potential links to various neurodegenerative and neuropsychiatric disorders such as Alzheimer's disease, attention deficit hyperactivity disorder, and major depressive disorder, it is important to understand if PFOS accumulation corresponds with brain regions associated with these conditions. Previous research has demonstrated that PFOS alters neurotransmitter levels in brain regions including the prefrontal cortex, dorsal striatum, ventral striatum, ventral midbrain, and hippocampus. We tested the hypothesis that PFOS accumulation in the brain leads to altered neurotransmitter levels, resulting in neurotoxicity. Here, we aimed to characterize the region- and sex-specific accumulation of PFOS to better understand its potential for neurofunctional disruption. We utilized Desorption Electrospray Ionizing-Mass Spectroscopy Imaging (DESI-MSI) to visualize PFOS accumulation in various regions of the brain. **Methods:** Three-month-old male and female Sprague-Dawley rats were dosed with a single bolus of 10 mg/kg PFOS or 10% DMSO vehicle via oral gavage. Brain, liver, blood, and cerebrospinal fluid (CSF) were collected 1, 3, 7, 28, and 112 days post-treatment. One hemisphere of the brain was flash-frozen in a 2-methylbutane bath surrounded by dry ice and stored at -80°C until cut. The remaining tissue was analyzed with high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) for PFOS quantification. The other hemisphere was sectioned coronally at 50 µm on a cryostat for DESI-MSI imaging conducted on a SYNAPT XS mass spectrometer (Waters Instruments) paired with a DESI XS Ion Source. Data were analyzed qualitatively using MassLynx Waters software v.4.2 to determine relative PFOS intensity across brain regions. **Results:** PFOS accumulated in multiple brain regions of male and female rats with distinct region- and sex-dependent patterns. The dorsal striatum exhibited the highest overall PFOS accumulation in both sexes, corresponding to its role in movement control and habit learning. Males displayed higher PFOS concentrations in the ventral striatum, a region associated with reward and motivation via the mesolimbic pathway, while females exhibited greater accumulation in the hippocampus, amygdala, thalamus, and hypothalamus; regions involved in memory encoding, emotional regulation, sensory relay, and hormone control. Females also exhibited reduced PFOS elimination over time, indicating greater retention relative to males. Notably, PFOS preferentially accumulated in gray matter rather than white matter, suggesting region-specific vulnerability of neuronal tissue. These results collectively demonstrate that PFOS distribution in the brain is both region- and sex-dependent, with implications for distinct neurofunctional outcomes. **Conclusions:** This study expands the characterization of PFOS distribution in the brain, confirming region-specific and sex-dependent accumulation patterns. The dorsal striatum, hippocampus, and hypothalamus exhibited particularly high PFOS retention, with marked differences between males and females. The preferential accumulation in gray matter suggests vulnerability of neuronal regions involved in motor, emotional, and cognitive regulation. Future studies will analyze additional brain regions of interest (prefrontal cortex, ventral tegmental area, substantia nigra), increase sample size, perform quantitative PFOS analysis in micro-dissected brain tissue (UHPLC), determine PFOS half-life, and compare findings to chronic PFOS exposure models. These data provide a foundation for understanding how persistent PFOS exposure may contribute to sex-specific neurotoxic mechanisms and neurodegenerative risk.

PS 4517 Perfluoroalkyl Substances (PFAS) Exposure Exacerbates Metabolic Dysfunction in Alcohol-Associated Liver Disease

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Background and Purpose: Polyfluoroalkyl substances (PFAS) are persistent environmental pollutants that are widely found in industrial applications and consumer products. Due to their strong perfluoroalkyl bonds, these substances bioaccumulate in humans and have been shown to disrupt hormone production, impair reproduction and development, and promote tumorigenesis. In the liver, studies showed that PFAS disrupt hepatic metabolism, increase oxidative stress, inflammation, and lipid accumulation. Similarly, alcohol consumption leads to alcohol-associated liver disease (ALD), by impairing mitochondrial fatty acid oxidation, VLDL secretion, and elevating lipid deposition. Nevertheless, approximately 35% of alcohol drinkers develop to advanced stages of liver disease, suggesting additional factors- including environmental exposures- further contribute to its progression. Emerging evidence suggests that environmental toxicants, including PFAS, further exacerbate alcohol-induced hepatotoxicity and ALD. However, the distinct and similar impacts of PFAS exposure and alcohol alone, or their combination, in inducing liver disease remains under characterized. **Methods:** Wild-type C57BL/6J mice are pre-exposed to a mixture of PFAS compounds containing PFOA (1mg/L), PFOS (1mg/L), and GENX (2mg/L) at the concentration of occupational doses in drinking water for 28 days. They are then subjected to a chronic plus binge alcohol model with concurrent PFAS mixture maintained throughout. Serum and liver tissues are collected for histological analysis, evaluation of liver injury markers, assessment of lipid metabolism, and fibrosis. **Results:** PFOA and PFOS accumulate in both serum and liver, whereas GENX is detected only in serum at minimal levels. PFAS exposure does not affect food intake and body weight during pre-exposure and alcohol feeding. Both alcohol consumption and PFAS exposure induces liver injury. PFAS-exposed mice exhibit enlarged livers and exacerbate alcohol-induced steatosis by further elevating hepatic triglyceride and cholesterol, while significantly reducing serum cholesterol. Sirius

Red staining reveals increased portal myofibroblast in both alcohol- and PFAS-fed groups. **Conclusions:** Occupational-level PFAS exposure accumulates in the liver and exacerbates ALD by increasing hepatic lipids and fibrosis.

PS 4518 Effects of PFOA on Physiology and Locomotor Behavior on *Drosophila melanogaster*

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are characterized by a hydrophobic perfluorinated alkyl chain—typically 2 to 16 carbon atoms in length—attached to various functional groups. These compounds are highly persistent in the environment and have been shown to bioaccumulate in brain regions such as the hippocampus, hypothalamus, and pons/medulla, where they are associated with disruptions in neural and hormonal homeostasis across multiple species. However, the long-term behavioral and physiological consequences of PFAS accumulation remain poorly understood. *Drosophila melanogaster* serves as an ideal model organism for addressing these knowledge gaps due to its genetic tractability, conserved neurobiological pathways, and the availability of well-characterized and reproducible behavioral assays. We hypothesized that PFAS accumulation in the brain induces neurotoxic effects by impairing neurotransmitter signaling, thereby disrupting overall fly physiology. To test this hypothesis, the present study investigates the effects of a long-chain PFAS, perfluorooctanoic acid (PFOA), on survival, reproductive capacity, and locomotor activity in *Drosophila melanogaster*. **Methods:** For the survival assay, male and female *Drosophila melanogaster* (Canton S strain), 2-5 days old, were exposed for 28 days to PFOA concentrations ranging from 1 µM to 1 mM in their culture media. Flies were transferred to fresh food vials every 7 days, and the number of surviving flies per vial was recorded. Reproductive capacity was assessed by placing 10 mating pairs in vials containing food supplemented with 100 µM, 300 µM, 700 µM, or 1 mM PFOA for 3 days. The number of offspring was counted over 7 days following the first egg hatching. Locomotor performance was evaluated using the Rapid Iterative Negative Geotaxis (RING) assay after 14 days of exposure to PFOA concentrations ranging from 1 µM to 1 mM. The number of flies reaching the 4 cm mark within 3 seconds was recorded as a measure of climbing ability. **Results:** Our findings indicate that PFAS exposure affects survival in a concentration- and sex-dependent manner. Reproductive output decreased significantly with increasing PFAS concentrations. No egg hatching was observed at 700 µM. Interestingly, at 1 mM, egg hatching was delayed rather than completely inhibited. Locomotor ability declined in a dose-dependent fashion, with the most pronounced reduction observed at 300 µM; however, no significant sex differences were detected. **Conclusions:** These results suggest that PFOA may impair survival, reproductive capacity, and negative geotaxis behavior in *Drosophila melanogaster* in a concentration- and exposure time-dependent manner. Further research is warranted to validate these preliminary findings and to elucidate the molecular and physiological mechanisms underlying PFOA-induced neurotoxicity.

PS 4519 Exploring the Role of SIRT1 in PFAS Mediated Steatosis

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are widespread environmental contaminants found in contaminated groundwaters that are often located near industrial manufacturing and military airfield sites. Commercial and industrial use of next-generation (next-gen) PFAS, such as perfluoroalkyl sulfonamides (FASAs) and fluorotelomer sulfonic acids (FTSAs), has replaced legacy PFAS (e.g., perfluoroalkyl sulfonic acid [PFSA], perfluorooctane sulfonic acid [PFOS]). Despite this replacement approach, there remain gaps in our knowledge related to the adverse effects of next-gen PFAS on the liver. Further, Sirtuin 1 (SIRT 1), a protein deacetylase that regulates a wide variety of biological processes, including lipid metabolism, has been noted as a target of PFOS. Thus, it is our working hypothesis that next-gen PFAS (FASAs, FTSAs, and additional PFASs) can inhibit SIRT 1 prompting alterations in the post-translational acetylation landscape, leading to increased lipid burden in hepatocytes. **Methods:** To test this hypothesis, differentiated hepatocyte-like cells (HepaRG) were exposed to an occupational exposure concentration of 25 µM PFAS for 72 hours, and neutral lipids were quantified using Nile Red. Total protein acetylation and SIRT 1 were measured by Western Blot. SIRT1 activity was determined using modified methods from Mitchem et al. *Redox Biochemistry and Chemistry* 2025. *In silico* modelling was utilized to model sites of PFAS-SIRT 1 interaction; screening was performed using FASA (perfluorobutane sulfonamide [FBSA], perfluorohexane sulfonamide [FHxSA]), perfluorooctane sulfonamide [PFOSA]), FTSAs (perfluorobutane sulfonic acid [PFBS], perfluorohexane sulfonic acid [PFHxS], PFOS) and FTSA (1,1,2,2 perfluorohexane sulfonic acid [4:2 FTS], 1,1,2,2 perfluorooctane sulfonic acid [6:2 FTS], 1,1,2,2 perfluorodecane sulfonic acid [8:2 FTS]) PFAS (Autodock vina v1.2, UCSF Chimera X). The SIRT 1 inhibitor EX-527 (10 µM) was utilized as a positive control in all experiments. **Results:** Neutral lipids increased 2-fold following PFSA, FASA, FTSA, and EX-527 exposure in HepaRG cells. Total protein acetylation increased between 13-25% following PFSA exposures while next-

gen FASA and FTSA increased acetylation between 27.5-34%. EX-527 increased acetylation by 50%. Making use of Autodock vina molecular docking software, we find that FASA, FTSA, and PFSA interact within the NAD⁺ cofactor binding pocket of SIRT 1, suggesting PFAS competitively inhibit SIRT 1. Further, we demonstrate that the predicted binding affinities of PFSA, FTSA, and FASA to the cofactor binding pocket positively correlate with PFAS carbon backbone length. These observations were noted independent of functional group. All PFAS docked in the NAD⁺ binding pocket of SIRT 1 with similar orientations, whereby the sulfonic acid and sulfonamide functional groups localized near the conserved adenine ribose binding moiety of NAD⁺ (1-6 Å). Hydrogen bonds and Van der Waal (VDW) interactions were characterized for each PFAS to SIRT 1 pairing and prevailed throughout the NAD⁺ binding pocket. **Conclusions:** Overall, these data suggest that FASA, FTSA, and PFSA negatively impact SIRT 1 at occupationally relevant exposure levels. Our data describe, for the first time, that next-gen PFAS may have greater lipogenic potential compared to legacy PFAS, warranting further research in this area. *In silico* modelling of PFAS with SIRT 1 suggests PFAS may competitively inhibit NAD⁺ cofactor binding, preventing the deacetylation process. The impact of next-gen PFAS on SIRT 1 and additional sites of SIRT 1-regulated protein acetylation remains uncharacterized *in vitro*, making it important for future studies to profile functional outcomes of PFAS exposure and acetylation on metabolic processes that are monitored by SIRT 1, such as *de novo* lipogenesis and fatty acid oxidation. Additionally, other studies should explore the contribution of PFAS exposure on other SIRT1s to better define the PTM landscape associated with steatotic phenotypes in the liver. Overall, our data elucidates a novel mechanism in which emerging and legacy PFAS classes may act to cause metabolic stress in human hepatocytes. This work is supported, in part, by NIH/NIEHS grant R21ES032529 to JL/RM and a T32 ES029074 awarded to JW.

PS 4520 Drosophila melanogaster as a Research Model to Study the Impact of PFAS on Neuroplasticity of Complex Motor Behaviors

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are persistent environmental pollutants known for their stability and bioaccumulative properties, raising concerns about their potential to disrupt biological systems. They tend to notably accumulate in brain regions such as the hippocampus, hypothalamus, and pons/medulla. Despite increasing evidence that PFAS disrupts homeostasis of the nervous system, the long-term behavioral consequences of their accumulation remain poorly understood. This study utilized *Drosophila melanogaster*—an ideal model organism due to its genetic tractability, conserved neurobiological pathways, and well-established behavioral assays. We hypothesized that PFOA accumulation within the brain induces changes in complex motoric behavior. To test our hypothesis, this study investigates to evaluate the effects of exposure to a long-chain PFAS, perfluorooctanoic acid (PFOA), on grooming behavior and acute ethanol tolerance development, two traits associated with neuroplasticity. **Methods:** To assess acute alcohol tolerance development, 2 to 5 days old *Drosophila melanogaster* (Canton S) males and females flies were exposed to 300 µM, 700 µM, and 1 mM PFAS for 14 days. On day 1 of the ethanol tolerance assay the flies were separated on control and experimental group. Control flies were exposed to water vapor while experimental group was exposed to 50% ethanol vapor. On day 2 all flies were exposed to 50 % ethanol vapor. The difference in time to reach 50% of sedated flies was scored. To assess the effect of PFOA on grooming behavior females' flies were exposed for 14 days to 10 nM, 100 nM, 1 µM, and 10 µM concentrations. After exposure, a dusting assay was performed in which flies were coated with Reactive Yellow 86 to induce self-grooming behavior. The amount of dust remaining on each fly after cleaning was measured to assess potential induced neurotoxicity that disrupts circuits responsible for grooming. **Results:** Ethanol tolerance development was significantly affected by PFOA exposure, as indicated by the increase in time to reach 50% sedation in both sexes. Preliminary data also suggest that PFOA exposure impairs normal grooming behavior in a dose-dependent manner, with the most pronounced reduction observed at higher concentrations, particularly at 10 µM. **Conclusions:** These results suggest that PFOA disrupts complex motor behaviors in *Drosophila melanogaster* in a concentration-dependent manner, with no significant differences observed between sexes. Further research is warranted to confirm these preliminary findings and to elucidate the molecular and cellular mechanisms underlying PFOA-induced neurotoxicity.

PS 4521 High-Throughput Prediction of PFAS Binding Affinities with Human Liver-Fatty Acid Binding Protein Using Machine Learning and QSAR Model

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are ubiquitous environmental contaminants known for their capacity to bioaccumulate in humans. Exposure to legacy PFAS, such as PFOS and PFOA have been associated with adverse health effects. However, a far larger number of structurally diverse

novel PFAS are currently in use, yet their bioaccumulation potential remains largely unknown. Experimental evidence shows strong correlations between PFAS binding affinity to liver fatty acid-binding protein (L-FABP) and hepatic PFAS concentrations across species, and pharmacokinetic modeling has further identified this binding affinity as a key parameter governing liver burden. Nevertheless, the vast number of PFAS (>12,000 registered in the U.S. EPA Master List), coupled with the limited availability of commercial analytical standards, presents a major challenge for experimentally determining their binding. Machine-learning-based quantitative structure-activity relationship (QSAR) models provide a promising pathway to bridge this data gap by predicting binding affinities ($K_{d, FABP}$) directly from molecular descriptors. **Methods:** An integrated experimental-computational framework was developed to quantify and predict the K_d FABP. Using an ultrafiltration-based assay, we first conducted a high-throughput experimental evaluation of $K_{d, FABP}$ for both PFAS analytical standards and PFAS extracted and semi-quantified from environmental samples through suspect-screening analysis. These experimentally derived K_d FABP values were then used to construct QSAR models through model comparison, feature selection, and hyperparameter optimization. Key physicochemical properties and structural descriptors were identified. Finally, the optimized model was applied to predict $K_{d, FABP}$ for PFAS listed in the U.S. EPA Master List as well as PFAS generated by artificial intelligence generated PFAS (PFAS-AI). **Results:** The ultrafiltration assay successfully enabled high-throughput measurement of $K_{d, FABP}$ values for a wide range of PFAS, including many compounds lacking commercial analytical standards. Among the machine-learning algorithms evaluated, extreme gradient boosting regression demonstrated the best predictive performance, achieving an R^2 of 0.77 on the test set. Feature importance analysis revealed that descriptors about atomic electronegativity, van der Waals volume, and ionization potential, were key determinants of binding affinity. Applying the optimized model to 9,117 PFAS from the U.S. EPA Master List within the model's applicability domain showed that 14% of these PFAS exhibit binding comparable to or stronger than PFOS and PFOA. Notably, DTXSID40896722, a compound containing three perfluorinated branches, was predicted to have the strongest binding. Evaluation of 76,216 PFAS-AI structures further identified more than 3,500 AI-generated PFAS with exceptionally strong predicted binding, signaling potential concern for future environmental or commercial use. Conversely, 7,567 PFAS-AI demonstrated extremely weak binding affinities, and 94% of these compounds were predicted to be synthetically less complex than current PFOA alternative, following additional evaluation, may serve as promising environmentally benign PFAS replacements. **Conclusions:** This study establishes a high-throughput experimental-computational framework for evaluating PFAS-L-FABP binding affinity. By integrating ultrafiltration measurements with machine learning models and expanding coverage across broad chemical space, including EPA-listed PFAS and AI-generated structures, we identified key molecular descriptors and structural motifs that govern PFAS-L-FABP interactions. The resulting QSAR models offer a powerful tool for PFAS prioritization and for accelerating the screening of emerging and novel PFAS. Moreover, the mechanistic insights gained from this work can inform the rational, *de novo* design of PFAS with reduced bioaccumulation potential, supporting the development of safer and more sustainable fluorinated chemistries.

PS 4522 Toxicological Perspectives on PFAS Exposure Shift: Hepatotoxicity and Gut-Liver Axis Disruption in Adult Zebrafish

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Background and Purpose: When unexpected toxicity is discovered in existing compounds, they are replaced with less toxic alternatives. This substitution strategy creates a unique exposure scenario: rather than isolated exposure to individual compounds, organisms in real-world environments experience continuous transitions from existing chemicals to substitute chemicals. A prominent example of this phenomenon is the replacement of perfluorooctane sulfonate (PFOS), a legacy per- and polyfluoroalkyl substance (PFAS), with shorter-chain alternatives such as perfluorobutanesulfonate (PFBS) and perfluorobutanesulfonamide (FBSA) following global regulatory restrictions. However, studies on existing substitute substances have focused on the single toxicity of the substitute or the combined toxicity with the original substance. Research elucidating the biological effects caused by exposure transition has yet to be established. **Methods:** Using the adult zebrafish model, we evaluated how exposure shift from perfluorooctane sulfonate (PFOS) to its alternatives governs the biological responses in overall hepatotoxicity and gut-liver axis. Four exposure groups were used: a solvent control, PFOS-only, PFOS to perfluorobutanesulfonate (PFBS) transition, and PFOS to perfluorobutanesulfonamide (FBSA) transition. The overall exposure lasted 21 days, and the exposure transition was designed as 1 week of exposure to the PFOS followed by 2 weeks of exposure to the alternatives (i.e., PFBS or FBSA). Dissected liver samples were analyzed for hepatosomatic index (HSI), liver-specific enzyme activities, and histopathology. Individual changes in liver gene expression and intestinal microbiota community profiling were characterized using RNA-seq and 16S rRNA-seq. To elucidate the correlation between changes in gut microbiota composition and liver gene expression, a multi-omics integrated analysis was performed. **Results:** In all exposure groups, a decrease in ALT and AST activity was observed alongside increased HSI, and histopathological analysis confirmed hepatocyte necrosis and lipid accumulation. The PFOS-only group exhibited the most severe liver damage, while the FBSA conversion group showed similar damage; in contrast, the PFBS conversion group demonstrated relatively mild damage. The decrease in ALT/AST suggests reduced the

enzyme production capacity due to extensive hepatocyte damage. Transcriptomic analysis revealed downregulation of genes related to lipid metabolism and the PPAR signaling pathway in both transition groups, indicating that the observed effects in PFOS-only and exposure transition groups are associated with metabolic disruption. Intestinal microbiota dysbiosis occurred in all exposed groups. A multi-omics integrated analysis showed that the PFOS-only group and the FBSA transition group primarily exhibited microbial-gene networks associated with metabolic dysfunction, whereas the PFBS conversion group displayed an integrated profile related to barrier function recovery. These multi-layered changes at the gut-liver level suggest that PFAS toxicity may cause not only direct liver damage but also disruption of the gut-liver axis. **Conclusions:** This study is the first to elucidate the biological effects of PFAS exposure conversion from various perspectives, and show that the gut-liver effects of PFAS exposure switching using multi-omics. Notably, despite switching to PFBS, a relatively less toxic substance, the reduction in toxicity was limited. PFBS exhibited similar levels of gut-liver axis disruption and liver damage as PFOS. This raises the possibility that switching to less toxic alternatives alone may not fully guarantee toxicity reduction. As the results of this study may vary depending on the exposure period and substance, further research is needed.

PS 4523 Chronic apple juice administration enhances per- and polyfluoroalkyl substances (PFAS) elimination and lowers systemic and hepatic burden

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Background and Purpose: Apple juice (AJ) administration can cause a clinically significant reduction in drug bioavailability. This is attributed to inhibition of Organic anion transporting polypeptide (Oatp)-facilitated uptake in the gastrointestinal tract GIT by polyphenols present in AJ (i.e. naringin and hesperidin). Per- and polyfluoroalkyl substances (PFAS) are a class of persistent amphiphilic pollutants that pose serious human exposure concerns, due to impacted drinking water, and have oral bioavailability. Mechanisms by which the GIT absorbs PFAS are unclear, but Oatp-related transport is likely. Previous studies indicate that PFAS are rat Oatp substrates. Our preliminary data indicate that human OAT2B1 transports PFBS and 6:2FTS in HEK293 transfected cells, and uptake is inhibited by rifamycin sv, naringin, and hesperidin. Thus, we hypothesized that administration of a 4X AJ concentrate (AJC) can decrease PFAS absorption in mice, presumably through Oatp inhibition. **Methods:** C57Bl/6J male mice (n=5-6/ treatment group) were administered a single oral dose of a PFAS cocktail (0.1mg/kg of PFOS, PFOA, PFHxS, PFBS and 6:2FTS at equal ratios) followed by 2 daily doses of either AJC or buffer for 7 days. Blood was collected via the submandibular vein 2hrs, 5hrs, 24hrs, 48hrs, and 72hrs post PFAS dosing. Blood and tissues were collected 7 days post PFAS administration. Additional C57Bl/6J male mice (n=6/treatment group) were administered a morning dose of PFAS cocktail prepared in either AJC/0.5%Tween20 or buffer/0.5%Tween20, followed by an evening dose of the same vehicle every day, for 7 days. Blood and tissues were collected after 7 days of PFAS dosing. All samples were processed utilizing QuEChERS extraction method for LC/MS analysis. **Results:** Following the administration of a single PFAS cocktail dose, AJC administration decreased PFOS and PFOA area under the curve (AUC), over 7 days, to 73% and 62% of the controls, respectively. AJC administration did not reduce PFHxS, 6:2FTS, or PFBS AUC, rather delayed absorption. PFBS concentrations decreased 2 hours after PFAS administration to 48% of the controls. PFHxS concentrations decreased 2 and 5 hours after PFAS administration to 30% and 61% of the controls, respectively. 6:2FTS concentrations decreased 2 and 5 hours after PFAS administration to 36% and 59% of the controls respectively. Following the administration of a PFAS cocktail dose, daily for 7 days, AJC administration decreased PFOS and PFOA plasma concentrations to 89% and 67% of PBS controls, respectively. PFBS, PFHxS, and 6:2FTS plasma concentrations were not impacted by AJC administration. In mice that received a single PFAS dose, AJC reduced liver PFOS and PFOA concentrations to 80% and 58% of PBS control, respectively, while in mice that received multiple PFAS doses, liver PFOS and PFOA concentrations were decreased to 75% and 59%, respectively. **Conclusions:** These findings suggest AJC likely impairs transporter mediated absorption and tissue distribution of PFAS, lowering the systemic exposure of PFOS and PFOA in mice. Overall, this study demonstrates the potential of AJC to decrease or delay PFAS absorption, suggesting that Oatp inhibition may be a critical mechanism to interfere with absorption in the GIT, decreasing PFAS bioavailability.

PS 4524 Half-life determination for oral perfluorooctanesulfonic acid (PFOS) in rat brain, cerebrospinal fluid, liver, and serum

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are a group of persistent organic pollutants, termed "forever chemicals" that are ubiquitously found in the environment and virtually in all living organisms, including humans. Exposure to PFAS has been correlated with sex-specific effects including endocrine disruption and motor behavior deficits. Perfluorooctanesulfonic acid (PFOS), the most prevalent PFAS pollutant, is estimated to have a systemic blood half-life of about 3-6 years in humans and 30-60 days in rodents. Although PFOS levels in the blood have been decreasing over time in the USA, the half-life of PFOS and other PFAS in the brain is unknown. Because PFOS and other PFAS have potential links to Alzheimer's disease, attention deficit hyperactivity disorder, and major depressive disorder; it is important to understand if PFOS accumulation corresponds with brain regions associated with these neurological diseases and disorders. Our previous research demonstrated that PFOS changes neurotransmitter levels in a brain region-specific manner in mice. We hypothesized that PFOS accumulation in the brain leads to altered neurotransmitter levels, which result in neurotoxicity. Here, we aim to characterize how PFOS is retained in the brain and cerebrospinal fluid (CSF), which is excreted from the brain, and compare them to each other and to that of the liver and serum. This will determine if CSF and serum PFOS levels are viable biomarkers for PFOS levels in the brain. **Methods:** Three-month-old male (M) and female (F) Sprague-Dawley rats were given a single bolus dose of 10 mg/kg PFOS or 10% DMSO vehicle via oral gavage (n=4 per sex-treatment-timepoint). Brain, liver, blood, and CSF were collected 1, 3, 7, 28, and 112-d post-treatment. Only CSF that had no visible blood contamination was used for analysis. One hemisphere of the brain and one lobe of liver were frozen on dry ice for PFOS quantification. The other hemisphere of the brain was flash-frozen to be used for analyzing the distribution of PFOS in the brain via DESI-MSI. All tissues were stored at -80°C until analysis. Preliminary half-life analysis was conducted using non-linear regression modeling and was used to estimate half-life for each tissue and sex. Data were analyzed using 2-way ANOVA with a Holm-Sidak post-hoc analysis. **Results:** Preliminary analysis of PFOS content (n=3) demonstrated sex-dependent and tissue-dependent deposition and retention of PFOS. Vmax was detected at the d 1 timepoint for serum and was at d 3 for brain, CSF, and liver. At Cmax, the liver had the highest concentration of PFOS and was higher in males compared to females (p-value=0.0234). Serum had the second highest levels of PFOS at Cmax was higher in females compared to males (p-value=0.0096). The brain and CSF did not have sex effects. There were only sex effects in the liver for the 112-d timepoint with males having higher PFOS levels compared to females (p-value=0.0299). The preliminary half-life was highest in the liver for males (42d M, 32d F) and highest in the serum for females (35d M, 41d F). The half-life of the brain (20d M, 26d F) and CSF (21d M, 28d F) were similar and was longer for females compared to males. **Conclusions:** These results demonstrate that PFOS has sex-specific distribution and retention in different organs in the body. The liver has been previously determined to have the highest accumulation of PFOS and our results show that in male rats PFOS levels in the male liver may remain elevated, even after systemic PFOS levels decrease over-time. Interestingly, the half-life of PFOS in the CSF is slightly longer compared to the brain, suggesting the secretion of CSF from the brain may be a mechanism of PFOS removal from the brain and may indicate CSF could be a viable biomarker of PFOS levels in the brain. Future analysis will determine correlations between brain and CSF, liver, and serum levels. Importantly, these findings will allow for better characterize the accumulation of PFOS in the human brain and aid epidemiological studies in assessing how PFOS exposure correlates with different neurological diseases and disorders.

PS 4525 Effects of Early-Life Exposure to PFAS on Adolescent Neuroimmune Activity

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Background and Purpose: Per-/polyfluoroalkyl substances (PFAS) are persistent organic pollutants commonly used in non-stick, oil-resistant, and water-proof food packaging as well as textiles; they are detected in more than 99% of human serum. Perfluorooctane sulfonic acid (PFOS), one of the most ubiquitous PFAS, is associated with altered lipid metabolism, immunotoxicity, and neurodevelopmental disorders. These outcomes all implicate microglia, resident macrophages in the brain critical for development and sensitive to lipid signaling, as key mediators of toxicity. However, developmental effects of PFOS on microglia are not well-characterized, nor is it clear how PFOS alters microglial inflammatory responses. We hypothesized that early-life PFOS exposure alters microglial responses to secondary inflammatory challenges later in life. **Methods:** To test this, pregnant Sprague Dawley dams were exposed to PFOS (0.5 ug/kg BW/day) or vehicle daily via Nilla wafers from gestation (22 days) until offspring weaning (postnatal day, P21). Offspring were weighed daily and development was monitored until euthanasia in adolescence (P42-47). Two

hours before euthanasia, adolescent offspring were given an inflammatory challenge (lipopolysaccharide, LPS, 0.5 mg/kg BW i.p.); sickness behaviors were monitored for two hours until euthanasia. Brains were collected and microglia were isolated from one cerebral hemisphere using CD11b+ magnetic beads. RNA was isolated from both the microglial and non-microglial pools. Five control and six PFOS litters were born over two cohorts, each contributing 2-4 animals per sex that were then distributed across the two inflammatory challenge groups. Final sample sizes ranged from 5-9 animals per sex, PFOS exposure, and LPS challenge. **Results:** Animals exposed to PFOS exhibited reduced weight gain from P7 to P21 relative to their P1 weight. Conversely, PFOS-exposed animals had greater weight gain after weaning, from P28 to P42 relative to P21. Because 'catch-up' weight gain in early development is linked to metabolic dysfunction in humans, lipid and thyroid hormone analyses are planned. Targeted gene expression analysis of non-microglial cells did not detect PFOS effects on expression of genes related to thyroid (Dio2), dopamine (Drd1a), and calcium (Camk1a) signaling or on synaptic regulation (Syt). While previously shown to be altered by PFOS exposure, the lack of PFOS-mediated alterations in expression of these genes in the current study may reflect region-specific responses undetectable in our whole brain samples. Unbiased analyses of the CD11b+ microglial transcriptome is currently underway, with preliminary results indicating widespread gene upregulation in response to LPS that is blunted with PFOS exposure among male rats. **Conclusions:** Our results indicate that perinatal PFOS exposure alters early-life body weight trajectories as well as inflammatory responses during adolescence. Ongoing work explores changes in brain and systemic lipid metabolism. Knowledge generated in these studies may inform regulatory approaches, risk reduction strategies, and/or treatments to address PFAS's significant threat to public health.

PS 4526 Assessment and Determination of Toxicological Endpoints of Sevoflurane Exposure in *C. elegans*

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are a group of nearly 15,000 synthetic chemicals that are often described as "forever chemicals" due to their persistent nature created by strong carbon fluorine bonds. PFASs are known to bioaccumulate in organisms and biomagnify in food webs. Sevoflurane is a volatile PFAS compound used for general anesthesia. Repeated exposure in childhood to sevoflurane has been associated with neurological complications. To investigate the molecular mechanism of action we assessed changes in gene expression in SOD3, a marker for oxidative stress, and several nuclear hormone receptors associated with lipid metabolism in *C. elegans*. **Methods:** Synchronized *C. elegans* were exposed to sevoflurane gas at clinically relevant concentrations (1.3% and 1.7% volume) for 3 hours within sealed glass containers. Worms were then collected at various timepoints (0, 1.5, and 3 hrs) post exposure and RNA was extracted, converted to cDNA, and analyzed via qPCR (Sybrgreen). Expression was normalized to the housekeeping gene, act-1. **Results:** Our data shows that immediately following sevoflurane exposure there was immediate decrease in relative expression of SOD3 gene (0.488±0.128) at the higher concentration of 1.7% and an immediate increase (1.91±0.856) at the 1.3% concentration. NHR-49 had a decreased expression immediately post exposure at both 1.3% (0.749±0.108) and 1.7% (0.614±0.176) sevoflurane concentrations. There was also an initial increase in NHR-80 (1.44±0.113) at the 1.3% concentration. Throughout the three-hour recovery period there was no statistically significant fluctuations in gene expression between time points. **Conclusions:** The data suggests that there is some disruption of lipid metabolism and oxidative stress genes during and hours after exposure to sevoflurane. The decrease in SOD3 and NHRs at higher concentrations suggests a suppressive effect of sevoflurane in lipid regulation and oxidative stress protection. At lower concentrations, increases in these genes suggest a threshold at which the suppressive effects take over from the body's physiologic response to the exposure.

PS 4527 Assessment of Aqueous Film-Forming Foam (AFFF)-Based Mixture of 27 PFAS Compared to the Mixture of Six Regulated PFAS on the Development and Toxicokinetics in Larval Zebrafish (*Danio rerio*)

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Background and Purpose: Concerns regarding unintentional, although inevitable, exposures to chemical mixtures have been mounting. One of the widely known mixtures of per- and poly-fluorinated substances (PFAS)—known to exert adverse effects on environmental and human health—is aqueous film-forming foams (AFFF). Used extensively at airports and military bases, they are a primary source of drinking water contamination with PFAS. PFAS encompass approximately 15,000 chemicals, but only 0.001% of these have restrictions or maximum contaminant levels (MCLs) regulations. Although individual PFAS have been previously studied, less is understood about the effects of PFAS mixtures. The aim of this study is to assess the developmental toxicity and toxicokinetics of a mixture of environmentally-relevant PFAS in larval zebrafish, *Danio rerio*. As many PFAS have been shown to cause oxida-

tive stress, we also investigated the role of Nrf2, a transcription factor that controls the adaptive response to oxidative stress, in mediating these effects. **Methods:** A PFAS mixture was synthesized to mimic the composition and ratios of 27 PFAS previously identified in a legacy AFFF sample acquired through the Massachusetts Department of Environmental Protection, and another mixture with 6 PFAS commonly monitored (PFBS, PFHxS, PFOS, PFHxA, PFOA, PFNA). Homozygous Nrf2a wild-type and mutant (*nrf2a^{h318/-}*) with a mutation in the DNA binding domain crossed with *Tg(ptf1a:GFP)* and *Tg(iris:GFP)* fish were statically exposed to three different sub-lethal concentrations of the 27 PFAS and 6 PFAS mixture equivalent to 0.00005% of the 3% AFFF application concentration (197.52 ppb-low), 0.001% (4.11 ppm-medium), 0.0025% (10.07 ppm-high) and 0.1% methanol as a solvent control. At 4 days post-fertilization (4 dpf), larvae were assessed for fish length, spine shape, craniofacial malformations, pancreas length, islet area, yolk sac area, and pericardial area using brightfield and fluorescence microscopy. Blinded images were analyzed using Image J. PFAS concentrations in stock solutions, exposure media, and the 0- and 4-dpf larvae (normalized to wet weight) were measured by Sciex QTRAP 7500 with ¹³C₁₂ PFOS surrogate standard and respective corresponding internal standards. **Results:** Nrf2a wild-type and mutant zebrafish larvae showed dose-dependent decreases in fish length (*p* < 0.001) and endocrine islet area (*p* < 0.001). Nrf2a mutant fish had a greater decrease in the ratio of exocrine pancreas length to fish length with exposure to the 27 PFAS mixture, but not with 6 PFAS mixture. Internal larval tissue concentrations showed no significant difference between the wild-type and mutant fish, indicating that Nrf2a did not affect PFAS uptake. The PFAS body burdens were highest for sulfonated PFAS than carboxylated PFAS in both mixtures with PFOS, PFHxS, PFHpS, PFNS and PFOA, PFHxA, PFHpA, PFEA in the 27 PFAS mixture while in 6 PFAS mixture were PFOS, and PFHxA. Surprisingly, PFNA (in both mixtures) showed negligible internal concentration in zebrafish larvae. In the 27 PFAS mixture, the exposure media and internal concentrations were both negligible for PFNA, PFTA, and PFTDA—suggesting that carboxylic acids with higher molecular weight may potentially be less likely to cause PFAS-induced toxicity in a mixture context. **Conclusions:** Zebrafish, as a model organism, can shed light on the impacts of PFAS toxicity and toxicokinetics. PFAS mixtures affect pancreatic organogenesis in larval zebrafish, with Nrf2a playing a protective role in growth of the exocrine but not the endocrine compartment. The mixture of 27 environmentally-relevant PFAS was more toxic than the 6 PFAS mixture of those more commonly monitored for, despite the total sum of PFAS in each being comparable. This indicates that the composition of PFAS in an exposure may be more significant than the total concentration of PFAS, suggesting that the current assessments that measure only a few PFAS likely underestimate risk.

PS 4528 Methodological framework for enhancing the reliability of polymer plastic quantification in toxicological research

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Background and Purpose: Micro(nano)plastics (MNP)s are ubiquitous in the environment and have recently been detected in human tissues, including the brain. Their ability to infiltrate biological systems raises concerns about toxicity and long-term health consequences. MNP exposure has been associated with organ dysfunction, reproductive and developmental toxicity, and neurotoxicity. Within the brain, lipids such as cholesterol, sphingomyelin, and docosahexaenoic acid (DHA) are critical for neuronal function. Alterations in lipid turnover or disruption of lipid balance are increasingly recognized as contributors to neurodegenerative disease. This study aims to simulate potential molecular mixtures between the major lipid classes in the brain and plastics by spiking cholesterol, sphingomyelin, and DHA with a mixed plastic polymer standard. We investigate how lipid-polymer mixtures influence molecular detections and how extraction techniques can be optimized to re-separate these components for accurate quantification by mass spectrometry. **Methods:** Our methodological approach employed sequential digestion using potassium hydroxide (KOH) followed by solvent washes (cyclohexane or nitric acid) to degrade lipids while preserving polymer integrity. Samples are then analyzed by pyrolysis gas chromatography-mass spectrometry (Py-GC/MS). **Results:** Our findings demonstrate that pure sphingomyelin generates up to 56% false-positive recovery of polyethylene (PE), which decreases to 7% following KOH digestion. Pure cholesterol produces up to 6% false-positive recovery, which decreases to 0.7% after KOH digestion. DHA increases from 1% false positive to up to 4% after digestion. **Conclusions:** Together, these results illustrate how endogenous brain lipids may interfere with MNP detection in biological matrices and provide a methodological framework for improving the reliability of polymer quantification in toxicological studies.

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Background and Purpose: Microplastics (MPs) are persistent environmental pollutants that have emerged as a growing global health concern. Although MPs are widespread, the biological consequences of MP exposure remain poorly understood, particularly regarding their effects on the immune system. Macrophages play a central role in innate immunity; however, emerging evidence suggests they have limited capacity to phagocytose MPs, potentially leading to cellular dysfunction and dysregulated immune responses. The objective of this study was to evaluate the effects of polystyrene MPs on macrophage polarization and function using human and mouse macrophage cell models. **Methods:** THP-1 human monocytes were differentiated into macrophages with phorbol 12-myristate 13-acetate (PMA) and were polarized toward either M1 (via lipopolysaccharide, LPS) or M2 (via IL-4) phenotypes. Cells were then exposed to 1 µm or 5 µm polystyrene MPs at concentrations of 0, 10, or 100 µg/mL for up to six days. Various endpoints related to monocyte function and polarization were assessed such as cellular metabolic activity, phagocytic capacity, cytokine secretion, and pro-inflammatory gene expression. **Results:** We found that the highest concentration of MPs significantly suppressed THP-1 monocyte growth. Interestingly, exposure to MPs enhanced the metabolic and phagocytic activity of M1 macrophages, suggesting a heightened proinflammatory phenotype. Consistent with this, MP-treated M1 macrophages exhibited increased secretion of IL-1β and TNF-α following LPS stimulation. Conversely, MPs suppressed the secretion of the anti-inflammatory cytokine IL-10 in M2 macrophages, indicating impairment of a pro-resolving phenotype. **Conclusions:** Together, these findings suggest that MPs exacerbate inflammatory responses while suppressing anti-inflammatory pathways, thereby disrupting macrophage polarization dynamics. Complementary studies are currently underway to validate these findings using primary mouse alveolar macrophage-like models. Results from the present study provide important insight into the immunotoxicity of microplastics and will serve as a foundation for future mechanistic investigations.

PS 4530 Allergic Contact Dermatitis Model for Assessing Contaminated Microplastics Isolated by Nanomembrane Technology

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Background and Purpose: Allergic Contact Dermatitis (ACD) is a prevalent inflammatory skin disease affecting approximately 15-20% of the U.S. population and is driven by allergen-specific T-cell activation. Although many allergens have been identified, the potential of microplastics (MPs) to influence allergic skin responses remains largely unexplored. MPs (1 µm-5 mm) are widespread environmental contaminants capable of adsorbing and transporting immune-activating agents such as persistent organic pollutants (POPs), heavy metals, and pathogens. While healthy skin serves as an effective barrier, MPs may penetrate through micro abrasions, follicles, or sweat glands, raising concerns about dermal exposure. Among these contaminants, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a potent Aryl Hydrocarbon Receptor (AhR) agonist that regulates xenobiotic metabolism and skin homeostasis, whereas chronic activation by dioxins can cause pathologies such as sustained inflammation and chloracne. Nickel (Ni²⁺) is a common environmental pollutant and a leading cause of ACD. It is capable of penetrating the skin and eliciting proinflammatory cytokine release. Understanding how these contaminants interact on microplastic surfaces is key to evaluating their role in skin immunotoxicity. **Methods:** To examine how metal-POP co-contamination alters MP toxicity, 2-µm silicon nitride nanomembrane chips (SiMPore, Inc.) were used as a dual filtration and cell-culture platform. Negatively charged 5 µm polystyrene (PS) beads were soaked and aged in mixtures of nickel chloride (NiCl₂) and 1 nM TCDD to create contaminated microplastic (cMP) models. The mixtures were filtered through the nanomembrane chips to retain intact particles while maintaining their chemical and physical characteristics. HaCaT keratinocytes were then exposed directly to the filtered cMPs for 24 and 48 hours to assess cellular effects. Cytotoxicity was evaluated using the PrestoBlue cell viability assay, which quantifies metabolic activity via reduction of resazurin to the fluorescent compound resorufin, serving as an indicator of cell viability and mitochondrial function. Additionally, Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was performed on filtered cMP samples to quantify the extent of Ni²⁺ adsorption (ng Ni²⁺/µg PS). **Results:** Preliminary data indicate that aged cMP containing PS + NiCl₂ + TCDD result in a significant decrease in cell viability, demonstrating enhanced cytotoxicity compared to PS + NiCl₂ alone or the negative control. This suggests that combined and aged pollutant exposure amplifies toxic effects on HaCaT cells. In parallel, preliminary ICP-MS analyses detected quantifiable levels of Ni²⁺ on the PS MPs which may contribute to the observed cellular responses. **Conclusions:** This work establishes a novel nanomembrane-based model for assessing how environmental contaminants interact on microplastic surfaces and influence skin cell responses. Ongoing analyses will clarify how Ni²⁺ and TCDD co-adsorption on MPs modulate AhR signaling, cellular viability, and potential pathways involved in allergic

contact dermatitis. Furthermore, these findings suggest that the surface chemistry of MP, combined with their ability to adsorb environmental pollutants like Ni²⁺ and TCDD, may alter their biological reactivity and potential to contribute to ACD pathogenesis. The nanomembrane filtration-cell culture system offers a powerful, loss-free platform to study microplastic-pollutant interactions under controlled conditions. Future studies will explore how these contaminated MP influence AhR activation, inflammatory cytokine release, and adaptive immune responses relevant to allergic skin disease in *in vivo* mouse models.

PS 4531 Microplastic exposure exacerbates atherosclerosis development in lean LDL receptor-deficient mice

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Background and Purpose: Microplastics (MPs) emerge as ubiquitous environmental pollutants but the adverse effects of MPs on human health are still poorly understood. MPs can be detected in human atherosclerotic plaques and have been associated with a higher risk of cardiovascular disease (CVD) and stroke in humans. However, the impact of MP exposure on the cardiovascular system remains elusive. This study aimed to investigate the effects of MP exposure on atherosclerosis development in low-density lipoprotein receptor-deficient (LDLR^{-/-}) mice. **Methods:** Four-week-old male and female LDLR^{-/-} mice were fed a semisynthetic low-fat diet and exposed to 10 mg/kg body weight MPs via daily oral gavage for 9 weeks before euthanasia. Atherosclerotic lesions in the aortic root and brachiocephalic artery of these mice were analyzed. Single-cell RNA sequencing (scRNA-seq) of the whole aorta was performed to characterize MP-induced changes in cell populations and transcriptional programs. *In vitro* assays using murine and human endothelial cells (ECs) were conducted to investigate the effects of MP exposure on EC gene expression. **Results:** Male and female LDLR^{-/-} mice fed the low-fat diet did not develop obesity phenotype and exposure to MPs did not affect adiposity and circulating lipid profiles in those lean mice. Intriguingly, MP exposure led to significantly increased atherosclerosis in male but not female LDLR^{-/-} mice. scRNA-seq analysis revealed that exposure to MPs affected the proportions and cellular processes of key atherosclerosis-related cell types, especially ECs. Consistently, MP exposure elicited pro-atherogenic gene expression in murine and human ECs *in vitro*. **Conclusions:** Our results demonstrate sex-specific atherogenic effects of MPs in a suitable laboratory animal model and the impact of MP exposure on EC atherogenic gene expression *in vitro*. These findings contribute to our understanding of the association between MP exposure and increased CVD risk in humans.

PS 4532 Microplastics in Edible Tissues of Commercial Fisheries sampled from Major Marine Fish Landing Sites across Coastal States in Nigeria

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Background and Purpose: There is a growing environmental and public health concern regarding plastic pollution, particularly the accumulation of microplastics (MPs) in marine ecosystems, which has significant implications for aquatic ecosystem and human health. In this study, MPs were examined in the muscle tissues of predominant commercial fish and benthic invertebrate species sampled from Nine (9) major marine fish landing sites across six (6) coastal states in Nigeria. **Methods:** Following ethics approval for the study, field expeditions were embarked upon in the wet season (May and June 2025) to identify and sample predominant commercial fish and benthic macroinvertebrate species landed from the ocean across major landing sites in Lagos (Epe, Apapa, Victoria Island and Badagry), Ogun (Iwopin), Ondo (Aiyetoro), Rivers (Oyokoroto), Akwa Ibom (Ibaka) and Cross River (Nsidung) states, Nigeria. The fisheries were transported to the laboratory on ice where taxonomic identification and morphometrics were done before extraction of the tissues. Thereafter, the tissues were digested, MPs therein were extracted, identified and counted with the aid of a stereomicroscope coupled with a digital camera following referenced protocols. The identified MPs were sorted by morphotypes, colour and quantified before spectroscopic identification of the polymers using ATR-FTIR. **Results:** The MPs count in the fish muscle tissues ranged from nd in the Blue Spot Mullet (*Mugil cephalus*) sampled from Aiyetoro, Ondo state to 96.6 particles/g muscle tissue in the Yellow Croacker (*Larimichthys polyactis*) sampled from Badagry in Lagos state. Fragments were the most preponderant MP morphotype followed by fibres and foam while the dominant colours were black, yellow, brown, white and blue. Sole fish (*Solea solea*) had the highest occurrence of fish species across four (4) (Epe, Apapa (Lagos), Nsidung (Cross River) and Ibaka (Akwa Ibom)) of the nine sites with corresponding highest MPs counts and colours (yellow, brown, white and black) in the tissues. MP counts in the macroinvertebrate muscle tissues ranged from 0.4 particles/g muscle tissues in the Guinean mantis shrimp (*Squilla aculeata*) - 43.9 particles/g muscle tissue in the Tiger prawn (*Penaeus monodon*). Fragments and fibres were the predominant MPs morphotypes while black, yellow and brown were the dominant colours in the macroinvertebrate species. Atlantic

Blue Crab (*Callinectes sapidus*) had the highest dominance of macroinvertebrates across seven of the nine sites while Tiger prawn (*P. monodon*) though less dominant, had the highest count of MPs observed across four sites. Polymer characterization of the MPs using ATR-FTIR are ongoing. **Conclusions:** Our results reveal for the first time, the occurrence of MPs in edible muscle tissues of commercial marine fisheries landed across coastal states in Nigeria. The high MPs counts, dominant colours observed (which points to single use plastic sources) poses a great concern to human and aquatic ecosystem health and calls for prompt action to curb plastic litter flows as well as curb dumping at sea.

PS 4533 Assessing the Influence of Nano-Plastic Particle Size on Developmental Toxicity, Bio-Distribution, and DNA Damage Using Chicken Embryos

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Background and Purpose: The World Health Organization emphasizes the importance of quantifying human exposure to micro/nanoplastics (MNPs) <10 µm because the negative health impacts of MNPs increase with decreased particle sizes. Most of the existing studies only address MPs >10 µm, but these MPs are probably excreted directly. MNPs have been detected in the human body, including in human placentas and infant feces, raising particular concern over their effects in sensitive developmental periods. The purpose of this study is to evaluate the developmental toxicity, biodistribution, and DNA damage with three different sizes of commercial fluorescence labeled polystyrene nanoplastics (PS NPs at 50, 100, 500 nm) in early life stage using a chicken embryo model. **Methods:** The PS NPs were injected at day 6 of chicken embryos at concentrations of 1 or 10 µg/ml, and the liver samples were collected on day 18 for measurement of oxidative stress and developmental toxicity endpoints, including LSI (Liver somatic index) and relative REEW (ratio to egg weight to embryo weight). Additionally, mortality was recorded. The biodistribution of the NPs in three different tissues (chicken liver, heart, and brain) was determined by injecting the NPs on day 16, and the tissue samples were collected 24 hours after injection and, fluorescence intensity of the NPs was measured using a plate reader. DNA damage was assessed by comet assay for PS NPs 50 nm and 100 nm. **Results:** The highest mortality (64%) is detected in the chicken embryos injected with PS NPs 50 nm at the concentration of 10 µg/ml. The PS NPs at 50 nm at the concentration of 1 µg/ml induced the highest oxidative stress compared to the control group. The dose dependent mortality of PS 50 nm treatments revealed that the embryonic mortality increased from 28% at 1 µg/ml to 64% at 10 µg/ml. The LSI was lowest in the embryos exposed to PS 100 nm at the concentration of 1 µg/ml, although this difference is not statistically significant. The REEW index determined for the PS 50 nm treatment at the concentration of 1 µg/ml is lowest compared to the control group. These NPs (PS 50 nm, 100 nm, and 500 nm) are mostly deposited in the brain tissues, with the relative abundance when compared to findings in other tissues. The PS NPs 50 nm treatment has induced the highest DNA damage (25.60%, statistically significant), while the PS NPs 100 nm has induced the second highest DNA damage around 22.52%. **Conclusions:** The smallest MNPs, PS 50 nm, caused higher toxicity concern. The PS 50 nm induced greater developmental concern by lowering the REEW. The dose-dependent mortality in the PS 50 nm group demonstrated proportional mortality in response to the doses. The PS 50 nm at the lower dose caused the highest oxidative stress. Additionally, PS NPs 50 nm have shown greater biodistribution in the liver, heart, and brain compared to the PS 100 nm and PS 500 nm. The PS 50 nm caused the highest DNA damage compared to PS 100 nm. The smaller the size, the higher the toxicity concern.

PS 4534 Assessing the toxicity of fragmented virgin and UV-aged polyamide microplastics on human tissue-derived intestinal organoids

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Background and Purpose: Ingestion is considered to be one of the main routes of human exposure to microplastics (MPs), and their potential impact on intestinal health has recently gained significant attention. While MPs have been detected in feces and shown to accumulate in the gut, their toxicity towards intestinal tissues remains poorly understood. Most of the studies often combine simplified models with pristine, uniformly spherical particles, limiting environmental and human relevance. In addition, toxicity testing usually focuses solely on the polymer itself, overlooking that these particles are complex mixtures containing multiple additives that may leach out and cause their own effects. **Methods:** In this study, the toxicity of fragmented polyamide (PA) MPs of two size classes (5 and 10 µm) and their leachates was investigated. Human tissue-derived intestinal organoids were exposed during their growth phase (6 days) to both virgin and UV-aged PA particles, in a dose-response setup (1-100 µg/mL). Copper (II) oxide (CuO) and titanium dioxide (TiO₂) particles served as reference particles. Changes in morphology were tracked through brightfield imaging, while their viability was assessed at the end of the exposure through a cell proliferation (MTS) assay. **Results:** Virgin particles exhibited a

greater overall toxicity compared to aged particles, significantly impairing organoid growth (in terms of size), morphology, and metabolic activity. A particle size effect was also observed. Notably, 5 µm virgin PA particles reduced metabolic activity by 25% even at the lowest concentration tested (1 µg/mL). No significant differences compared to the control group were observed at the same dose for the 10 µm PA. The highest potency of the smallest particles was evident for the aged particles as well, with the viability dropping to 65% at the highest dose (100 µg/mL), compared to 72% for the 10 µm particles. Since the aged particles appeared less potent, it was hypothesized that these effects were driven by chemical additives, which potentially are lost during the aging and milling process (wet milling in 1-propanol). To test this, both virgin and UV-aged PA particles were incubated in 1-propanol for 39 days at room temperature to create a worst-case leaching scenario. Preliminary data show that exposure to the leachate from the aged particles reduced metabolic activity without altering morphology, suggesting that particle-associated chemicals contribute to the observed toxicity. The observed toxicity of the leachate of the UV-aged MNPs suggests that the aging process increases the leaching of chemicals during milling, making the particles themselves less potent, as observed when testing the UV-aged MNPs. **Conclusions:** Our findings highlight the importance of approaching MPs as a mixture, giving emphasis on their physicochemical properties and aging state in order to assess their hazard potential and mode of action. Ongoing research is evaluating the leaching potency of the particles (virgin and aged) in cell culture medium, 1-propanol, and the role of genes associated with organoid development, such as the Hoxa family, on the effects observed.

PS 4535 Neurotoxic effects of pristine and aged micro- and nanoplastics in vitro

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Background and Purpose: Micro- and nanoplastics (MNPs) are increasingly recognized environmental contaminants to which humans are widely exposed through food, water, air, and consumer products. Their detection in human blood suggests systemic distribution and raises concerns about potential accumulation in sensitive organs, including the brain. Although emerging studies indicate that MNPs can reach the central nervous system, the extent, mechanisms, and consequences of brain exposure remain poorly understood. Environmental aging processes may further modify particle properties and toxicity, yet aged MNPs remain underexplored in a human-relevant context. This study therefore examines whether pristine and aged MNPs can interact with the blood-brain barrier (BBB) and affect neuronal function. **Methods:** A human *in vitro* BBB model using human induced pluripotent stem cell (iPSC)-derived brain microvascular endothelial cells (BMECs) cultured on transwell inserts was used to evaluate MNP interactions and barrier function following exposure to pristine and UV-aged PVC, PET, PA and PP particles of varying sizes (10 µg/mL). In parallel, fluorescent PS beads (200 nm and 1 µm) were included to visualize cellular uptake by confocal microscopy. BBB integrity and function were evaluated via transendothelial electrical resistance (TEER), sodium fluorescein permeability, and BMEC metabolic activity. Potential neurotoxic effects were assessed in primary rat cortical cultures exposed to the same MNPs (10 µg/mL), with neuronal activity monitored using multi-well microelectrode array (MEA) recordings after short-term (30 min) and prolonged (21 days) exposure. Neuroinflammatory responses were quantified in BV2 microglial cells by measuring tumor necrosis factor-alpha (TNFα) and interleukin 6 (IL-6) secretion using an enzyme-linked immunosorbent assay (ELISA). **Results:** PS beads (200 nm and 1 µm) were internalized by BMECs, indicating direct particle interaction with the BBB. Across four days of exposure, none of the tested pristine or aged MNPs altered barrier integrity, barrier permeability or metabolic activity. Acute MNP exposure produced no changes in neuronal activity for PVC, PET, PA or pristine PP. UV-aged PP (1-5 and 5-10 µm) reduced neuronal activity, however, responses remained within benchmark ranges. Following 21-day exposure, MNPs induced only minimal changes in network activity. Pristine and UV-aged PET (5-10 µm) and their leachates induced small increases in network bursting, yet all changes remained within expected biological variability. None of the tested MNPs induced TNFα or IL-6 secretion in BV2 microglia after 24-hour exposure. **Conclusions:** This work shows that selected pristine and aged MNPs interact with BBB and neuronal models, but do not cause significant disruption of barrier function, neuronal network activity, or microglial cytokine secretion at 10 µg/mL. Detected changes in neuronal activity were modest and not consistently indicative of toxicity. Collectively, these results provide early evidence that MNPs exert limited neurotoxic and neuroinflammatory effects under the conditions tested, while emphasizing the need for continued research into particle-specific responses and long-term exposure scenarios.

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Background and Purpose: Airborne micro- and nano plastics (MNPs), generated from synthetic textiles, vehicle tires and plastic wastes, are omnipresent in the environment and known to deposit in human lungs. Bioaccumulation of MNPs in lung tissues has been studied, but effect of tobacco smoking on deposition is not well understood. Tobacco smoke is known to contain heavy metals which can get adsorbed to the MNP surface, leading to increased environmental bioavailability. We hypothesized that smoking leads to enhanced chelation of heavy metals with airborne MNPs leading to accentuated lung bioaccumulation, thereby leading to lung pathologies. **Methods:** Age (20-75 years) and sex-matched archival lung tissue samples from smokers (n = 9) and non-smokers (n = 10) were procured and quantified to assess: (a) the levels of MNPs by pyro-GC/MS and (b) heavy metals by ICP-MS. Furthermore, to establish if there exists a correlation between MNP deposition and disease occurrence in current smokers, tissue samples from age-matched patients with COPD (n = 4) and IPF (n = 4) underwent pyro-GC/MS analyses. **Results:** A relatively higher level of MNPs were found in the lungs of smokers (43.67 mcg/gm) as compared to non-smoker (34.53 mcg/gm) upon pyro-GC/MS analyses. We observed a marked increase in the levels of polyethylene (PE; p < 0.0076), polycarbonate (PC; p < 0.0947) and nylon6 (N6; p < 0.0653) polymers in the lungs of smokers as compared to non-smokers. Metal analyses showed a significant (p < 0.0001) increase in the levels of cadmium (Cd) in the lungs of smokers with the levels of deposited Cd being more pronounced for female smokers. Importantly, we observed a strong correlation (p < 0.05) between the amounts of accumulated PE and levels of Cd in the lungs. Pyro-GC/MS analyses did not reveal any relation between MNP deposition and disease occurrence in our study. Furthermore, little to no change in the level of deposited MNPs was observed in the lungs of current versus ex-smokers in disease cohorts. **Conclusions:** Overall, our results show that smoking promotes bioaccumulation of certain MNPs (PE) with heavy metals (Cd) which may lead to adverse lung effects in smokers.

PS 4537 Utilizing Single Particle ICP-MS (spICP-MS) to Measure Uptake, Depuration, and Transfer of Polydispersed Nano-Microplastics (NMPs) in *Daphnia magna*

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Background and Purpose: Current nano- and microplastic (NMP) research often lacks environmental relevance because they rely on spherical, monodispersions rather than the irregular, polydispersed fragments common in nature. As a result, most NMP uptake toxicity studies rely on nominal exposure concentrations calculated from single size particle mass rather than true particle quantification. This analytical gap constrains interpretation of dose-response relationships of NMP based on particle number and hinders assessment of how environmentally-relevant polydispersions of NMPs influence uptake, translocation, and clearance within an organism. To address this, we synthesized a metal-doped tantalum ethoxide poly(methyl methacrylate) (Ta-PMMA) polydispersion (~150-3350 nm) allowing for individual particle measurements in media and organisms using single-particle inductively coupled plasma mass spectrometry (spICP-MS). The goal of this study was to quantify particle uptake, depuration, and maternal transfer of a polydispersed solution of Ta-PMMA NMPs in the aquatic filter feeder *Daphnia magna* utilizing single particle spICP-MS. We hypothesized that (i) polydispersed NMPs exhibit distinct uptake and depuration kinetics compared to monodispersed nanoparticles, (ii) uptake and depuration rates differ among particle size fractions within a polydispersion, and (iii) chronic (21-day) exposures to Ta-PMMA exhibit a dose-dependent increase in particle number in neonates (offspring) and decrease in neonate number. **Methods:** We utilized the novel technique of synthesizing the metal doped Ta-PMMA polydispersion, in which individual particles could be distinguished using spICP-MS. This allowed for quantification of particle number uptake through time across size classes in *D. magna*. First, we exposed *D. magna* to 50 and 0.5 µg/L of Ta-PMMA particles for 24 hours to measure active uptake and determine sizes up took. Second, we exposed of *D. magna* to 50 µg/L of either Ta-PMMA polydispersion or 1.5 µg/L 200 nm silver NP monodispersion for 24 hours, followed by a 24 h depuration in clean media. For this, a subset of *Daphnia* were collected at 3, 6, 12, 18, and 24 h (particle exposure), followed by 30, 36 and 48 h (depuration). This was to compare monodispersion versus polydispersions and individual versus grouped polydispersion exposures. Next, we wanted to explore a chronic low dose exposure and look at potential maternal transfer of particles to neonates. Thus, adult *Daphnia* were exposed for 21 days to 0.1-1 µg /L of Ta-PMMA, where average clutch number, particle size, and number per neonate clutch was measured. **Results:** In the first experiment, *D. magna* exposed to 50 and 0.5 µg/L PMMA ingested an average of 66,420 and 850 particles per organism, respectively. Evaluation of NMP distributions in egg water relative to *D. magna* suggest these organisms may be able to size select NMP particles from a polydisperse exposure as there was a difference between media relative to *D. magna* slopes with NMPs >1 µm. This difference indicates that

D. magna do not take up the expected proportion of large particles from egg water. Surprisingly, the 24 h uptake-depuration study indicated that uptake of the polydispersed Ta-PMMA peaked at 6 hours, suggesting that *Daphnia* ingested particles and then stopped eating to allow for depuration before exposure was complete. There was minimal size-specific differences ($k_d < 1 \mu\text{m}$: 0.0744 h⁻¹; $> 1 \mu\text{m}$: 0.0711 h⁻¹) in depuration rates in *D. magna* exposed to the polydispersion. Finally, the 21-d chronic study of showed that particles were visually detected in spawned neonates as low as 100 ppt and that neonate clutch production decreased at concentrations as low as 250 ppt (p = 0.0303). **Conclusions:** These findings demonstrate the effectiveness of metal-tagged NMPs and spICP-MS in quantifying NMP uptake in aquatic organisms at environmentally relevant concentrations. This highlights the importance of polydispersions in uptake and depuration studies as it demonstrated the differences between polydispersed and monodispersed uptake and depuration patterns. This work also provides insight into how realistic NMP exposures affect aquatic invertebrates and their progeny.

PS 4538 Oral Exposure to Polyethylene microplastics aggravates steatosis and metabolic dysfunction

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Background and Purpose: Microplastics (MPs) are increasingly recognized as a global health concern with health-related annual economic losses of approximately \$1.5 trillion. MPs can enter the human body through ingestion, inhalation, and dermal contact accumulating in various organs and leading to multiple organ toxicities. Previous studies have shown critical exposure effects, including inflammation, oxidative stress, neurotoxicity, and metabolic dysfunction. Polyethylene (PE) is the most abundant polymer detected in humans. It is also present in raw and treated drinking water, bottled mineral water and in various forms, including buckets, crates, bags, films, sheets, bubble wraps, and toys. However, its effect on hepatotoxicity and metabolic dysfunction has not been comprehensively unraveled. Our present study investigates the toxicopathological effects and mechanism of PE induced hepatotoxicity. **Methods:** We assessed the effects of seven commonly used MPs - Polyethylene (PE), Polyvinylidene chloride (PVDC), Polyethylene terephthalate (PET), Polypropylene (PP), Polyamide-6 (PA-6), Polystyrene (PS), Polyvinyl chloride (PVC) on cell viability using MTT assay in isolated mouse primary hepatocytes. Furthermore, the role of oral exposure to polyethylene (PE) was investigated using *in vivo* models. Male C57BL6 (WT) mice were fed daily with 2 mg PE (10 - 150 µm diameter) per day using oral gavage for 8 weeks. Additionally, to determine the effects of PE microplastics on metabolic dysfunction-associated fatty liver disease (MASLD), male mice fed with a high-fat, high-fructose, high-cholesterol diet (HFHFC diet) were orally administered with PE for 8 weeks. Metabolic studies were performed. Moreover, combined bulk and spatial transcriptomic analyses evaluated gene expression patterns and cell type heterogeneity. **Results:** PE treatment showed significantly reduced cellular metabolic activity, resulting in only 78% viability of mouse primary hepatocytes, as measured using the MTT assay. *In vivo* oral administration of PE caused a significant increase in normalized body mass and liver-to-body weight ratio in HFHFC diet-fed groups compared to the HFHFC diet-only cohort. PE ingestion resulted in aggravated steatosis, inflammation, and liver injury. Bulk and spatial transcriptomic analyses revealed that PE resulted in hepatic dysfunction in mice fed on a control or HFHFC diet, driving both globally and spatially distinct transcriptional alterations. Spatial transcriptomics uncovered the cell-type heterogeneity and gene expression patterns masked in bulk analyses. Spatial clustering uncovered inflammatory hotspots characterized by reduced cellular diversity and distinct PE-responsive gene signatures. Among these, nuclear receptor signaling specifically Ppara emerged as a key regulator. **Conclusions:** The present study demonstrated that oral exposure to PE microplastics resulted in hepatic injury and inflammation. Moreover, in a diet-induced MASLD model, PE treatment aggravated steatosis, inflammation, and liver injury. Mechanistically, PE exposure modulated nuclear receptor signaling, with Ppara as a key regulator of hepatotoxicity. These findings offer new insights into how PE disrupts hepatic homeostasis and underscore the value of spatial transcriptomics in elucidating environmental impacts on tissue organization and gene expression regulation.

PS 4539 Bayesian Physiologically Based Pharmacokinetic (PBPK) Modeling Integrated with Particle Dosimetry to Predict Inhalation Exposure and Biodistribution of Airborne Microplastics in Humans

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Background and Purpose: Airborne microplastics (MPs) have emerged as contaminants of increasing concern due to their widespread presence and potential human health impacts. While ingestion exposure has been relatively characterized, the internal distribution of inhaled MPs remains poorly understood. This study aims to develop a human inhalation PBPK model for MPs, extrapolated from the oral PBPK model previously developed in mice, to quantitatively predict absorption, distribution, and clearance following inhalation exposure. **Methods:** Atmospheric MP data were systematically compiled from peer-reviewed studies reporting particle number, size, shape, and polymer type characterized by μ FTIR, Raman spectroscopy, and pyrolysis-GC/MS. Particle number concentrations were converted to mass-based units using polymer densities and geometric assumptions. The MPPD model was applied to estimate size-dependent deposition efficiencies across the nasal, tracheobronchial, and alveolar regions, accounting for human breathing patterns and physiology. The resulting deposition fluxes were used as inputs to a Bayesian-calibrated PBPK model simulating absorption, systemic translocation, and clearance of MPs across major organs and tissues. Bayesian inference was employed for probabilistic parameter estimation, uncertainty quantification, and cross-route extrapolation from oral to inhalation exposure scenarios. **Results:** Integration of the MPPD and PBPK models enabled quantitative prediction of MP deposition and internal biodistribution across a wide range of particle sizes. The Bayesian calibration improved parameter convergence and uncertainty characterization under data-limited conditions. Model simulations produced physiologically consistent retention and systemic distribution patterns, with evaluation datasets showing observed data points largely within the 95% credible intervals of simulations. **Conclusions:** This integrative Bayesian PBPK modeling framework provides a mechanistic and quantitative approach for evaluating biodistribution and internal exposure of inhaled microplastics in humans. By combining particle dosimetry with Bayesian uncertainty analysis, the model enables extrapolation from oral to inhalation exposure and supports human health risk assessment for airborne microplastics.

PS 4540 The role of microplastic oxidation in promoting inflammation in lung cells

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Background and Purpose: Micro- and nanoplastics (MNP) are ubiquitous environmental contaminants and their accumulation has been reported throughout the body. While their precise contributions to adverse health outcomes remains unclear, the growing evidence of respiratory exposures highlights the urgent need to define MNP's effects on lung macrophages and epithelial cells. MNP have previously been reported to initiate ROS and inflammation in lung cells; however, dosing, plastic type, and duration of exposure often result in contradictory reports. As such, the mechanisms of MNP-caused cell dysfunction remain poorly defined. Here, the effects of four MNP and their ultraviolet A (UVA) irradiated counterparts was evaluated in both macrophages and epithelial cells to define mechanisms and highlight the importance of environmental oxidation in the bioactivity of microplastics. **Methods:** Polyethylene terephthalate (PET), low density polyethylene (LDPE), high density polyethylene (HDPE), and polystyrene (PS) of a 1-5 μ m size range were purchased from Nanochemazone, washed with 70% EtOH, and allowed to dry overnight. PET, LDPE, HDPE, and PS were exposed to UVA (365nm) for 72 hours to mimic environmental exposure to sunlight (α_{ox}). MNP and MNP $_{ox}$ were characterized by SEM and Raman microscopy. Cell uptake of MNP/MNP $_{ox}$ was confirmed with flow cytometry and laser scanning confocal microscopy in murine *ex vivo* cultured macrophages and murine C-10 epithelial cells. Bioactivity of MNP/MNP $_{ox}$ was evaluated in macrophages and epithelial cells by quantifying ROS, cell death, lysosomal function, and cytokine release. Cells were exposed to MNP/MNP $_{ox}$ with and without LPS. 1 μ g/mL, 10 μ g/mL, 100 μ g/mL, and 1,000 μ g/mL doses of MNP/MNP $_{ox}$ were used for 24 or 48 hours. mRNA expression of NF κ B and NQO1 were evaluated in MNP/MNP $_{ox}$ exposed cells and optimized doses. MNP/MNP $_{ox}$ uptake by macrophages and epithelial cells was prevented with actin polymerization disrupter, Cytochalasin D, to confirm necessity of uptake to observed biological effects. Lysosomal function of MNP/MNP $_{ox}$ treated macrophages was evaluated with Lysotracker Red. **Results:** Raman microscopy of MNP $_{ox}$ demonstrated chemical changes as compared to pristine MNP, with PET $_{ox}$ showing broadening and dampening of peaks compared to PET at the corresponding wavenumber. PS $_{ox}$ showed the next greatest change by Raman spectra, while LDPE and HDPE showed subtle but notable changes to spectra with oxidation. In contrast, SEM showed no evident structural differences between pristine and oxidized MNP. PET and LDPE plastic fragments showed irregular shapes with more fragmented edges, while HDPE and PS demonstrated a more spherical

structure. Macrophages showed a slower uptake of MNP compared to crystalline silica (1.5 μ m) as indicated by change in median side scatter with flow cytometry. Oxidation enhanced the biological effects of the MNP on macrophage function with demonstrable increases in cell death observed with PET $_{ox}$ and LDPE $_{ox}$ compared to pristine PET or LDPE respectively. **Conclusions:** As MNP are so widely distributed this work helps further refine the toxicological parameters for relevant evaluations of MNP effects in biological systems. This study highlights the necessity of examining a variety of MNP polymer types in toxicological studies. Furthermore, it shows that oxidation of MNP enhances the biological effects of MNP, demonstrating the need for proper oxidative protocols in MNP research to more closely replicate true to life exposure scenarios. The research shown here builds upon the current knowledge of MNP toxicity and enhances our understanding of dosing and duration, and the contributions of chemical structure to bioactivity.

PS 4541 Chylomicron-Mediated Translocation of Oxidized Nano- and Microplastics into the Circulatory System and Human Heart Tissue

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Background and Purpose: Rising global concentrations of environmental microplastics and nanoplastics (MNPs) raise significant concerns regarding human exposure and long-term health outcomes. Previous studies from our group demonstrated widespread MNP deposition across multiple organs. In this study, we investigated the mechanisms underpinning intestinal absorption and systemic transport pathways that enable MNP entry into the circulatory system. **Methods:** MNPs primarily enter the human body through ingestion of contaminated food and water. We hypothesized that nanoplastics, which have comparable chemical structure to free fatty acids and bind to monoglycerides, will become packaged within the small intestine into chylomicron complexes by enterocytes. Using fluorescently labeled MNPs of varying sizes, we observed that gavaged 500 nm polystyrene particles could be found co-localized in the chylomicron fraction of serum but not in VLDL/IDL/LDL fractions 4h post exposure. Interestingly, 1 μ m particles were not detected in any fraction of the serum. These findings suggest a size-dependent mechanism of MNP trafficking within chylomicrons. **Results:** Further work in human cadaver samples has been initiated to confirm that MNPs enter lacteals, pass through mesenteric lymphatic vessels, and merging into the thoracic duct before finally draining into the left subclavian vein, the gateway to the circulatory system. We quantified the MNP burden and confirmed their presence within lipid-dependent cardiac tissue samples obtained from 24 different human hearts using pyrolysis gas chromatography-mass spectrometry (Py-GC/MS). MNPs were further confirmed by complementary analytical techniques including Fourier-transform infrared (FTIR) spectroscopy, Raman spectroscopy, and transmission electron microscopy (TEM). **Conclusions:** Collectively, our data supports a novel mechanistic model in which oxidized nanoplastics exploit lipid absorption pathways to reach systemic circulation and partially accumulate in cardiac tissue. These findings underscore the need for further investigation into MNP-lipid interactions and their implications for cardiovascular health.

PS 4542 Microplastics Exhibit Region Specific Retention in Kidney Tissue and Are Detected in Human Urine

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Background and Purpose: Micro- and nanoplastics (MNPs) have been recently detected in human biofluids and organs. However, it is unknown whether the kidney functions solely as a clearance organ or also as a site of MNP retention and injury. Here, we evaluated the occurrence, polymer composition, and spatial distribution of MNPs in human urine and mammalian kidney tissue to determine region-specific deposition patterns relevant to renal function. **Methods:** Fresh human urine samples were processed using four isolation workflows (centrifugal ultrafiltration, quartz filtration with and without basic digestion, and combined methods). Microplastics were quantified using Pyrolysis-GCMS targeting polymer-specific thermal fragments and TEM. Porcine kidneys were dissected into cortex and medulla regions and analyzed using identical methods following contamination-controlled digestion. **Results:** Five major polymers, polyethylene (PE), polypropylene (PP), polyethylene terephthalate (PET), acrylonitrile butadiene styrene (ABS), and styrene-butadiene rubber (SBR), were consistently detected in urine above the limit of quantification (LOQ). PE was dominant, exceeding 20 μ g/mL in some individuals; PET was also routinely measured across multiple human samples. Kidney mapping revealed non-uniform distribution: PE concentrations were ~1.3-fold higher in cortex (564 μ g/mg) than medulla (435 μ g/mg), with similar cortical enrichment observed for PP and ABS. PET was detectable in cortex (2.6 μ g/mg) but absent in medulla thus far, suggesting selective retention of environmentally relevant polymers with adsorbed

coronas. **Conclusions:** These findings provide evidence that renal handling of MNPs is polymer-dependent and region-specific, consistent with glomerular filtration threshold effects and potential proximal tubule uptake. Combined with urinary excretion levels, these patterns position the kidney as both a sentinel organ and an active site of MNP interception. Ongoing work will evaluate correlations between regional polymer burden, glomerular filtration rate, and molecular markers of renal injury to define mechanistic pathways of plastics-induced nephrotoxicity.

PS 4543 Prenatal Exposure to Polyethylene Nanoplastics: Preliminary Evidence of Accumulation and Potential Neurodevelopmental Effects in Human Brain Organoids

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Background and Purpose: Nanoplastics are emerging environmental contaminants that are increasingly detected in human tissues and biological fluids. Their nanoscale size raises the possibility of placental transfer and fetal exposure during critical windows of development. However, their potential impact on human brain development remains poorly understood. This study aims to investigate whether polyethylene (PE) nanoplastics accumulate in developing neural tissues and whether such accumulation could perturb early neurodevelopmental processes. Specifically, we examined the distribution of PE nanoparticles within human brain organoids as a model for fetal brain development. **Methods:** Human pluripotent stem cell-derived cortical organoids were exposed to fluorescently labeled PE nanoparticles (50-65 nm). Nanoparticle localization was assessed using confocal microscopy on both optically cleared whole organoids and cryosections. Accumulation was quantified within neural rosette regions, representing early neuroepithelial structures. Organoid diameter and growth dynamics will be monitored to detect morphological changes following exposure. To confirm nanoplastic identity and aggregation, transmission electron microscopy (TEM) and dynamic light scattering (DLS) analyses are being conducted. Cytotoxicity and apoptosis markers will be analyzed to evaluate potential neurotoxic effects of PE exposure. **Results:** Preliminary imaging data indicate that PE nanoplastics are internalized by brain organoids and appear to accumulate within neural rosette-like structures. These findings suggest that developing neural tissues can uptake and possibly retain nanoplastics during early differentiation. Ongoing analyses will determine nanoparticle aggregation state and the degree of cellular stress and apoptosis. **Conclusions:** These preliminary results support the notion that fetal-like neural tissues are capable of accumulating nanoplastics during early developmental stages. Future studies will include both fluorescently labeled and non-tagged environmentally relevant plastics—collected from ocean samples and subsequently prepared—to better mimic real-world exposure scenarios. Together, these efforts aim to clarify how prenatal exposure to environmental nanoplastics may pose risks to human neurodevelopment.

PS 4544 Physicochemical Influences on Metal Interactions and Toxicity in Exposure to Real-World Micro- and Nanoplastics

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Background and Purpose: Micro- and nanoplastics (MNPs) are environmentally abundant as plastic production has increased. Degradation of plastic waste generates MNPs, which are commonly found in air, water, and soil, where interactions can occur with other environmental contaminants. Due to their environmental abundance and potential to interact with other contaminants, MNPs may pose a significant risk to human health. Epidemiology has reported the presence of MNPs in blood, feces, sputum, and placenta. Exposure to MNPs has been linked to respiratory diseases such as pulmonary fibrosis and intestinal diseases such as Crohn's Disease. Inhalation or ingestion of MNPs can initiate systemic inflammation, oxidative stress, and cytotoxicity, promoting cardiovascular diseases, reproductive disorders, and carcinogenesis. Traditionally, most studies use engineered polystyrene nanomaterials with uniform shape and size, however, environmental MNPs have a wide size distribution and irregular shapes. These physicochemical differences can impact interactions with environmental pollutants as well as toxicity. We hypothesize MNPs made from real-world plastic waste will differentially interact with environmental contaminants based on their physicochemical properties, resulting in altered toxicity compared to pristine MNPs and those without environmental contaminants. **Methods:** Mouse macrophages were exposed to 0, 0.01, 0.1, 1, 10, 25, 50, or 100 ug/ml of representative engineered polystyrene nanomaterials, MNPs generated from salt milling (PET, PS, PP), MNPs with adsorbed Pb, MNPs with an adsorbed metal mixture (Mn, Co, Zn, Cd, Pb), or to metal concentrations consistent with the amount adsorbed on MNP surfaces. Endpoints of toxicity, inflammation, and oxidative stress were evaluated 24- hours post-exposure. **Results:** Assessments of viability identified MNP-specific alterations where MNPs with adsorbed Pb or metal mixtures were more cytotoxic than Pb or metal mixtures alone, respectively. Gene expression analyses revealed significant upregulation of key inflammatory mediators following exposure. At 50 and 100 ug/ml all MNPs without metal induced expression of IL-6 and IL-1b without modifying CXCL2. Additionally, MNPs with heavy metal associated on their surface induced IL-6 and IL-1b expression that was less than MNP or metals

alone. Examination of HO-1 expression as a marker of oxidative stress demonstrated induction at 50 ug/ml in MNPs with metals adhered on their surface and at 100 ug/ml in MNPs with only Pb adhered on their surface. **Conclusions:** Overall, exposure to MNPs with or without surface adherence of heavy metals induce concentration-dependent cytotoxic responses and activate inflammatory pathways. This suggests investigating environmentally relevant MNPs with co-occurring exposures may enhance translatability of toxicity evaluations. Future assessments will aim to identify molecular signatures by assessing cellular uptake of MNPs after exposure via untargeted proteomics. Alterations in biological markers of ferroptosis and metal homeostasis will also be assessed. Together, these will be used to elucidate pathways of toxicity and disease.

PS 4545 Paternal exposure to microplastics alters sperm small non-coding RNA landscape and elicits offspring metabolic dysfunctions in mice

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Background and Purpose: Microplastics (MPs) are small plastic particles emerging as significant environmental pollutants and humans are ubiquitously exposed to microplastics. Recent studies have associated MP exposure with the increased risk of chronic diseases in humans. MPs can also be detected in both male and female reproductive tissues in humans. However, the impact of parental MP exposure on offspring health is unknown. This study aimed to investigate the impact of paternal exposure to MPs on the metabolic health of F1 offspring in mice. **Methods:** In this study, eight-week-old male C57BL/6J wild-type (WT) mice were exposed with 10 mg/kg body weight of MPs or vehicle control by daily oral gavage for 4 weeks before mating with unexposed female mice to generate F1 offspring. Total RNAs were isolated from sperm of F0 sires and subjected to PANDORA-seq analysis. F1 offspring from MP- or control-exposed F0 sires were fed a high-fat diet before euthanasia at 12 weeks of age. **Results:** We found that paternal MPs exposure had sex-specific effects on diet-induced obesity in F1 offspring. Female F1 offspring from MP-exposed sires had decreased lean mass and exacerbated insulin resistance. PANDORA-seq revealed that MP exposure altered sperm tRNA-derived small RNA (tsRNA) and rRNA-derived small RNAs (rsRNA) profiles. Furthermore, microplastic-stimulated sperm tsRNAs/rsRNAs can induce early transcription changes in mouse embryonic stem cells, potentially contributing to paternal MP exposure-elicited metabolic disorders in offspring. **Conclusions:** Our results suggest that MPs exposure may have intergenerational adverse impact on offspring metabolic health, also underscore the urgency of better understanding the health consequences of plastic exposure in humans.

PS 4546 Development and characterization of reference environmentally relevant micro-nano-plastics for risk assessment studies

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Background and Purpose: Most risk-assessment studies of micro-nanoplastics (MNPs) utilize commercial pristine, spherical, uniform primary MNPs. However, humans are primarily exposed to secondary MNPs produced by degradation of plastics in the environment, which have irregular shapes, varied sizes, and altered surface chemistries. The toxicological properties of these environmentally relevant MNPs may differ greatly from those of pristine spherical MNPs. **Methods:** We developed methods that replicate major degradation processes in the plastic life cycle, including mechanical fragmentation, photo-oxidation, and incineration. We employed cryomilling (mechanical fragmentation) and a UV-aging chamber (photo-oxidation) to generate secondary MNPs of polyvinyl chloride (PVC) and polyethylene terephthalate (PET). A dry and wet sieving protocol was used to collect the PM₁₀ size fraction. Multi-angle light diffraction (MALD) and scanning electron microscopy (SEM) were used to determine size distribution and morphology, and X-ray Photoelectron Spectroscopy (XPS) and Diffuse Reflectance Infrared Fourier Transform (DRIFT) were used to characterize surface chemistry. Incinerated MNPs of PVC, PET, high-density polyethylene (HDPE), acrylonitrile butadiene styrene (ABS), polycarbonate (PC), and polypropylene (PP) were created using our Integrated Exposure Generation System (INEXS). Emissions of CO and total volatile organic compounds (TVOCs) were monitored in real-time, and size analysis was conducted using a Scanning Mobility Particle Sizer (SMPS) and Aerodynamic Particle Sizer (APS). Emissions were size-fractionated using a Harvard Compact Cascade Impactor (CCI) to obtain PM_{0.1}, PM_{0.1-2.5}, PM_{2.5-10}, and PM_{>10} fractions for subsequent investigations. **Results:** The median sizes of the cryomilled and aged PVC and PET MNPs (by MALD) were ~10.7 and ~11.2 µm, respectively. The average sizes determined by SEM were 2.7 ± 1.4 µm and 4.8 ± 2.0 µm, respectively. SEM images showed that the particles maintained irregular morphology throughout the weathering process. The zeta potential for aged PVC and PET MNPs was -33.0 ± 0.8 mV and -36.3 ± 1.0 mV, respectively, indicating a stable

colloidal suspension. DRIFT analysis revealed the presence of oxygen-containing functional groups in UV-aged PVC MNPs, while XPS indicated a 5.5% increase in oxygen content compared to un-aged PVC. In contrast, no significant changes in surface oxygen content or functional groups were observed in 21-day UV-aged PET MNPs. During incineration, CO peaked for all plastics between 531°C and 715°C, with HDPE emitting the highest concentration (1240 ppm) and PVC the lowest (79.5 ppm). TVOC emissions reached ~26,400 ppb for most plastics, except for PC, which reached 1,440 ppb. SMPS measurements showed peak nanoscale particle concentrations ranging from 7.04×10^5 /cm³ (PVC) to 1.41×10^7 /cm³ (ABS). APS measurements showed peak micron-scale particle concentrations of 4.17×10^5 /cm³ (PVC) to 1.35×10^5 /cm³ (PP). Total aerosol concentrations ranged from 26.5 mg/m³ (PVC) to 60.6 mg/m³ (PET). EC/OC analysis revealed that PM_{0.1} MNPs were >99% organic carbon. PAH analysis identified multiple carcinogenic PAHs in all incinerated MNPs, with PC exhibiting the highest content (125.4 µg/kg). **Conclusions:** Using environmentally relevant secondary MNPs in toxicological studies will yield more accurate and applicable results compared to the pristine commercial MNPs commonly used. Thorough characterization of these reference materials will enable the identification of specific structure-activity relationships for environmentally relevant MNPs.

PS 4547 Assessing the Effects of UV Aging on Toxicity and Bioavailability of Inductively Coupled Plasma Mass Spectrometry (ICP-MS)-Traceable Core-Shell Polystyrene Nanoplastics in an *In Vitro* Triculture Model of Small Intestinal Epithelium

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Background and Purpose: Most studies of micro-nanoplastics (MNPs) toxicity have employed commercially available pristine MNPs, rather than environmentally relevant MNPs generated by fragmentation and photo-oxidation of plastics in the environment, or by incineration of plastic waste. An accurate assessment of the potential health impacts of MNPs requires understanding the complex physicochemical changes that occur during the aforementioned processes. Notably, photo-oxidation from UV exposure markedly alters the surface chemistry and morphology of MNPs, enhancing roughness and introducing reactive functional groups. Another challenge in assessing the risk of MNPs is the lack of robust analytical methods for precise and species-specific quantification, which is critical for evaluating their transport in biological systems. However, it is possible to engineer MNPs with a metallic (e.g., Au) core and a plastic shell, enabling highly accurate quantification in biological media and samples by inductively coupled plasma mass spectrometry (ICP-MS). **Methods:** In this study we investigated the impact of photo-aging on the physicochemical properties, toxicity, and bioavailability of ICP-MS traceable 25 nm gold-core shell polystyrene (AuPS25) in a triculture small intestinal epithelium (SIE) model. An aqueous suspension of AuPS25 was subjected to UV aging for 21 days in an accelerated UV-aging chamber (equivalent to 6 months of outdoor weathering). Dynamic light scattering (DLS), transmission electron microscopy (TEM), Fourier-transform infrared spectroscopy (FT-IR), and X-ray photoelectron spectroscopy (XPS) analysis were performed at days 0, 7, 14, and 21. The aged and un-aged AuPS25 was subjected to three-phase simulated digestion to replicate the physicochemical transformations during digestion, and the resulting digesta was applied to an *in vitro* transwell triculture SIE model. Toxicological assessments included oxidative stress, lactate dehydrogenase (LDH) release, trans-epithelial electrical resistance (TEER), and dextran permeability. AuPS25 uptake and translocation across the SIE were quantified via ICP-MS. **Results:** Physicochemical characterization revealed photo-oxidation of AuPS25 NPs at 14 days of UV-aging. TEM analysis revealed that the PS shell thickness remained unchanged after 7 and 14 days of UV aging; however, a significant reduction from ~4 nm to 0.80 nm occurred by day 21, indicating degradation. FT-IR analysis confirmed the formation of carboxyl groups after 7 and 14 days of UV exposure, signifying photo-oxidation. Similarly, XPS analysis revealed a 5.9% increase in oxygen content in 14-day UV-aged AuPS25 NPs, indicating the formation of oxygen-containing functional groups. Toxicological evaluations in the SIE model demonstrated that exposure to digestas of aged AuPS25 at oral concentrations of 4 and 40 µg/mL impaired barrier integrity by ~15% and ~18% and increased cytotoxicity by ~4.3% and ~5.4%, respectively, whereas exposure to digestas of un-aged AuPS25 had no significant effect. Although AuPS25 translocation rates were similar for aged and un-aged PS NPs, uptake of aged AuPS25 was significantly higher, reaching 72.2% at 4 µg/mL and 59.2% at 40 µg/mL. In contrast, less than 0.5% of the un-aged PS NPs were taken up at either concentration. **Conclusions:** UV aging significantly impacts the physicochemical properties of PS NPs, leading to photo-oxidation, increased cytotoxicity, disruption of the epithelial barrier, and enhanced SIE uptake. Using ICP-MS-traceable core-shell particles, we achieved NP detection at environmentally relevant concentrations and established a sensitive quantification framework. These results highlight the necessity of including aged and relevant MNPs in risk assessments to improve the accuracy of toxicological evaluations and inform public health decisions regarding this emerging contaminant.

PS 4548 Micro(nano)plastic (MNPs) in sinus tissue: A methodology comparison

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Background and Purpose: The human nasal cavity represents a potential deposition site for micro(nano)plastics (MNPs), including polymer fibers. The efficiency of MNPs deposition in the upper and lower respiratory tracts depends on their size, aspect ratios, and density, and the geometry of the nasal cavities. Synthetic and semi-synthetic fibers, widely generated from textiles, clothing, carpeting, and other sources, predominate the composition of indoor dust particles. Recent studies have raised concern about microplastic contamination in the nasal cavity, with alterations in microbial communities, and have associated the presence of microplastics with upper respiratory infections and the development of nasal polyps. **Methods:** For this study, 500mg of sinus tissue (4 samples) was tested for the efficacy of the MNPs isolation and quantification methodology using pyrolysis gas chromatography-mass spectrometry. Duplicates of two tissue samples were split into two treatments: The first group was digested with nitric acid and then 10% Potassium Hydroxide (KOH), and the second group was digested with 10% KOH only. Digestants from both groups were incubated for 24 hours in cyclohexane and then filtered through a quartz micro-fiber filter with a 50-nanometer pore size. **Results:** Preliminary results show that samples that didn't contain cartilage or bones didn't show significant concentrations of MNPs. In contrast, samples containing cartilage or bones contained polyethylene terephthalate (PET) (21.4-40.4 µg/g) and polyethylene (PE) (32.9-90.6 µg/g). **Conclusions:** More sample tissues will be analyzed to confirm how bone presence affects the bioaccumulation and quantifiability of MNPs in the sinus tissues.

PS 4549 Lifecycle implications of polyvinyl chloride (PVC) micronanoplastics (MNPs): Interactions with co-exposed environmental pollutants (EPs) and impact on their toxicity and bioavailability

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Background and Purpose: Most risk-assessment studies on MNPs rely on commercially available, pristine, spherical MNPs, which differ substantially from environmentally relevant MNPs produced by mechanical fragmentation and photo-oxidation in the environment or incineration of plastic waste. Environmentally relevant MNPs have irregular morphologies, varied sizes, and distinct surface chemistries. This oversight has perpetuated significant knowledge gaps regarding the impacts of plastics life cycle and associated MNPs. MNPs have been found to sorb a range of environmental pollutants (EPs), including toxic metals and organic compounds. However, due to the lack of studies with environmentally relevant MNPs, critical questions remain unanswered, including the capacity of relevant aged and incinerated MNPs to sorb EPs, and the potential synergistic effects on gastrointestinal (GIT) toxicity and bioavailability of both MNPs and EPs. **Methods:** We investigated the biointeractions, toxicity, and bioavailability of PVC MNPs throughout the plastic waste lifecycle, alone and with EPs, in an *in vitro* transwell triculture small intestinal epithelium (SIE) model. Model MNPs included mechanical-fragmented/cryomilled and un-aged PM₁₀ (PVC-UA), cryomilled and UV-aged PM₁₀ (PVC-A), and incinerated PM_{0.1} (PVC-I) PVC MNPs. EPs tested included As(V), Cr(III), Pb(II), boscalid, and PFOS. We also assessed the sorption of each EP by different PVC MNP types in each phase of simulated digestion, the toxicity of PVC MNPs, alone and with EPs, and translocation of EPs and MNPs, alone or combined, in the SIE model. Toxicological assessments included oxidative stress, LDH release, trans-epithelial electrical resistance (TEER), and dextran permeability. Translocation of EPs through the SIE was quantified by inductively coupled plasma mass spectrometry (ICP-MS) and liquid chromatography-mass spectrometry (LC-MS), and bioavailability of PVC MNPs was assessed by pyrolysis-gas chromatography-mass spectrometry (Py-GCMS). Effects on gene expression were assessed by RNA-seq. **Results:** Sorption of most metals was MNP lifecycle-stage- and EP-specific, with aged-MNPs exhibiting a higher affinity for EP than unaged cryomilled and incinerated-MNPs. Whereas exposure to unaged MNPs and EPs alone or combined did not cause cytotoxicity in the SIE, aged-MNPs and incinerated-MNPs in the presence of EPs triggered significant oxidative stress. The presence of MNPs significantly increased the bioavailability of EPs and vice versa. The enhanced translocation influenced by PVC-A, PVC-I, and EPs correlated with the downregulation of tight junction, adherens junction, gap junction, intercellular adhesion, and focal adhesion genes. **Conclusions:** The lifecycle stage of PVC MNPs plays a significant role in the sorption of EPs, with aged MNPs exhibiting a higher affinity for EPs throughout the GIT. Additionally, exposure to UV-aged and incinerated PVC MNPs induces oxidative stress in the SIE and facilitates the enhanced translocation of EPs, and vice versa, by causing significant alterations in cell junction genes, which suggests leaky gut effects. Collectively, our findings offer significant toxicological insights into the lifecycle of ecologically relevant PVC MNPs, their interactions with EPs, and the consequent implications for the integrity of human intestinal epithelial structures.

PS 4550 Characterization of tire microplastic leachates and their impacts to human epithelial lung cells via *in vitro* studies and multi-omics analysis

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Background and Purpose: Microplastics (MPs) are small plastic particles of less than 5 millimeters in length that are generated from primary sources (manufactured at this size) or secondary sources (when large synthetic polymer products degrade into smaller pieces or are intentionally fragmented through mechanical or chemical recycling processes). These risks stem not only from their physical properties but also from the leaching of toxic chemical additives and their capacity to carry microbial contaminants. Tire wear particles represent one of the largest yet often overlooked sources of MPs, accounting for an estimated 26-74% of global MP pollution. Recycled tire materials used in recreational spaces further contribute to exposure, releasing complex mixtures of additives such as antioxidants, vulcanizers, and plasticizers with poorly characterized toxicities. In this study, we employed a multi-omic approach to investigate the biological effects of tire-derived MPs leachates on two human upper-respiratory cell models: lung carcinoma (A549) and bronchial epithelial transformed (BBM) cells. **Methods:** Tire MP particles (<45 µm) were leached in culture media shaken at 100 RPM for 24 hours at 37 °C and applied at concentrations of 0, 50, 100, and 150 µg/mL. These filtered (0.2 µm) and unfiltered MP leachate samples were dosed to both A549 and BBM cells. Over three days, we evaluated cell proliferation via Incucyte live-imager and viability using an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay at 24 h, 48 h and 72 h. Media was collected at the end of the experiment to determine inflammatory cytokine release using a multiplex cytokine assay. In addition, control and 150 µg/mL treatment groups were analyzed for cellular uptake of tire-associated chemicals using inductively coupled plasma mass spectrometry (ICP-MS) for metals and liquid chromatography-high-resolution mass spectrometry (LC-HRMS) for organic tire additives and transformation products. LC-HRMS was also employed for both metabolomic and proteomic analyses. **Results:** Exposure to tire particle leachates induced concentration- and cell type-dependent increases in proliferation (*P≤0.05, **P≤0.01, ***P≤0.001), and cytokine release (*P≤0.05, **P≤0.01, ***P≤0.001). Metal analysis revealed in 150 µg/mL samples had significant cellular uptake of zinc, copper and lead. Non-targeted analysis revealed cellular uptake of diverse organic tire additives, including corrosion inhibitors, process regulators, antioxidants, adhesives, and transformation products. Notably, N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine quinone (6PPD-quinone)—a toxic environmental transformation product of a common tire antioxidant—was detected in exposed cells. Reported hazards of the additional chemicals structurally annotated included potential toxicity to aquatic life, acute toxicity for human health, skin irritation, and reproductive harm. Additional structurally annotated chemicals were linked to hazards such as acute toxicity, skin irritation, and reproductive harm. In addition to upregulation of specific cellular metabolites with tire leachate dosing, bottom-up proteomic analysis identified a novel protein biomarker of airway exposure to quinone-derived tire-associated chemicals. In total, 2,332 unique proteins were identified across the BBM and A549 cells. Proteins hits with significant expression level changes were identified using a p-value cutoff of 0.01 and a Z-score of 1 resulting in the identification of 317 protein hits in the BBMs and 181 protein hits in the A549 with 46 hits overlapping in both. Many of the 46 protein hits are classified as heat shock and histone proteins that are important for gene expression and cell growth. **Conclusions:** We observed that Tire derived MP leachates can drive cellular responses to growth, metabolic activity, and inflammation in cell- and concentration-dependent ways. Several chemicals, including metals and transformation products of tire additives, were identified within exposed cells and may contribute to these observed effects. Collectively, these findings underscore the potential human health risks posed by tire-derived MPs as an emerging global and regional air pollutant.

PS 4551 Role of ApoEε3/4 variants in modifying endothelial uptake and toxicity of microplastics derived from the Pacific Gyre

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Background and Purpose: **Background:** Micro- and nanoplastics (MNPs) are emerging environmental pollutants with the potential to affect human health and contribute to diseases. Increasing evidence shows that MNPs accumulate in human organs, especially the brain, and their levels are rising over time. MNPs can cross the blood-brain barrier and stay as nanoscale fragments within brain tissue. Notably, concentrations of MNPs are higher in the brains of people with dementia, including Alzheimer's disease, compared to those without neurodegenerative conditions. This specific buildup may disrupt cerebrovascular walls and impair immune cell function, raising important questions about the role of environmental exposure in disease development. There is an urgent need to understand how MNPs enter brain cells, a process that is still being clarified. ApoEε4, the strongest genetic risk factor for Alzheimer's disease, not only disrupts lipid metabolism but also decreases the clearance of amyloid-beta and promotes neuroinflammation. Over recent decades, research has focused on how ApoEε4 interacts with lifestyle factors, but there is

a lack of studies on how MNPs interact with ApoEε4. **Aim of the Study:** The objective of the study is to investigate the effect of aged and unaged microplastics on primary human brain microvascular endothelial cells and how ApoEε3 or ApoEε4 proteins may modify these interactions. **Methods:** Environmental micro-nanoparticles (MNPs) derived from plastics obtained from the Pacific Gyre were labeled with Rhodamine B dye. Primary human brain microvascular endothelial cells were exposed to 10 µg/ml of either UV-aged and unaged MNPs, with or without dispersion media containing ApoEε3 or ApoEε4 lipoproteins. After 24h exposure, the integrity of the cell barrier and wound healing were monitored using the Electrical Cell Impedance System (ECIS). Localization of the MNPs was confirmed with confocal microscopy using Z-Stack analysis. The cellular uptake of Rhodamine B-dyed **Results:** MNPs was quantified using the AMNIS flow cytometry imaging system. **Results:** Confocal imaging showed that cells uptake Rhodamine B-dyed microplastics, and Z-stack analysis verified their presence inside the cells. The cell behavior analysis indicated that aged microplastics in the presence of ApoEε4 impaired cell migration in the wound healing assay than microplastics with ApoEε3 or microplastics alone. Additionally, the uptake of aged microplastics was higher in the presence of ApoEε4 compared to either ApoEε3 or microplastics alone in primary human brain microvascular endothelial cells. **Conclusions:** This study concludes that the ApoEε4 apolipoprotein, which likely carries microplastics in the circulation, may influence the biological effects of MNPs on neurovascular endothelial cells as well as the passage into the brain itself. This creates a novel GxE paradigm that could, in part, explain the dementia risk incurred by the ApoEε4 variant. **Funding:** National Institute of Aging 5U01AG088557-02.

PS 4552 Physicochemical Interaction Dependent Transportation of Pollutant Adsorbed Nanoplastics (PANs) in Lung Cells

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Background and Purpose: Microplastics are well established vectors for pollutants, absorbing harmful substances like pesticides and industrial chemicals, posing risks to human health and sparking regulatory initiatives against them. Compared to microplastics, smaller **nanoplastics (<1 µm)** can travel over long distances in the atmosphere and possess altered health risks via lung inhalation. Moreover, their increased surface area-to-volume ratio enhances their ability to adsorb pollutants, forming a complex material termed **pollutant-adsorbed nanoplastics, or PANs**. Nanoplastics have been linked to cancer progression by inducing pro-inflammatory responses, endocrine disruption, and oxidative stress, yet the alteration of these cellular functions because of nanoplastic interplay with small molecule pollutants found in complex atmospheric particulate matter (i.e., formation of PANs) is poorly understood. Current research on PAN toxicity provides limited characterization of their formation kinetics and affinity. The physicochemical interactions between pollutants and plastics drive PAN formation and their transportation and toxicity in the body. **Methods:** This research applies enhanced darkfield hyperspectral imaging (EDF-HSI) as a novel, label-free approach for single-particle chemical and spatial mapping of PANs, revealing how PANs exacerbate pollutant toxicity and carcinogenicity by resolving their composition and desorption in cells. **Results:** EDF-HSI was used to chemically detect and spatially map spectral shifts in 220 nm polystyrene diaziron (PSDZN) PANs compared to pristine PS controls, revealing significant adsorption heterogeneity (Fig. 1C). Principle component analysis (PCA) revealed clustering of PSDZN compared to pristine PS. Changes in cell uptake of PSDZN PANs compared to PS and PS incubated in methanol solvent (PSMeOH) were evaluated in human lung adenocarcinoma (A549) cells. Quantification of particle counts within cells showed trends of increased uptake for PSDZN compared to controls. Mean particle spectra were stratified by exposure condition and in/outside of cells for each exposure condition. PCA analysis revealed similar clustering of all groups, within and outside of cells except for PSDZN, which exhibited blue spectral shifts. **Conclusions:** This data established the feasibility of EDF-HSI for non-destructive, label-free, single-particle spatial and chemical characterization of PAN formation kinetics and interactions in cells and highlights the significance of pollutant adsorption on nanoplastic transportation in lung cells.

PS 4553 Real-World Micro/Nanoplastics and Pristine Nanoplastics Differentially Disrupt Microglial Mitochondrial and Redox Homeostasis

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Background and Purpose: Global plastic production now exceeds 390 million tons per year, resulting in pervasive environmental contamination and increasing human exposure to micro- and nanoplastics (MNPs). These particles have recently been detected in multiple human tissues, including the brain, raising concerns about their potential impact on neurological health. Emerging evidence indicates that MNP accumulation in brain tissue may contribute to neuroinflammation and cognitive decline, with preferential accumulation in microglia. Despite these concerns, most toxicological studies rely on commercially available pristine nanobeads, which inadequately reflect the physicochemical diversity of real-world MNPs. To address this gap, the present study investigated the subcytotoxic effects of MNPs generated

from common consumer items —disposable cups and forks (Cup-MNPs and Fork-MNPs) —on oxidative stress pathways and mitochondrial parameters in immortalized human fetal microglia (HMC3). These responses were directly compared with those elicited by pristine 50 nm polystyrene nanoplastics (PS-50), as they are known to reach the brain and induce neurotoxic and neurodegenerative markers *in vivo*. **Methods:** Disposable plastic cups and forks were manually cut, ground, and sieved to generate MNPs. The resulting particles were characterized by FT-IR spectroscopy, zeta potential analysis, and SEM imaging. Both consumer-derived MNP preparations consisted mostly of polystyrene. SEM showed mean particle sizes of $35.7 \pm 20 \mu\text{m}$ (Cup-MNPs) and $47.5 \pm 22 \mu\text{m}$ (Fork-MNPs), while hydrodynamic analysis revealed a larger nanoscale fraction in Fork-MNPs. Pristine polystyrene nanoplastics (PS-50; ~64 nm) were sourced commercially. All particle types exhibited slightly negative zeta potentials in cell culture media. HMC3 human microglial cells were exposed to 0.1 or 1.0 $\mu\text{g/mL}$ of each MNP type for 72 h. Endpoints included cell viability (Trypan Blue exclusion), intracellular and mitochondrial ROS (DCFDA and MitoROS by flow cytometry), antioxidant enzyme activity and glutathione redox status (GSH/GSSG) (colorimetric assays), mitochondrial membrane potential (TMRE), mitochondrial respiration (Seahorse XF), and mitophagy (MtPhagy probe by flow cytometry). **Results:** Although none of the particles were cytotoxic, Fork-MNPs and PS-50 at 0.1 $\mu\text{g/mL}$ significantly increased total ROS. All materials, including Cup-MNPs, elevated SOD activity without affecting catalase levels. At this low concentration, Fork-MNPs also reduced the GSH/GSSG ratio, indicating redox imbalance. At 1 $\mu\text{g/mL}$, all materials increased intracellular ROS, particularly PS-50. Fork- and Cup-MNPs further decreased the GSH/GSSG ratio, whereas PS-50 continued to enhance SOD activity. None of the treatments altered MMP or mtROS. However, PS-50 exposure reduced all mitochondrial oxygen consumption rate (OCR) parameters and lowered ATP levels, consistent with impaired mitochondrial function. In contrast, Fork-MNP-exposed cells showed increased OCR across all parameters, suggesting a compensatory upregulation of oxidative phosphorylation to meet energy demands under stress. Both Fork-MNPs and PS-50 induced mitophagy, as demonstrated by flow cytometry using a fluorescent probe and by co-localization analysis showing increased mitochondria-lysosome interaction. **Conclusions:** Both “real-world” MNPs and pristine NPs induced cellular stress at low, subcytotoxic doses, but the magnitude and nature of the responses differed markedly. Particle size, particularly the nanoscale fraction, emerged as a major driver of these effects. Pristine nanoplastics produced pronounced suppression of mitochondrial respiration, whereas Fork-MNPs triggered adaptive, compensatory mitochondrial responses at the same doses. Given the central role of microglia in maintaining neuronal homeostasis, such mitochondrial and redox disruptions could compromise brain energy balance and potentially increase vulnerability to neurodegeneration. Importantly, the divergent biological responses observed between pristine nanobeads and “real-world” particles underscore that pristine models may not accurately represent environmentally relevant exposures and should be interpreted with caution. Overall, these findings highlight the critical need to incorporate “real-world” MNPs into nanosafety assessments to capture biologically meaningful effects.

PS 4554 Methodological Approaches for Food Digestion in Micro(nano) plastic (MNP) Analysis and Quantification to Assess Human Dietary Exposure

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Background and Purpose: Microplastics and nanoplastics (MNPs) are now recognized as widespread environmental contaminants, with increasing evidence pointing to their presence in agricultural soils and food chains. Despite their growing detection, little is known about the extent to which MNPs accumulate in plant-based food sources that are regularly consumed by people worldwide. Rice and beans—staple crops in many global diets—have shown potential for MNP uptake, but current studies fall short of quantifying the levels humans might be exposed to through consumption. One of the significant roadblocks to accurate analysis lies in sample preparation: plant cell walls are difficult to break down using traditional MNPs digestion protocols, which employ lightly destructive chemical methods to preserve the structural integrity of MNPs. **Methods:** This study investigates the use of enzymatic digestion and diluted nitric acid (HNO_3) as pre-digestion methods, aiming to enhance the breakdown of plant tissues while preserving MNP integrity. **Results:** We found that nitric acid was sufficient to break down rice, but beans required the introduction of enzymes (amylase, protease, cellulase, and pectinase). Initial data reveal the occurrence of polyethylene, polystyrene, polyvinyl chloride, and Styrene-Butadiene Rubber in estimated total mass concentrations above 100 mg/kg per dry weight. **Conclusions:** By developing a more effective pre-treatment approach and quantifying MNPs in rice, beans, fruits, and vegetables, this research aims to determine human exposure through diet.

PS 4555 Mechanism of Pulmonary Toxicity by Inhaled Polyester Microplastic Fibers in Mice

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Background and Purpose: Human respiratory exposure to microplastic fibers (MPF) can occur in occupational settings and in everyday life through numerous sources, including textiles. While toxicity of sphere-shaped microplastics in the respiratory system are relatively well-studied, our mechanistic understanding of MPF toxicity in the lung is lacking. In this study, we aimed to identify symptoms and mechanisms of pulmonary injury caused by oropharyngeal aspiration of lab-generated, size-standardized polyester MPF, with and without azobenzene disperse dyes (ADD), in mice. **Methods:** Anesthetized, 12-week-old male mice were exposed by oropharyngeal aspiration to 5,000, 14 μm diameter, 45 μm length clear polyethylene terephthalate (PET) filament MPFs, which were either undyed (UMPF) or dyed (DMPF) with ADD violet 93 (DV93) daily for 5 days/week for 4 weeks. A separate group received DV93 only (no fibers), and controls received equal volume of vehicle. At the end of the exposure period, six mice per group were anesthetized, lung function was analyzed by FlexiVent at baseline and with methacholine challenge (MC; 0-50 mg/mL), bronchoalveolar lavage fluid (BALF) was collected and, after euthanasia, then lungs were excised and RNA was isolated from the tissue. BALF was analyzed for total and differential cell counts. Following quality control and quantification RNA sequencing (RNAseq) was performed and analyzed using CLC Genomic Workbench (Qiagen) and by using R. Differences between groups were tested by one way analysis of variance (ANOVA) followed by Dunnett's post-hoc test for normally distributed data, and by Kruskal-Wallis ANOVA followed by Dunn's test for non-normal data using Graphpad Prism (Version 9.5.1 (733)). Significance was accepted at $p < 0.05$. **Results:** Mice exposed to either UMPF or DMPF had significantly reduced inspiratory capacity at baseline and increased tissue dampening and elastance with 20 and 50 mg/mL MC challenge compared to the control group. Additionally, DMPF had significantly increased total tissue and Newtonian resistance after 50 mg/mL MC. Total cell count, neutrophils and eosinophils were significantly increased in both UMPF and DMPF, while monocytes were only significantly increased in DMPF and lymphocytes were unchanged. No significant changes were observed in respiratory function parameters or differential cell counts in mice exposed only to the dye DV93. RNAseq performed on total lung RNA revealed that there were 194 and 200 transcripts that were significantly modulated compared to control in UMPF and DMPF, respectively, with 135 transcripts being common and 59 vs 65 transcripts being unique for UMPF and DMPF, respectively. The top ten most upregulated genes were identical in both UMPF and DMPF and included CLCA1, a calcium sensitive Cl⁻ channel, GP2, a cell surface glycoprotein, CYP4A11 with CYP22 CYP4A11, two paralogs in the cytochrome P450 superfamily and REG3G, a C-type lectin. Gene enrichment and over representation analysis performed using the list of significantly regulated genes revealed that in gene ontology (GO), Kyoto encyclopedia of genes and genomes (KEGG), Reactome and Wiki pathway terms pertaining to smooth muscle growth and contraction dominated the top enrichment terms and also included cAMP signaling and IL-17 signaling (in KEGG), PPAR signaling (KEGG and Wiki) and various terms related to cytochrome P450 function (KEGG, Reactome and Wiki). **Conclusions:** Exposure of mice to MPFs results in moderate changes in respiratory function, mild pulmonary inflammation, and transcriptomic changes indicative of increased smooth muscle proliferation and reactivity, PPAR signaling and cytochrome P450 activity. The relatively minute differences between dyed and undyed MPFs, suggest a primary role for the fibers rather than DV93 leaching into surrounding tissues. Regardless, RNA sequencing analysis revealed molecular pathways and networks that have not been widely described responses to MPFs in the lung. This is one of the few studies to evaluate biological responses in a model of chronic low dose respiratory exposure to MPFs that may more closely mimic ‘real-world’ exposures. These first-time data will provide the basis for more specific and probing studies of defined mechanisms of action.

PS 4556 Chemical Additive Leaching and Human Exposure Potential from PVC and PE Microplastics in U.S. Drinking Water

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Background and Purpose: Microplastics are increasingly detected in surface water, groundwater, and municipal drinking-water supplies, raising concerns about their role as vectors for human chemical exposure. These particles (<5 mm), originating from degrading larger plastics or the direct release of primary microplastics, can leach additives such as plasticizers, stabilizers, and antioxidants. Many of these substances exhibit persistence, bioaccumulation potential, or toxicological activity, including potential endocrine or developmental effects. As a result, ingestion of microplastics through drinking water may represent a low-level but continuous source of chemical exposure. This study evaluated potential oral exposure and chemical risk from microplastics in drinking water by quantifying particle occurrence, size distribution, and the leaching potential of representative polymer additives. **Methods:** Microplastic particles present in drinking-water samples collected in five U.S. states on two different days at two different times were analyzed using Confocal Raman Microscopy (CRM) to characterize particle morphology and identify polymer

types. Mosaic optical images were first captured to locate particles, after which Raman spectra were acquired under confocal conditions to improve compositional accuracy. Polymer identification was performed by comparing sample spectra with a validated reference database, with an High Quality Index (HQI) score $\geq 60\%$ used as the acceptance threshold. The method reliably identified particles between $\sim 20\ \mu\text{m}$ and $500\ \mu\text{m}$. Ten common polymer categories (including PE, PP, PS, PVC, PET, PC, PMMA, PA, PU, and PTFE) were included in the verified spectral library. To evaluate potential chemical migration, a diffusion-based model was applied to estimate additive release into water over 24 hours, assuming Fickian transport through spherical particles. Polymer-specific diffusion coefficients were applied, and the fraction of additive released was calculated as a function of particle radius and diffusion rate. Predicted water concentrations were combined with the U.S. EPA's adult drinking-water ingestion rate to estimate daily oral intake for each additive. These exposure estimates were then compared with established health-based reference values to determine the relative contribution of microplastic-associated leaching to overall human oral exposure. **Results:** PVC and PE microplastics were detected in all drinking-water samples collected across five U.S. states. PVC additives such as DEHP (di-(2-ethylhexyl) phthalate), DINP (diisononyl phthalate; mixture), DIDP (diisodecyl phthalate; mixture), and TOTM (tri-(2-ethylhexyl) trimellitate) produced estimated exposures of $\sim 20\text{--}22\ \mu\text{g/day}$, while PE additives resulted in $\sim 1\ \mu\text{g/day}$, corresponding to doses in the 10^{-5} to 10^{-4} mg/kg-day range which are well below toxicological benchmarks. Risk Characterization Ratios for nearly all additives fell between 10^{-3} and 10^{-4} , indicating low concern under the modeled conditions. The only notable exception was the organotin stabilizer Dioctyltin bis(2-ethylhexyl thioglycolate) (DOTE), which produced a higher RCR (~ 0.15). Overall, despite the widespread presence of PVC and PE microplastics in drinking water, modeled additive exposures remain far below health-based limits, with organotin stabilizers representing the primary substances warranting closer scrutiny. **Conclusions:** PVC and PE microplastics were consistently detected across drinking-water systems in multiple U.S. states and can contribute small amounts of additive-related chemical exposure. Modeled 24-hour leaching indicated that resulting adult oral intakes for most additives were well below health-based reference values. Only DOTE approached a higher level of concern due to its low toxicological threshold rather than elevated migration. Overall, estimated microplastic-associated chemical exposure from drinking water appears low, though continued monitoring of polymer additives and toxicity data remains important. These findings quantify the contribution of microplastic-associated chemicals to overall oral exposure and support future risk assessment and mitigation strategies for drinking-water systems.

PS 4552 Microplastic And Nanoplastic Testing Framework: Shortfalls and Recommendations Based on A Structured Review of Recent Research

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Background and Purpose: Microplastics and nanoplastics (MNPs), particularly polystyrene (PS) and polyethylene (PE), have emerged as a global concern due to their widespread presence in the environment. Because of this, MNPs are now widely studied for their influence on various health outcomes, including potential genotoxic and carcinogenic effects. Authoritative agencies such as the World Health Organization (WHO) have developed tiered approaches to score the extent by which existing MNP research complies with testing standards, with emphasis placed on relevance of the exposure, rigor in experimental design, and the applicability for the study and its observed effects to existing risk assessment frameworks. However, the extent to which existing published studies adhere to these standards and employ robust analytical techniques is unclear. This investigation systematically evaluates the current body of literature on MNP genotoxicity and carcinogenicity, highlighting the need for harmonized protocols to strengthen the reliability of MNP hazard characterization. **Methods:** First, structured literature reviews were done in PubMed. A *priori* search terms and screening criteria were defined per PRISMA guidelines. A multi-level screening approach was applied to titles and abstracts to identify potentially relevant publications evaluating (i) PS and PE administered as an isolated test material and (ii) endpoints aligned with current testing guidance from the OECD for genotoxicity (gene mutation, chromosomal effects, DNA damage) and carcinogenicity (neoplasms, tumors) in relevant test systems. A quality assessment was then conducted to determine whether the qualitative and quantitative analytical techniques used to assess the MNPs were appropriate and sufficient. Test material deemed suitable for further review included materials sourced as analytical reference material, or synthesized test materials compositionally characterized by several acceptable techniques (e.g., spectroscopy, mass spectrometry, etc.). All test material was considered adequate if assessed for concentration, size, and shape of the particles dosed in their respective test systems. **Results:** A total of 701 publications were found, 471 when using key search terms for genotoxicity and 231 for carcinogenicity. Post title/abstract screening, 51 and 7 publications were identified as potentially relevant by test material and endpoint for genotoxicity and carcinogenicity, respectively. The majority of excluded studies did not assess the key toxicological endpoints for genotoxicity and carcinogenicity or if measured, were investigated in models not recognized by existing testing guideline frameworks for human health risk assessment. Of those that were included, the majority were *in vitro* or *in vivo* investigations using a Comet assay, a genotoxicity indicator test, to evaluate oxidative stress-mediated DNA damage. Fifteen genotoxicity and three carcinogenicity

publications had sufficient test material analytical information. However, the remaining experimental designs still had limitations including non-validated endpoints to assess human health risks and were deemed insufficient to incorporate into a risk assessment framework. **Conclusions:** Despite recent enthusiasm to understand the toxicological potential of MNPs, many publications suffer from methodological limitations that limit their usefulness in current risk assessment frameworks. Such limitations include non-compliance with existing testing guidelines (i.e., OECD), inadequate characterization of test models, which compromises the reliability of the observed effects, as well as analytical inadequacies (e.g., failure to characterize test material pre- and post-administration). Overall, this work provides a tiered approach for the evaluation of existing MNP research in accordance with a standardized review framework. It also highlights critical shortcomings in the current research landscape that hinder integration of published data into existing risk assessment frameworks.

PS 4558 Mechanistic Insights into Nanoplastic Uptake and Barrier Dysfunction in Human Cell Lines

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Background and Purpose: Nanoplastics (NPs) are emerging contaminants of global concern due to their widespread distribution and potential to infiltrate biological systems. Unlike larger microplastics, NPs can cross cellular membranes, accumulate in tissues, and disrupt essential processes. However, the dynamics of NP, cell interactions remain poorly characterized, and most studies have focused on hepatic and intestinal models. Other critical cell types, including endothelial cells, microglia, and keratinocytes, are underexplored despite their central roles in barrier function and immune defense. This study exposed seven human cell lines to NPs to evaluate cytotoxicity, rates of interaction, accumulation patterns, and uptake mechanisms. Additionally, Caco-2 monolayers were used to assess whether NPs compromise epithelial barrier integrity. **Methods:** Seven cell lines, Caco-2, MRC-5, HEK001, HepaRG, HEK293-hTLR2, HMEC-1, and HMC3 were exposed to 0.01-100 $\mu\text{g/mL}$ of polystyrene NPs ranging from 0.07 to 1.35 μm , as well as a mixed-size suspension, for 24-48 hours. Cytotoxicity was measured with PrestoBlue™ assay. Dynamic imaging was conducted with the Lionheart Automatic Microscope at 30-minute intervals for 48 hours. Fluorescent Nile Red-labeled polystyrene NPs were used to quantify uptake, with cytochalasin D applied to evaluate actin-dependent endocytosis. Barrier integrity was assessed in Caco-2 cells using transepithelial electrical resistance (TEER). **Results:** Cytotoxicity was observed only in endothelial cells (HMEC-1), which showed decreased viability with 1.35 μm NPs and mixed suspensions. Other cell lines maintained viability across exposures. Live-cell imaging revealed minimal NP uptake in Caco-2 and HepaRG cells, though HepaRG showed delayed accumulation. HEK001, HEK293, HMC3, HMEC-1, and MRC-5 actively internalized NPs, with perinuclear accumulation evident after 48 hours. Fluorescence analysis confirmed significant uptake in HepaRG, HEK293, MRC-5, and size-selective accumulation in HMEC-1, HMC3, and HEK001. Cytochalasin D reduced uptake in several models, confirming actin-mediated pathways, but paradoxically increased NP association in Caco-2 and MRC-5. Barrier testing showed that 1.35 μm NPs disrupted Caco-2 integrity, reducing TEER values comparably to Triton X-100, while smaller particles had no measurable effect. **Conclusions:** This study demonstrates that NPs interact with human cells in complex, cell-specific ways. Endothelial cells are especially vulnerable, while many other cell types internalize NPs via actin-dependent mechanisms. Larger NPs disrupt epithelial barriers, while smaller particles accumulate intracellularly, raising concerns about long-term, sublethal impacts. Together, these findings highlight NPs as biologically active contaminants and emphasize the need for chronic exposure studies targeting barrier dysfunction, immune activation, and subtle toxicity mechanisms.

PS 4559 Proquinazid: In Vitro Metabolism of an Agrochemical Fungicide to support Mechanistic Toxicology Testing and PBK Model Development

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Background and Purpose: The mammalian metabolism of a crop protection chemical is an essential component to understanding relevance to human health risk assessment. The comparative *in vitro* metabolism of proquinazid-¹⁴C was investigated in human, rat, mouse, dog and rabbit hepatocytes. This type of study is a regulatory requirement in Europe; however, it can also support the interpretation of NAM mechanistic studies, and aid building *in vitro* to *in vivo* concordance for application to human PBK modeling. Here, the main objective was to identify metabolites unique to the human species at 5% of dose or greater relative to the other test species. Metabolites observed *in vitro* can be compared with *in vivo* ¹⁴C plasma metabolites in rats that formed the bases for PBK modeling of liver metabolism. **Methods:** Pooled cryopreserved hepatocytes were from male human (n=10), male Sprague Dawley rats (n=18), male CD-1 mouse (n=36), male beagle dogs (n=3) and male New Zealand white rabbit (n=4). Experiments were performed using proquinazid-phenyl-UL-¹⁴C (proquinazid-¹⁴C) prepared in acetonitrile stock solution. The cell viability by Trypan blue exclusion prior to the start of incubations ranged from 76 to 93%. The hepatocytes were suspended in an incubation volume of 1 mL containing 2×10^6 cells/mL at 37°C. Reactions were started by addition of 5 μL of dose stock solution (organic

solvent 0.5% of the incubation volume) for a starting incubation concentration of 18 μ M. Incubations were terminated at 15, 60 and 120 min by the addition of 1 mL of ice-cold acetonitrile. Specific endpoints included percent ^{14} C-recovery, extraction efficiency, and identification and quantitation of metabolites as percent of applied radioactive dose. The profile of metabolites was characterized in the extracts by HPLC mass spectrometry in conjunction with in-line ^{14} C-radiochromatography and retention time match with available metabolite standards. **Results:** The metabolite profile of proquinazid- ^{14} C in the human was compared with profiles for the rat, mouse, dog and rabbit species. Proquinazid and 12 metabolites (13 components total) were identified in the human species across all time points. Only one metabolite radiochemical peak was quantified in the human species at a level of 5% or greater of the applied radioactive dose. This radiochemical peak consisted of two coeluted metabolites, X12649845 and X12649855-a, that could not be fully separated. For all other species (rat, mouse, dog, and rabbit), this same peak consisting of coeluted X12649845 and X12649855-a metabolites was detected and quantified at a higher percent of the applied dose in at least one of the three incubation time points. The *in vitro* metabolism of proquinazid was extensive. Overall, the metabolic reactions involved a series of mono- and di-hydroxylations, ether hydrolysis (O-dealkylation), and glucuronide conjugation. The hydroxylated metabolites were further oxidized to ketone and/or carboxylic acid moieties. Three metabolites (X12649845, X12649801, and X12649859) in the rat species were subsequently identified in plasma of a ^{14} C *in vivo* study, two (X12649845 and X12649801) of these three were also observed in the current *in vitro* study with human hepatocytes. **Conclusions:** The proquinazid metabolite profile provides weight-of-evidence that metabolite formation maintains parity across toxicology test species and the human and between *in vitro* and *in vivo* test systems. Concordance with an *in vivo* rat study was observed and supported selection of 4 metabolites for PBK modeling. The overall results provide improved confidence for application to human health risk assessment.

PS 4560 Neurotoxicity Risk Modeling of Oral-Mucosal BPA Exposure from Dental Resin Composites: Implications for Reassessing the U.S. EPA Standard

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Background and Purpose: Bisphenol A (BPA) is a ubiquitous organic monomer with well-documented endocrine-disrupting and neurotoxic effects. Dental materials frequently contain BPA derivatives, such as bisphenol A-glycidyl methacrylate (bis-GMA) in resin-based composite fillings, which can release BPA into the oral cavity upon degradation. Although BPA exposure from dental resin composites (DRCs) has been considered safe based on regulatory thresholds, these existing ingestion-based safety levels do not account for oral-mucosal absorption, a critical exposure route for dental materials that remain in the oral cavity for extended periods. Furthermore, the recent substantial downward revision of the BPA safety guideline by the European Food Safety Authority (EFSA) suggests the need for reevaluation. Despite the widespread use of DRCs—utilized in 96.1% of caries restorations in the U.S.-insured population in 2023—the neurotoxicity risk associated with BPA absorption through the oral mucosa remains uncharacterized. **Methods:** To evaluate neurotoxicity risk from BPA oral-mucosal absorption, we derived a novel serum reference dose (SRfD) by integrating data from a murine BPA neurotoxicity study and cross-species BPA metabolic studies. A one-compartment risk assessment model was used to estimate serum BPA levels resulting from chronic BPA mucosal absorption (BMA) in modeled U.S. pediatric (ages 6-11) and adult (ages 20-64) populations undergoing simulated caries treatment with 5% or 30% bis-GMA composites. Model parameters were obtained from published sources, including dental material fact sheets, population statistics, and human serum BPA measurements. **Results:** All BMA exposure scenarios, including 1% absorption from DRCs containing 5% bis-GMA, exceeded the neurotoxicity SRfD of 0.000328 ng/ml in both populations, regardless of other BPA sources. Whereas only 0.1% of ingested BPA enters systemic circulation as bioactive BPA, BMA was estimated to result in 4-5% steady-state serum concentration of bioactive BPA, suggesting greater bioavailability and potential for toxicity. The neurotoxicity risk associated with the current U.S. Environmental Protection Agency (EPA) reference dose exceeded the SRfD by at least 2,250-fold, whereas the EFSA guideline remained more than 100-fold below the derived SRfD. **Conclusions:** The derived SRfD enables neurotoxicity risk assessment across exposure routes, particularly oral-mucosal absorption, which is highly relevant to dental materials. The elevated risk from BMA highlights the importance of careful safety evaluation of dental materials, as substances retained in the oral cavity may serve as chronic sources of exposure to neurotoxicants, such as BPA. Reassessment of the EPA safety threshold is urgently warranted, given that it has remained unchanged since 1988, exceeds the updated EFSA guideline by 250,000-fold, and far surpasses the derived neurotoxicity SRfD.

PS 4561 A Key Biodistribution Study, with Fluorescent Acquisitions in Mice, for the Development of a Therapeutic Candidate for ALS Patients

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Background and Purpose: K-ENV is present in affected brain neurons and cerebrospinal fluid (CSF) of sporadic amyotrophic lateral sclerosis (ALS) patients. A study was performed at ERBC Dommartin to evaluate biodistribution of GNK301, an IgG4 humanized antibody neutralizing the HERV-K ENV (K-ENV) neurotoxic endogenous retroviral protein. **Methods:** A fluo-labelled version of GNK301 was injected IV to 2 groups of mice previously injected intracerebrally by stereotaxy with K-ENV protein or its solution buffer. A third group of 4 mice served as untreated sham control. Clinical follow-up and fluorescent imaging using an IVIS Spectrum were performed over 5 days post administration. **Results:** A specific accumulation of fluo-GNK301 was observed in the cerebral hemispheres of treated mice. A significantly elevated signal was present in the left hemisphere, at the site of K-ENV stereotaxic injection, in comparison to the signal obtained in mice not injected with K-ENV. Sham mouse brains displayed no signal. **Conclusions:** These results indicate the passage of fluo-GNK301 across the blood brain barrier and its accumulation at the site of K-ENV antigen injection in the left brain hemisphere. This work highlights the usefulness of fluorescence detection in preclinical animal studies, in particular when exploring biodistribution in the central nervous system.

PS 4562 Analytical method development and dermal absorption of Pyranine Conc(G204), a cosmetic ingredient

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Background and Purpose: Pyranine Conc (G204) is utilized as a coloring agent of cosmetics at a maximum concentration of 0.01% in Korea and it is not allowed to be used around the eyes or on the lips. As no reports exist regarding the dermal absorption of G204 in cosmetic products, an *in vitro* dermal absorption study was conducted in compliance with the guidelines according to the Ministry of Food and Drug Safety (MFDS) of Korea. **Methods:** Prior to conducting the dermal absorption study, analytical methods were established using high-performance liquid chromatography with ultraviolet detection (HPLC-UV) technique for the quantification of G204 in various matrices, including swabs, stratum corneum (SC), skin (dermis and epidermis), and receptor fluid (RF). The G204 analytical method used in this study demonstrated excellent linearity ($r^2 = 0.9911$ - 0.9998), accuracy (81.1-109.1%), and precision (0.5-7.2%) in compliance with the validation guidelines. Considering the LLOQ values for each matrix, dermal absorption studies were conducted using 0.3% and 0.5% G204 formulations, concentrations higher than the maximum permitted level, in order to ensure the analytical accuracy of the study. To evaluate the skin absorption of G204 in coloring agent, a Franz diffusion cell was utilized. Cream formulation of G204 (0.3 and 0.5%) was applied to mini-pig skin at a dose of 10 μ l/cm². After 24 hours of application, the skin was wiped with a swab, and the stratum corneum (SC) was collected using tape stripping. Subsequently, the mini-pig skin was sectioned into eight pieces, and receptor fluid (RF) samples were collected at 0, 1, 2, 4, 8, 12, and 24 hours. **Results:** As a result, the total dermal absorption rate of G204 was determined to be 0.60 ± 0.39 μ g/cm² ($10.11 \pm 6.49\%$) and 1.38 ± 1.00 μ g/cm² ($13.75 \pm 9.90\%$) for the cream formulations of 0.3 and 0.5%, respectively. **Conclusions:** These results can be utilized for the exposure assessment of G204 when used as a cosmetic ingredient.

PS 4563 Hepatotoxic Mechanisms by Drug-Induced Cholestasis and Reactive Metabolites in Human Hepatocyte Co-Culture System

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Background and Purpose: Hepatotoxicity is a critical concern in drug development because the liver is the primary organ responsible for drug metabolism and detoxification. Drug-induced cholestasis (DIC) represents a major subtype of drug-induced liver injury (DILI) that arises when compounds disrupt bile acid synthesis, uptake, or efflux in hepatocytes. This leads to bile acid accumulation and liver injury. Early detection of cholestatic potential is essential, as DIC is often species-specific and not reliably predicted by conventional animal models. Reactive drug metabolites are unstable intermediates formed during xenobiotic biotransformation, primarily mediated by hepatic cytochrome P450 (CYP) enzymes. These metabolites can covalently bind to cellular macromolecules such as proteins, lipids, and DNA, causing oxidative stress, mitochondrial dysfunction, and immune-mediated hepatocellular injury. Traditional liver toxicity assessments relying on animal models and isolated liver tissues have limitations including high cost, low throughput, interspecies variability, ethical concerns, and limited predictivity for human outcomes. We previously developed an advanced hepatocyte co-culture system that maintains long-term viability (>4 weeks) and stable metabolic function. These models (human, dog, and rat) were

validated with 29 reference compounds, demonstrating dose-dependent hepatotoxicity, species-specific responses, and improved sensitivity (83-92%) compared to hepatocyte monocultures (50%). In the present study, we utilized the human hepatocyte co-culture system to evaluate compounds associated with DIC and determine the effect of reactive metabolites on hepatotoxicity. **Methods:** The human hepatocyte system was generated by co-culturing cryopreserved primary human hepatocytes with stromal fibroblast cells in collagen-coated 96-well plates for one week prior to test compound exposure. For the DIC study, cholestatic compounds (cyclosporin A and bosentan) were tested at seven concentrations in the presence and absence of a bile acid (BA) mixture to mimic physiological cholestatic conditions. For the reactive metabolite study, compounds known to produce bioactivated intermediates (cyclophosphamide and aflatoxin B₁) were tested at seven concentrations with and without a broad pan-CYP inhibitor, aminobenzotriazole (1 mM). Hepatotoxicity was assessed using an ATP cell viability assay after 7-day exposure to the test compounds. TC₅₀ values were calculated from non-linear regression curves fitted with GraphPad software. **Results:** The results demonstrated dose-dependent hepatotoxic effects for the test compounds. In the DIC study, TC₅₀ values for both cyclosporin A and bosentan were significantly lower with BA supplementation, indicating bile acid associated hepatotoxicity. In the reactive metabolite study, co-treatment with the CYP inhibitor significantly reduced the cytotoxicity of both cyclophosphamide and aflatoxin B₁. TC₅₀ values for these compounds were significantly higher with the CYP inhibitor, confirming that their hepatotoxicity is mediated by reactive metabolite formation. **Conclusions:** The human hepatocyte co-culture model provides a robust and predictive *in vitro* platform for elucidating hepatotoxic mechanisms associated with cholestasis and reactive metabolites. These models enable identification of cholestatic risk, mechanistic evaluation of bioactivation and detoxification pathways, and detection of delayed or cumulative hepatotoxicity from repeated exposure. Overall, these models reduce reliance on animal testing and improve human-relevant risk assessment, supporting the design and development of safer drug candidates.

PS 4564 Multi-Method, Interpretable Prediction of Oral Bioavailability and Formulation Vehicle Effects

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Background and Purpose: Both active pharmaceutical ingredients (APIs) and trace chemical impurities can vary widely in oral bioavailability (BA), driven by differences in physicochemical properties, metabolism, transport mechanisms, and formulation characteristics. Solvent or vehicle choice, such as aqueous suspensions, co-solvent systems, or lipid carriers, can influence systemic exposure; however, practical tools for assessing overall BA and its sensitivity to vehicles remain limited. In addition to APIs, predicting the bioavailability of leachable or extractable impurities is critical for assessing systemic exposure related toxicological risks arising from container closure systems and medical devices. This work presents a novel, transparent and multi-method prediction framework that integrates data-driven QSAR models, read-across, and expert rules to (i) estimate baseline oral BA and (ii) evaluate how formulation vehicles may shift that baseline. **Methods:** The framework integrates several complementary components: 1. **Curated database:** A dataset of 1,594 compounds with experimentally measured BA from peer-reviewed literature. 2. **Baseline BA Prediction System:** Three independent methods provide qualitative BA classifications (Very Low, Low, Medium, High) with associated uncertainty estimates: i. **Analog-based prediction:** Using top analogs, based on structural, physicochemical and BA specific feature similarity. ii. **QSAR model:** Built using physicochemical and fragment-based molecular descriptors. iii. **Expert rule-based prediction:** Applies interpretable thresholds for molecular weight and LogP. 3. **Supplementary ADME Modifiers and Vehicle Sensitivity Module:** i. **ADME Modifiers:** QSARs for metabolic stability, CYP substrate likelihood, and MDR1 (P-gp) substrate potential. ii. **Vehicle Sensitivity:** Expert rules that evaluate LogP, LogS, TPSA, and HBD to characterize solubility-permeability balance and Biopharmaceutics Classification System (BCS) class. Logical criteria for aqueous CMC (0.5%), aqueous co-solvent (≤5% DMSO), and lipid or surfactant systems predict qualitative BA changes (significant, modest, or minimal) with mechanistic rationale. **Results:** Across diverse chemotypes, the multi-method framework produced consistent and interpretable outcomes. Analog-based and QSAR predictions generally agreed within uncertainty margins, while the rule-based method provided a transparent baseline and mechanistic rationale. In cross-validation, the analog-based approach achieved 47% strict and 89% within-one-tier accuracy, the QSAR model 45% and 88%, and the expert rules 39% and 79%, respectively. Supplementary ADME screens identified cases where rapid metabolism or transporter interactions restricted achievable BA despite favorable solubility-permeability profiles. Predicted formulation-property relationships aligned with experimental behavior—for example, lipid-based vehicles produced the largest BA gains for highly lipophilic, poorly soluble molecules (LogP > 5; LogS < -2) with adequate permeability (TPSA < 120 Å²; HBD ≤ 5). **Conclusions:** This integrated framework provides a mechanistically interpretable and validated approach for predicting oral bioavailability and its modulation by formulation vehicles. By combining analog-based inference, probabilistic QSARs, and expert rules within an ADME-aware context, it delivers both predictive confidence and mechanistic insight. Beyond APIs, the approach extends to predicting the bioavailability and systemic exposure of extractable and leachable impurities, supporting evaluation of their potential human health impact. Overall,

the framework unites empirical formulation science and computational modeling to inform early-stage formulation design, impurity risk assessment, and regulatory decision-making.

PS 4565 Proquinazid: Quantitative Blood and Plasma Metabolite Identification in the Sprague-Dawley Rat to Support an Improved Evaluation of Internal Dosimetry

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Background and Purpose: Proquinazid is a crop protection fungicide for which an improved understanding of metabolism in the rat is needed to support safety testing and regulatory assessment. Previous absorption, distribution, metabolism and excretion (ADME) studies in the rat investigated proquinazid-¹⁴C metabolites in excreta (urine and feces), plasma pharmacokinetics, tissue distribution, and biliary elimination following single oral dose administration. A follow-up study was conducted with repeated oral dose administration of proquinazid-¹⁴C; however, neither study investigated the metabolite profile of proquinazid in the systemic circulation, a key matrix for advanced kinetic modeling to facilitate interpretation of toxicological findings. The current study identified and quantified the profile of proquinazid metabolites in plasma and blood of female Sprague Dawley rats, the species used for the existing regulatory toxicology package. **Methods:** The profile of metabolites in the blood and plasma of female Sprague Dawley (CrI:CD(SD)) rats was investigated following single oral gavage administration of proquinazid-Ph-UL-¹⁴C (proquinazid-¹⁴C) at 20 mg/kg bw (body weight). Female rats were selected since the metabolism and pharmacokinetic parameters were similar between sexes in previous ADME studies. The dose level matched that previously used. The ¹⁴C dose of 500 µCi/kg bw was prepared in 0.1% Tween 80 in 0.5% aqueous methylcellulose at a dose volume of 10 mL/kg bw. Blood and plasma samples were collected in a staggered design distributed among 4 rats to provide a ¹⁴C concentration time course at 0.25, 1, 2, 4, 6, 8, 10, and 12 h after administration. After total ¹⁴C counting, the parent proquinazid-Ph-UL-¹⁴C radiolabeled test substance and metabolites were quantified in the rat plasma and blood samples using HPLC with in-line radioactivity detection and identification was performed using high resolution accurate mass (HRAM) spectrometry with confirmation using available reference standards. Kinetic analysis of the plasma and blood time-course results included determination of T_{max}, C_{max}, area-under-the-curve (AUC), and metabolites as a percentage of the total ¹⁴C AUC. **Results:** Undifferentiated proquinazid-¹⁴C equivalents were reliably quantified through 12 h post dose administration. Parent proquinazid was extensively metabolized and was not quantifiable in either plasma or blood. Eleven metabolites were observed in rat plasma and blood. The metabolic pathway involved: 1) mono- and di-hydroxylation, 2) O-dealkylation, 3) N-dealkylation, 4) oxidation of terminal N-propyl carbon to carboxylic acid, 5) glucuronidation, 6) sulfate conjugation, and 7) combinations thereof. Ranked in order of plasma C_{max} values, the most abundant metabolites were X12649845, X12649801, X12688723, and X12649859, with similar rankings in blood. The overall T_{max} for ¹⁴C equivalents was 2 h, while T_{max} values for metabolites ranged from 1 to 10 h. AUC values of the 11 measured metabolites, summarized as percent of the total ¹⁴C equivalents, accounted for 97.2% and 98.0% of the total ¹⁴C AUC in plasma and blood, respectively. The four most abundant metabolites accounted for 84.3% and 86.8% of the total radioactivity in plasma and blood, respectively. The remaining 7 metabolites in plasma and blood accounted for ≤12.9% of the total ¹⁴C AUC. None of these 7 metabolites individually accounted for greater than 2.7% except for X12649849 and a tentative hydroxylated metabolite of X12649801 (MW346) which co-eluted and could not be distinguished from each other. **Conclusions:** Overall, systemic exposure was well characterized by the percent AUC rank order of the four most abundant metabolites in plasma and blood as X12649845 > X12649801 > X12688723 > X12649859. This data provides internal dosimetry of metabolites for proquinazid-induced liver effects and serves a key role for PBK modeling development in the rat for subsequent extrapolation to the human liver dosimetry.

PS 4566 iPSC-derived Intestinal Epithelial Cells Form a Functional, Relevant Barrier Model of the Human Gut

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Background and Purpose: Intestinal epithelial cells (IEC) make up the single layer lining of the small intestine. These cells act as a barrier to prevent entry of harmful products and regulate uptake of nutrients and fluid. The intestinal epithelial layer is composed of numerous cell types with varied roles, including goblet cells for mucin secretion and M-cells for the uptake of antigens and intestinal bacteria. Human iPSC-derived IEC offer a renewable source of cellular material with consistent intestinal function and are available for use in FDA-recommended New Approach Methodologies (NAM). **Methods:** iCell Intestinal Epithelial Cells (FUJIFILM Cellular Dynamics) were differentiated from two different donors (01279 and 21527) and cryopreserved at a density of >3M cells per vial. Upon thaw, cells were characterized by flow cytometry, ICC, and RNA expression using IEC identity markers CDX2, EpCAM, VIL1, and KRT20. Cells were cultured on Transwell® Inserts in both 24- and

96-well format. Barrier function was assessed over time by TEER assay and under inflammatory conditions +/- TNF α (100 ng/mL) and IFN γ (50 ng/mL). CYP activity was measured by P450-Glo™ Assays (Promega) and LC-MS/MS and correlated to human *in vivo* Fg. iCell IEC were assessed for proliferation by Ki-67 flow cytometry. Intestinal subtypes were identified by ICC. **Results:** Human iPSC-derived IEC express CDX2, EpCAM, and VIL1 at >80% by flow cytometry and KRT20 around 70% for both donors. After a 5-day maturation period post-thaw, iCell Intestinal Epithelial Cells showed TEER values >600 ohms x cm² and stably maintained that level for at least another 5 days. During this time frame, P-gp activity was assessed and iCell IEC exhibited an efflux ratio >2, indicating functional transporter activity. CYP3A4 activity from iPSC-derived IEC was equivalent to primary intestinal cells. Moreover, the estimated *in vitro* Fg for Felodipine, Midazolam, Verapamil, and Sildenafil were highly correlated to Human *in vivo* Fg. All of these suggest iCell IEC is a reliable system for studying intestinal drug metabolism and absorption. iCell IEC were nearly 50% positive for the proliferation marker Ki-67 by flow cytometry at thaw but decreased to less than 20% after 7 days of culture. Intestinal cell subtypes were identified by ICC, with a majority of enterocytes (KRT20) and presence of Goblet (MUC2), Paneth (LYZ), Enterendocrine (CHGA), Tuft (AVIL), and Microfold (GP2) cells at physiologically relevant levels. **Conclusions:** Nutrient and drug absorption and metabolism of the small intestine is critical to understanding drug efficacy and toxicity. iPSC-derived IEC provide a biologically relevant alternative to immortalized cancer lines without the inherent variability of primary tissue. Implementation of iCell IEC into assays for drug metabolism, uptake, and inflammatory response provides a novel platform for interrogating intestinal function in toxicology workflows with more human relevance.

PS 4567 Analyses of delta9-tetrahydrocannabinol and the endocannabinoids in mouse plasma and brain

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Background and Purpose: Adolescence represents a critical period of neuromaturation, particularly within realms governing cognitive and behavioral function. Importantly, the endocannabinoid system (ECS) achieves peak expression and activity during this period. Delta9-tetrahydrocannabinol (THC) binds with high affinity to cannabinoid receptor 1 (CB1R) within the ECS, disrupting EC signaling and dysregulating neurotransmitters. A mouse model was utilized to test the hypothesis that vaporized THC dominant cannabis use causes sex- and age-dependent effects on the ECS. **Methods:** A commercially available THC dominant cannabis vape cartridge (75% THC) or vehicle (50:50 propylene glycol:vegetable glycerin, PG/VG) were administered once-daily to mice over 20 minutes via a SCIREQ In-Exposure inhalation system as part of an IACUC approved study. 8-week-old mice (adolescent) received either 1 or 5 weeks of cannabis, while 12-week-old mice (adult) received one week. Mice were sacrificed by decapitation at specified time points to collect plasma and brain, which were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine concentrations of THC/metabolites and the endocannabinoids anandamide (AEA) and 2-arachidonylglycerol (2-AG). Data analysis utilized Phoenix WinNonLin v8.5.2.4 and GraphPad Prism v10.6.1. **Results:** First-dose mean (standard deviation, SD) peak plasma concentrations of THC were 27.5 (14.0) and 25.8 (7.95) ng/mL for 8- and 12-week-old mice, respectively, and did not differ by sex. However, 8-week-old mice treated for 5 weeks had significantly lower peak THC plasma concentrations (mean (SD): 11.3 (2.57) ng/mL) than first dose in either 8- (p=0.0034) or 12-week-old (p<0.0001) mice. THC plasma half-life was 2.1 hr. OH-THC mean peak plasma concentrations were higher in males than females in both 8-week (3.45 vs. 2.00 ng/mL) and 12-week-old mice (3.37 vs. 2.13 ng/mL), though these values did not reach significance. Frontal cortex AEA in animals treated for one week did not differ by age or sex, but was significantly lower than vehicle treated animals (48.1 vs. 68.8 ng/g; p<0.0001). Similarly, frontal cortex 2-AG was lower in mice treated for one week compared to vehicles (3283 vs. 5273 ng/g; p=0.0003), but did not differ by age or sex. After 5 weeks of treatment, AEA (55.1 ng/g; p=0.01) and 2-AG (4334 ng/g; p=0.0004) were increased relative to a single week of treatment and similar to values observed in mice administered PG/VG vehicle for 5 weeks (AEA: 61.0 ng/g; 2-AG: 4593 ng/g). **Conclusions:** In summary, plasma PK of THC and its metabolites did not differ by age or sex, nor did frontal cortex concentrations of either AEA or 2-AG. Continued THC use over five weeks resulted in significantly lower THC plasma concentrations, likely indicating induced metabolism following chronic use. One week of THC dosing significantly reduced both AEA and 2-AG in the frontal cortex relative to vehicle controls, regardless of age, though decreases were attenuated after 5 weeks. These data suggest that acute THC dominant cannabis use has the potential to dysregulate neural function regardless of initiation age, while chronic use results in adaptations in metabolism of both exogenous and endogenous cannabinoids. Further work is needed to determine if ECS changes following the use of vaporized THC dominant cannabis drives changes in cognitive and/or behavioral function.

PS 4568 MSQ-DTR: A targeted proteomics tool for transporter quantification and validation of blood-brain barrier *in vitro* model systems

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Background and Purpose: Efflux transporters at the blood-brain barrier (BBB) play a critical role in protecting the central nervous system by actively transporting compounds out of the brain and into the surrounding vasculature, thereby limiting exposure to potentially harmful substances. The main classes of BBB efflux transporters include ATP-binding cassette (ABC) transporters namely P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), multidrug resistance proteins (MRPs), and solute carrier transporters such as organic anion transporting polypeptides (OATPs) and organic anion transporters (OATs). Current efforts to quantitatively characterize efflux transporter function at the BBB are often constrained by *in vitro* models that inadequately replicate the complex physiology of the BBB. A key limitation is the lack of accurate data on the proteomic abundance of transporters in these models, which hinders the translation of findings to *in vivo* contexts. For drug developers and toxicologists utilizing physiologically based pharmacokinetic (PBPK) models, this gap presents a significant challenge. Without reliable and translatable transporter expression data, predictions of drug disposition and CNS exposure remain uncertain, limiting the utility of these models in guiding therapeutic development and safety assessments. The objective of this study was to develop and validate an LC-MS assay tool kit (MSQ-DTR) for quantifying the abundance of efflux transporter proteins at the BBB. **Methods:** The MSQ-DTR kit includes unique and sensitive stable isotope-labeled (SIL) surrogate peptides for P-gp, BCRP, MRPs, and MATE1, as well as solute carrier transporters such as OATPs, OCTs, and OATs. Surrogate peptides were chosen to be conserved across multiple species to enhance translational relevance. We performed a simultaneous quantification of the target protein levels in commercially available samples of tissue and brain microvascular endothelial cells (BMECs) in humans and key preclinical species. Raw data was acquired using nanoLC coupled with a Q-Exactive HF mass spectrometer in the parallel reaction monitoring (PRM) mode and analyzed using Skyline software. **Results:** Validation of the MSQ-DTR quantification kit. The panel of surrogate peptides was shown to be linear, accurate, and reproducible across a wide range of concentrations, with coefficient of variation (%CV) values less than 20%. Surrogate peptides are stable in the matrix of human liver microsome digest for up to 48 hours at 6 °C, showing an 85-115% response at both the upper and lower limit of quantification (ULOQ and LLOQ). **Quantification of efflux transporters in samples.** The MSQ-DTR kit was used to analyze commercially supplied BMEC lots from single donors. In these test samples the expression levels of P-gp and BCRP in human, mouse, and monkey BMECs were found to be less than 1 pmol/mg. This value is significantly lower than previously published values and underscores a key challenge - technical variability in commercially available *in vitro* models. These results show that future efforts to improve reliability and translatability should always include samples from multiple donors and include rigorous validation across platforms. **Conclusions:** While there are numerous ongoing efforts to develop *in vitro* models of the BBB, incorporating transporter proteomics should be an integral piece of the qualification of such models. Our future work includes utilizing the MSQ-DTR kit to characterize *in vitro* and experimental models such as microphysiological systems and additional preclinical animal models, as well as assessment of donor and lot-to-lot variability of cell lines and other *in vitro* reagents. We envision the MSQ-DTR kit will provide researchers with a tool to collect robust abundance data on efflux transporters, ensuring the translational relevance and reliability of the data. A better understanding of BBB models allow for improved *in vitro* to *in vivo* extrapolation predictions and ultimately improved accuracy of drug metabolism and toxicity predictions.

PS 4569 Development and Application of a Physiologically-Based Kinetic Model for Evaluation of Internal Dose in the Rat and Human

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Background and Purpose: Proquinazid is a fungicide used on food crops. The regulatory toxicological package identifies the liver and thyroid as target organs in rats following exposure to proquinazid. The pattern of effects suggest that effects on the thyroid are likely due to higher thyroid hormone turnover as a consequence of liver induction, in particular increased UGT clearance of T4. A conservative reference point of 5 µM was taken from a study comparing UGT-T4 activity in Sprague-Dawley (SD) rat and human hepatocytes *in vitro*. A PBK model was used to determine if the human liver concentration of proquinazid and the sum of its metabolites would reach a 5 µM liver concentration *in vivo*, from estimated dietary exposure. **Methods:** A fit for purpose PBK model was created in R, using assumptions derived from available proquinazid *in vivo* and *in vitro* ADME studies. The PBK model was used to make predictions assuming an estimated, worst-case chronic dietary exposure of 0.84 µg/kg bw/day in humans (predicted from the EFSA PRIMo v3.1 dietary exposure model). **Results:** The predicted sum of proquinazid and its major metabolites in liver was 0.00240 and 0.00266 µM for male and female subjects, respectively. These results suggested that the margin-of-safety approaches 2000-fold relative to the much lower

estimated dietary exposure in humans. **Conclusions:** The human liver internal dose would not be expected to approach a level that would be associated with biologically relevant liver mediated thyroid effects, following worst-case human exposure estimates.

PS 4570 A Physiologically Based Pharmacokinetic (PBPK) Model to Align Dosimetry of the Isobutyl Metabolic Series in Rats and Humans

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Background and Purpose: Traditional chemical risk assessment often involves evaluating toxicity data for individual chemicals in laboratory animals. However, for metabolically related chemicals, the metabolic series approach offers an alternative method to assess toxicity of downstream metabolites after exposure to a parent compound, provided that the chemical dosimetry is well understood. In this study, we developed a physiologically based pharmacokinetic (PBPK) model in rats and humans for the isobutyl metabolic series, which includes isobutyl acetate, isobutanol, isobutyraldehyde, and isobutyric acid. **Methods:** To construct the isobutyl PBPK model, we leveraged a previously developed PBPK model for the propyl metabolic series as a framework. To support model development, we measured *in vitro* metabolism of isobutyl acetate in rat and human blood and liver S9 fractions. **Results:** Our findings indicated that humans exhibited faster isobutyl acetate hydrolysis in liver S9 fractions compared to rats, while hydrolysis rates in blood were similar between the two species. Experiments involving closed chamber exposures of rats to isobutyl acetate or isobutanol revealed higher isobutanol concentrations in blood compared to other isobutyl compounds. Using these data to parameterize the model, the PBPK model accurately simulated available time-course concentrations of isobutyl compounds in rats and humans. **Conclusions:** The isobutyl PBPK model enables comparisons of internal dose metrics across various isobutyl compound exposures and species and allows for calculation of equivalent external exposures that result in the same dose metric. Regulators can employ this PBPK model to predict and align internal dose metrics of isobutyl compounds for risk assessment purposes.

PS 4571 MASH Impairs Renal Anion Transporter Function and Alters Cefazolin Disposition

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Background and Purpose: Metabolic dysfunction-associated steatohepatitis (MASH), a progressive form of liver disease, affects up to 7% of the global population. While hepatic transporter alterations in MASH have been well characterized, far less is known about the effect of MASH on renal transport processes. In particular, the renal handling of organic anions remains poorly understood. Herein, we used cefazolin (CEF), an anionic antibiotic primarily used to prevent surgical site infection, as a model compound to investigate MASH-induced alterations in renal transporter function and their role in pharmacokinetics. **Methods:** MASH was induced in mice using fast food-dieted/thioacetamide (FFDTH) which has been shown to accurately model the renal transporter changes seen in human MASH. Transporter expression was evaluated using LC-MS/MS proteomics and plasma, urine, and tissue concentrations of CEF were determined. **Results:** Expression of Oat3 and Oat5 were significantly reduced (50% and 80%, respectively) in MASH animals compared to controls. In response, plasma concentrations of CEF increased to 183% in MASH compared to controls, while hepatic accumulation increased to 180% of control. No differences in renal accumulation or excretion of CEF were observed between MASH and healthy mice. **Conclusions:** This data suggests that MASH-induced OAT transporter reduction increases both systematic CEF concentration along with accumulation in liver, without differences in renal or urinary excretion. Overall, these findings highlight the risk of increased systemic exposure in MASH patients, ultimately suggesting that it would be beneficial to explore dose adjustments in these patients.

PS 4572 PCB-11 Induces Brain CYP2B10 Expression and Activity in Mice: Implications for Neurotoxicity and Drug Interaction Potential

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Background and Purpose: 3,3'-Dichlorobiphenyl (PCB-11) is a persistent environmental contaminant unintentionally produced during the synthesis of diarylide yellow and other organic pigments, which are used in printing inks and other applications. It enters the environment through air, water, and consumer products, and has been detected in human tissues, raising concerns about its potential health risks. Experimental evidence suggests that PCB-11 and its metabolites can disrupt neuronal connectivity, implicating possible neurodevelopmental and neurotoxic

outcomes. Cytochrome P450 (CYP) enzymes are key mediators of xenobiotic and endogenous compound metabolism. Among them, CYP2B6 (and its murine homolog CYP2B10) plays an important role in metabolizing neuroactive drugs, thereby influencing both therapeutic efficacy and the risk of adverse effects. CYP enzymes also metabolize PCBs into hydroxylated metabolites (OH-PCBs), which can be more toxic than the parent compound. The aim of this study is to understand how PCB-11 influences CYP2B10 expression in the brain, which is essential for evaluating the potential of PCB-11 to alter drug metabolism and neurotoxic outcomes. **Methods:** Wild-type C57BL/6 mice were exposed to PCB-11 (500 µmol/kg body weight) or vehicle (peanut oil) via oral gavage for three consecutive days. Brain membrane preparations and liver microsomes were prepared 24 hours after the final exposure. CYP2B10 protein levels were quantified using Western blotting, and CYP2B enzyme activity was assessed using bupropion as a substrate. **Results:** The PCB-11 oral exposure significantly increased CYP2B10 protein expression in mouse whole brain membrane preparations, compared with vehicle controls. Enzyme activity assays using bupropion as a substrate confirmed a substantial increase in brain CYP2B10 activity. Consistent with these findings, liver microsomes from the same animals also showed a robust increase in CYP2B10 activity following PCB-11 treatment. These findings indicate that PCB-11 exposure upregulates CYP2B10 protein levels and activity in the brain. **Conclusions:** PCB-11 exposure induces significant increases in CYP2B10 expression and activity in both brain and liver of mice. This induction suggests potential for auto-induction of its own metabolism and formation of hydroxylated PCB metabolites that may contribute to neurotoxicity. Moreover, upregulation of CYP2B10 in the brain highlights the possibility of altered metabolism of neuroactive drugs, raising concern for PCB-related drug-environment interactions. Together, these findings identify a novel mechanism by which PCB-11 exposure may modulate brain metabolism, offering new insight into its neurotoxic and pharmacological implications. (supported in part by NIH grant ES014901).

PS 4573 Translating Internal Dosimetry of an Environmental Mixture of Polycyclic Aromatic Hydrocarbons (PAHs) from Zebrafish to Humans

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Background and Purpose: New Approach Methodologies (NAMs), such as high-throughput screening with zebrafish models, enables rapid assessment of chemical hazards. However, translating toxicity data from zebrafish experiments to humans poses significant challenges due to complexities associated with correlating zebrafish nominal exposures to human internal dosimetry. This study aims to address these challenges by using pharmacokinetic modeling to translate zebrafish exposures to Supermix-10, the ten most abundant PAHs found at the Portland Harbor Superfund Site, into corresponding human exposures. **Methods:** To facilitate cross-species comparison, we developed a generalized human physiologically based pharmacokinetic model (PBPK) to simulate the disposition of PAHs in humans. This model builds on a previous PBPK model framework developed in our lab for high molecular weight PAHs. We parameterized the model with measured human metabolism rates of naphthalene, 2-methylnaphthalene, chrysene, benzo(a)anthracene, pyrene, fluorene, fluoranthene, phenanthrene, and retene in humans and estimated partition coefficients using algorithms. A published zebrafish PBPK model estimated key internal dose metrics (e.g. peak concentration in zebrafish embryos and area under the curve (AUC)) for Supermix-10 exposures. Subsequently, we applied reverse dosimetry techniques to translate zebrafish internal dose metrics into human external dose equivalents, linking zebrafish toxicity outcomes to human exposure scenarios. **Results:** All Supermix-10 PAHs demonstrated rapid parent compound disappearance in human hepatic microsomes. PAH metabolism rates grouped into three patterns: high metabolism activity and capacity, where rapid metabolism did not saturate under the tested conditions (2-methylnaphthalene, acenaphthylene, fluorene, naphthalene, and retene); high affinity metabolism that rapidly achieves low-level saturation (benzo[a]anthracene and chrysene); or moderate metabolic activity and capacity (fluoranthene, pyrene, and phenanthrene). The zebrafish PBPK model accurately predicted 83% of 48-hour post fertilization (hpf) body burdens within a factor of two. Zebrafish PBPK models simulations of AUC of concentrations in zebrafish resulted in altered hazard rankings from nominal exposure concentrations. Reverse dosimetry using the human PBPK model predicted human exposure levels that would lead to toxicity threshold observed in zebrafish. **Conclusions:** Here, we demonstrate the utility of dosimetry modeling to translate nominal exposure levels in a zebrafish NAM system to external exposures in humans. This suite of dosimetry models can assist risk assessment and cleanup efforts at contaminated sites. This study demonstrates the utility of dosimetry modeling approaches for translating zebrafish NAM exposure data into human-relevant external exposures. By bridging the gap between nominal zebrafish exposures and human dosimetry, this framework enhances the utility of zebrafish systems for risk assessment and informs remediation strategies for contaminated sites. Supported by NIH Grant P42 ES016465.

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Background and Purpose: Urinalysis is one of the non-invasive methods in the toxicity assessment of drug candidates and is required by the ICH safety guidelines. The amount of urinary electrolyte excretion is typically calculated by multiplying the concentration of the urinary electrolyte by the urine volume (volume-based calculation method). However, in cases where only a limited volume of urine can be collected, such as in toxicity studies using small mice, this method is not applicable because of drying of the samples during collection or retention of the urine in the cage, resulting in incomplete collection. In the clinical field, a method for evaluating electrolyte excretion using correction based on urinary creatinine (CRN) (CRN-corrected method) has been established. However, the validity of this method remains unclear in small mice including the BALB/c strain. In this study, empagliflozin was administered orally for 10 days to ICR and BALB/c mice to evaluate the validity of the CRN-corrected method for assessing urinary electrolyte excretion. Using ICR mice, the validity of CRN-corrected electrolyte excretion was evaluated by comparing it with the volume-based calculation method. Furthermore, in BALB/c mice, changes in electrolyte excretion were assessed using the CRN-corrected method. By comparing the CRN-corrected results obtained from both strains, the applicability of this method in mice was examined. **Methods:** Empagliflozin was administered orally, once daily, to CrI:CD1(ICR) (ICR) and BALB/cJ (JLJ) male mice (9 weeks of age, 6 animals per group of both strains) at the dose levels of 100 and 1000 mg/kg for 10 days. The animals in the matched control groups (6 animals of both strains) were administered only the vehicle (0.5% hydroxyethylcellulose aqueous solution). The animals were placed in metabolism cages after dosing on Day 10 and urine samples were collected for approximately 24 hours. The urine concentrations of the following parameters were measured using an automatic analyzer: glucose (GLU), calcium (Ca), inorganic phosphorus (IP), sodium (Na), potassium (K), chloride (Cl) and creatinine (CRN). In ICR mice the amounts excreted of the urinary electrolytes and glucose were calculated by the volume-based calculation method and the CRN-corrected method (amount excreted per gram of CRN). In BALB/c mice the amounts excreted of the urinary electrolytes and glucose were calculated using only the CRN-corrected method. The electrolyte and glucose excretion levels calculated by this method were compared between the BALB/c and ICR mice. **Results:** When comparing the electrolyte excretion calculated using the urine volume-based calculation method with that calculated using the CRN correction method in ICR mice, there was a positive correlation for all the parameters of Na, K, Cl, Ca, IP, and GLU. The degree of change in the electrolyte and glucose excretion calculated by the CRN-corrected method at 100 mg/kg and 1000 mg/kg was comparable between ICR and BALB/c mice when compared with each control group. In ICR mice a positive correlation was observed between the electrolyte and glucose excretion calculated by the volume-based method and the CRN-corrected method, indicating that the CRN-corrected approach is also applicable for evaluating urinary electrolyte and glucose excretion in mice. In addition, since the CRN-corrected results were comparable between the two mouse strains, CRN correction was considered to be applicable for excretion assessment even in small mice, where use of the urine volume-based calculation is problematic due to the limited amount of urine collected. **Conclusions:** Although this approach may show slight differences in the rate of change compared with urine volume-based calculation, this method provides a useful alternative when measurement of the urine volume is problematic.

PS 4575 Considerations for Appropriate Application of One-Compartment Toxicokinetic Models to Assess Per- and Polyfluoroalkyl Substances (PFAS) Exposures

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Background and Purpose: A one-compartment (OC) model is the simplest form of a toxicokinetic model where the entire body is considered a single, homogenous unit. This is unlike physiologically based (PB) models that characterize the movement of substances between different compartments within the body (e.g., blood, bone, organs). A key advantage of OC models is the reduced data needs compared to PB models, allowing for broader applications. A good example of this is with PFAS OC models, which have been developed to characterize PFAS exposures in impacted communities. These models have been useful for PFAS research and in public health contexts; however, their limitations can be overlooked, and such models can be used inappropriately. In this analysis, we review several OC models that have been used for risk assessment, compare the models' strengths and limitations, demonstrate how specific inputs can impact model results, and propose a list of considerations to inform whether use of the OC model is appropriate. **Methods:** We identified OC PFAS models for inclusion based on the following considerations: 1) the model was developed for human exposure characterization; 2) the model was developed/updated in the last 15 years; 3) data used in development and calibration were well described; 4) model validation had been performed; and 5) the model has been applied in risk assessment. We compared model features and evaluated how differences between them impact model outputs by running them with the same hypothetical exposure scenarios. **Results:** We reviewed publications that developed or applied OC

models, including models developed as part of the C8 Health Study, by the Minnesota Department of Health, and by the Agency for Toxic Substances and Disease Registry. All models include variables for intake dose, clearance rate (calculated based on half-life [$t_{1/2}$]), and volume of distribution (V_d). The simplest OC models reflect steady-state conditions, while dynamic models include changes in parameters over time. Some models incorporate additional variables (e.g., placental transfer, lactation, menstruation) while maintaining an OC structure. A few models incorporate background PFAS exposures in their calculations. The choice of values for input variables such as V_d and $t_{1/2}$ can have significant impacts on model outputs. For individuals, behavioral (e.g., use of PFAS-containing consumer products) and physiological (e.g., kidney function, lactation) factors can impact PFAS intake and clearance rates, and this information is often unavailable. Implementations of OC models rely on averages published in scientific literature for input values, which individually can vary by up to two-fold. We demonstrate that, together, these choices can have compounding (i.e., four-fold or higher) impacts on model predictions. **Conclusions:** While OC models all share a fundamental structure, the differences between models highlight the importance of careful planning when selecting which model to use. Key considerations include: 1) What is the study question that the user wants to address (e.g., characterizing exposure for a particular community vs. establishing exposure limits)? Does the study question align with the original objectives driving model development? 2) What information is available about the target population being modeled? Does this population share characteristics (e.g., age range, pregnancy history, degree of exposure) with the population that was used in model development? 3) How will the model be used to draw conclusions about exposure? Given the simplicity of OC models, they are better suited to estimate a plausible range of values (e.g., 5th-95th percentile) rather than a specific point estimate. If there are characteristics of the target population that deviate significantly from underlying model assumptions, then the model may not produce valid results. We identify here examples of invalid applications, such as: using a model developed for a community impacted by drinking water contamination to characterize a different population where water concentrations are low compared to other sources; applying central tendency physiological parameters to individuals with very high or low values; and using a steady-state model when parameters are in flux over time. Careful consideration of model design and data needs is essential to obtaining outputs that are sufficiently accurate to address the study question.

PS 4576 A Physiologically Based Pharmacokinetic Model for Tyrosine Kinase Inhibitor Zongertinib in Adults

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Background and Purpose: Non-small cell lung cancer (NSCLC) is one of the most common cancer types, accounting for approximately 80% of all lung cancer cases in the United States. Approximately 2% - 4% of NSCLC cases have mutations in the ERBB2 gene, which encodes ErbB family receptor tyrosine kinase HER2. HER2 alteration leads to resistance to existing HER2 targeted therapies (i.e., Trastuzumab-Deruxtecan/T-DXd). Zongertinib (HERNEXEOS®/BI 1810631) is an irreversible HER2 selective tyrosine kinase inhibitor (TKI) developed by Boehringer Ingelheim. It was granted accelerated approval by the US FDA in August 2025 for HER2-mutant, unresectable or metastatic non-squamous NSCLC. It provides a selective and strong covalent binding with ATP binding sites in receptor tyrosine kinase (RTK) and a sustained inhibition of aberrant HER2-mediated signaling in HER2 mutant NSCLC. This study aimed to build a physiologically based pharmacokinetic (PBPk) model to predict the plasma concentration profiles of Zongertinib for regimen design optimization. **Methods:** The PBPk model was developed using Gastroplus® (Version 9.9.3). The development of the zongertinib PBPk model follows a pipeline of parameter calibration, model validation, and drug-drug interaction (DDI). For the model calibration, the plasma concentration data from a clinical PK study of both intravenous injection (IV) and oral tablet (PO) were used. Parameter sensitivity analysis (PSA) was conducted to assess the parameter impact. To build mechanistic clearance, CYP3A4 was introduced to the model, and enzyme kinetics parameters (V_{max} , K_m) were predicted by the AMDET predictor (Version 11.0). The model validation was performed by an independent oral tablet dose escalation PK dataset of the plasma concentration profile. The physiological system parameters were set to reflect reference human physiology reported in the baseline PK studies. **Results:** The established zongertinib PBPk model was evaluated by an independent dataset with R^2 of 0.98 in the calibration dataset, and an overall R^2 of 0.82 in the model validation datasets in human subjects. **Conclusions:** The PBPk model provides a mechanistic framework that successfully captures the observed plasma concentrations and PK profiles in the target population. It's also an analytical approach to evaluate enzyme-mediated drug-drug interactions (DDIs). However, further refinement is needed due to existing data gaps. The lack of *in vitro* experimental data for key enzymes and transporters results in the use of predicted parameters instead, which may reduce the model's prediction accuracy and sensitivity. Moreover, zongertinib exposure is not linearly proportional to the administered dose, indicating non-linear PK likely driven by saturation of certain biological processes at higher dose levels.

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are persistent organic pollutants known to accumulate in the liver, where they undergo little-to-no metabolism. Previous studies *in vitro* showed that certain PFAS can inhibit human CYP enzymes. In recombinant assays, PFOS and perfluorooctanoic acid (PFOA) inhibited CYP2D6, 2C19, 2E1, and 3A4, with half-maximal inhibitory concentration (IC_{50}) values ranging from ~5 to 100 μ M. Perfluorononanoic acid (PFNA) and perfluorodecanoic acid (PFDA) inhibited all tested CYP isoforms except CYP2E1, while short-chain PFAS showed weak to no inhibition. In immortalized HepaRG cells, PFOS and PFOA downregulated CYP1A2, 2C19, and 3A4 mRNA and activity after 48 h at ≥ 50 ng/L, whereas CYP3A4 was induced at low μ M levels in a PXR-independent manner. Short-chain PFAS ($npfc \leq 6$) such as perfluorohexanesulfonic acid (PFHxS) and 6:2 fluorotelomer alcohol (6:2 FTOH) modulated CYP1A2 activity when using β -naphthoflavone as a substrate. Interestingly, PFOS potentially inhibited CYP2C9 ($K_i = 0.04$ μ M) in human liver microsomes (HLM), an effect over two orders of magnitude stronger than in immortalized cell-based assays, underscoring the need for physiologically relevant *in vitro* models such as HLM to assess PFAS-mediated CYP inhibition. **Methods:** We assessed the reversible inhibition of seven CYP isoforms - CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2C6, and CYP3A4/5 - using selective probe substrates for each isoform. The PFAS tested as potential CYP spanned six to 12 perfluorinated carbons (n_{pfc}), including fluorotelomer and ether-substituted structures. These compounds comprised Perfluorooctanesulfonamide (FOSA), N-ethylperfluorooctanesulfonamide (EtFOSA), N-methylperfluorooctanesulfonamide (MeFOSA), PFOS, 8:2 Fluorotelomer Sulfonic Acid (8:2 FTS), perfluoroundecanoic acid (PFUnDA), Perfluorododecanoic acid (PFDoDA), PFOA, Perfluorodecanoic acid (PFDA), perfluorononanoic acid (PFNA), perfluoroheptanoic acid (PFHpA), Perfluorohexane sulfonic acid (PFHxS), Perfluoro(2-ethoxyethane) sulfonic acid (PFEESA), and an equipotent mixture of all 13 PFAS. Metabolite formation of each probe substrate was quantified and normalized to a solvent control to determine half maximal inhibitory concentration (IC_{50}) values. A C18-based solid-phase micro-extraction assay was used to determine the unbound fraction ($f_{unbound}$) of each PFAS in human liver microsomes (HLM) to derive unbound IC_{50} values. Equivalent unbound concentrations were derived from previously measured PFAS concentrations in 211 human liver samples and compared to unbound IC_{50} for individual PFAS and mixtures to evaluate potential for CYP inhibition across real-world human liver burdens. **Results:** CYP2C enzymes were most susceptible to PFAS inhibition, with IC_{50} s ranging from 23 nM ($n_{pfc} = 8$ sulfonamide) to 31 μ M ($n_{pfc} = 8$ fluorotelomer) for CYP2C9. QIVIVE compared inhibitory values to PFAS concentrations measured in 211 human livers revealed that 19% of individuals fall within 10-fold of the 10% inhibitory concentrations for PFOS, while 61% had hepatic PFAS concentrations within 100-fold of this threshold. Notably, certain sulfonamides and sulfonates exceeded the inhibitory potency of the CYP2C9 reference inhibitor sulfaphenazole, as well as several clinically relevant CYP2C9 inhibitors. The equimolar PFAS mixture yielded an unbound IC_{50} of 35 nM, with measured hepatic mixture burdens approaching these inhibitory levels. **Conclusions:** These findings indicate that PFAS can inhibit CYP2C enzymes at human-relevant exposure levels, potentially compromising both drug clearance and endogenous fatty acid metabolism. This is particularly relevant for clinical trials involving CYP2C9 substrates, where unrecognized environmental exposures may confound drug efficacy and lead to trial failure or suboptimal dosing. Incorporating PFAS and other environmental chemicals as covariates in clinical trial design or pharmacogenomic models may enhance precision medicine strategies by accounting for altered drug metabolism, exposures, and toxicity risk.

PS 4578 Access Rich Toxicokinetics Data through the Chemical Effects in Biological Systems (CEBS) Platform

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Background and Purpose: Absorption, distribution, metabolism, excretion and toxicokinetics (TK) data serve as the cornerstone of understanding the systemic effects within biological systems following exposure to putative toxic substances. These data not only illuminate the toxicology profile of substances but also play a pivotal role in informing risk assessment and decision-making processes. The National Toxicology Program/Division of Translational Toxicology (DTT) at the National Institute of Environmental Health Sciences has played a significant role in generating toxicity data, using animal models, on environmental agents with potential adverse human health impacts. In support of these toxicity studies, it has conducted over 200 TK studies. Most of the study data have been annotated at both individual animal (concentration versus time data) and group (TK parameters) levels. The comprehensive TK parameters and metadata accompanying these annotated data enhance interpretability and utility of TK data and aligns with FAIR (Findable, Accessible, Interoperable, and Reusable) standards. **Methods:** Not applicable. Abstract is for non-laboratory or non-field Studies. **Results:** The original published annotated individual animal plasma or tissue concentration versus time data and group TK parameter data, as well as summary data files, are available through the Chemical Effects in

Biological Systems (CEBS, <https://doi.org/10.22427/NTP-DATA-1>), an open-source platform tailored to the needs of environmental health scientists. CEBS offers a new option for accessing TK data using the Toxicokinetics Data Collection that is presented here. This data collection boasts rich standardized metadata, such as test article, dose, dose frequency, species, strain, route, and modeling methods and has a user-friendly interface to facilitate easy data searching, filtering, and downloading that enables scientists to easily access data of interest. This dataset is also accessible through the CEBS DTT Integrated Data Resources, which offers bulk download options for both individual animal and group parameter data. Data from TK studies are also seamlessly integrated into their respective CEBS test article pages, giving researchers a comprehensive view of all studies and publication data related to a specific substance that are available in CEBS. **Conclusions:** Not applicable. Abstract is for non-laboratory or non-field Studies.

PS 4579 Beyond the kinetically derived maximum dose: a holistic view of nonlinear pharmacokinetics

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Background and Purpose: In chemical risk assessment, an understanding of pharmacokinetics (PK) is essential for analyzing and interpreting dose-response data, as well as for effectively guiding the design of animal studies. Historically, the kinetically derived maximum dose (KMD) concept was introduced to incorporate nonlinear PK data into dose selection or response interpretation. The goal of this approach is preventing the use of excessively high doses irrelevant to human exposure. KMD has been defined as an inflection point slightly above the onset of nonlinear PK. However, this definition is both inaccurate and impractical. Nonlinear PK does not produce a distinct inflection point in the internal to external concentration (IEC) relationship. Additionally, nonlinear PK alone does not inherently signal the onset of toxic responses. The objective of this study is to compare the KMD approach with an integrated, biologically based strategy that incorporates all available PK data to elucidate the underlying shape of the IEC relationship. This approach allows for a quantitative evaluation of the presence and extent of PK nonlinearity to address risk assessment questions. **Methods:** This study illustrates the use of both animal data and new approach methods (NAMs) to investigate the impact of nonlinear pharmacokinetics (PK) on dose-response interpretation, using the pesticide afidopyropen as a case study. Uterine adenocarcinomas were identified in female rats at doses of 50 and 150 mg/kg body weight (bw)/day. To explore whether tumors occurred only at doses where afidopyropen clearance was saturated, a 28-day dietary study was conducted to measure afidopyropen concentrations in plasma at doses of 0.1, 0.5, 2.5, 12.5, 50, and 150 mg/kg bw/day. Instead of identifying a KMD based on this single dataset, our analysis used a physiologically based pharmacokinetic (PBPK) model to integrate all available PK data, such as oral absorption fraction and the absence of renal clearance. The model was then optimized against area under the curve (AUC) data from the 28-day study to determine whether the assumption of linear or nonlinear metabolism more accurately describes the data. **Results:** Although initial analysis suggested that the nonlinear metabolism assumption offered a slightly better fit to the plasma AUC data, the linear assumption also provided a reasonable fit. The linear and nonlinear models subtly diverged at the 12.5 mg/kg bw/day dose, but the absence of reliable plasma data at doses of 50 and 150 mg/kg bw/day limits further evaluation of PK linearity beyond 12.5 mg/kg bw/day. Additionally, the variability in the observed AUC data at higher doses, though problematic, is larger than the difference between the simulated linear and nonlinear curves. It is unclear whether the minor divergence of the two models reflects true nonlinear PK or is simply due to data variability. Consequently, the plasma AUC data cannot be used to exclude the relevance of the tumors observed at 50 and 150 mg/kg bw/day in rats to human exposure. This conclusion differs from the original study, which determined a KMD between 2.5 and 12.5 mg/kg bw/day and deemed the rat tumors irrelevant to human exposure. **Conclusions:** Although the KMD can indicate potential nonlinear PK that merits further examination, it overemphasizes identifying a single dose where PK transitions from linear to nonlinear. As demonstrated in the case example, such deviation is often too subtle to significantly impact dose-response interpretation. Risk assessment decisions should not depend solely on a single piece of evidence, such as a shift in PK behavior in one dataset, without understanding its biological or toxicological implications. Instead of focusing on identifying a KMD, it is essential to integrate data on exposure, PK, and hazard to guide dose selection and response interpretation, without preconceived assumptions about the impact of nonlinear PK. NAMs, such as predictive modeling, offer opportunities to bridge data and knowledge gaps without additional animal studies.

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Background and Purpose: The U.S. EPA is focused on increasing the pace and reducing the cost of endocrine disruption screening for hundreds of pesticide chemicals. Quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) is a New Approach Method that extrapolates *in vitro* bioactive concentrations to an external exposure level comparable to *in vivo* responses in whole organisms. Toxicokinetic (TK) models are a key component of mammalian IVIVE approaches. However, similar approaches are being developed for ecologically relevant species (e.g., fish), and species-specific differences between *in vitro* TKs are still poorly understood. Therefore, the objective of the current study is to conduct *in vitro* plasma binding and intrinsic metabolic clearance studies across 3 species (human, rat, and rainbow trout) and evaluate species-specific differences for each chemical. **Methods:** Plasma binding (fraction unbound (fUB)) was assessed using rapid equilibrium dialysis, while intrinsic metabolic clearance rates (Cl_{int}) were determined using substrate depletion assays. In the assays, mixed sex pools for human, rat, and juvenile rainbow trout plasma and hepatocytes were used. A list of reference chemicals for *in vivo* endocrine screening assays that span a diverse range of physiochemical properties were compiled. Chemicals were pre-screened for stability in the test systems to optimize test concentrations and timepoints. After preliminary evaluation, 40 chemicals were chosen for definitive testing in both assays. For the definitive plasma binding assays, the concentrations tested were between 1 and 10 µM with sampling times of 8 or 24 hours. For the definitive metabolic clearance assays, the concentrations tested were between 0.1 and 1 µM with six sampling times between 0 and up to 90 minutes. Each test chemical run included a concurrent negative control (inactive hepatocytes) replicate at the same test chemical concentration which was sampled at the same six time points.

Results: Overall, the fUB values were similar across all three species for most chemicals. Trout tended to be most different among the three species with diethylstilbestrol, estril, and fenarimol showing the highest variance of fUB across species. For example, fUB values for diethylstilbestrol were 0.008, 0.014, and 0.312, in human, rat, and trout plasma, respectively. The Cl_{int} values between species were more variable than that of the plasma binding data with the trout data commonly being different from the human and rat data. Rat or human Cl_{int} values were at least an order of magnitude or more than trout Cl_{int} values for 17β-trenbolone, diazinon, flutolanil, linuron, mifepristone, and propargite. For example, the Cl_{int} values of flutolanil were 62.1, 189, and 8.66 µL/min/106 hepatocytes for human, rat, and trout, respectively. **Conclusions:** This study has built upon previous efforts to add more empirical *in vitro* information to the endocrine relevant chemical space. The data generated in this study will be critical for developing QIVIVE approaches for endocrine disruption screening assays, such as the Hershberger assay, the female pubertal assay, and the fish short-term reproduction assay. This abstract does not necessarily reflect US EPA policy.

PS 4581 MCSimMod: Updates and Improvements to an R Package for Implementation and Application of PBPK Models

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Background and Purpose: Pharmacokinetic (PK) and physiologically based pharmacokinetic (PBPK) models, which are commonly used in human health risk assessments for chemicals, are generally implemented as computer programs. Historically, many PBPK modelers favored the programming language ACSL and its associated environment, acslX. However, in 2015, the company responsible for acslX announced it would cease support and sales of the software. Before acslX's decline, some PBPK modelers used the open-source tools R and MCSim, often separately, for computational PBPK modeling tasks. Since then, many have adopted a combined approach for implementing PBPK models using R and MCSim together. Each of these software tools has its advantages and limitations. R is a flexible and widely used language and environment for data analysis and graphics generation. However, because R is an "interpreted" language, R commands must be translated into machine code each time they are executed. This leads to slower performance for complex calculations, such as those involved in PBPK model simulations. MCSim, on the other hand, is a specialized tool for implementing and calibrating mathematical models that uses "compiled" languages to execute model simulations swiftly. To use R and MCSim together, one writes model descriptions in the MCSim model specification language, uses an MCSim utility to automatically translate and compile them, and then conducts simulations efficiently through R scripts that leverage the compiled code. Unfortunately, installing R, MCSim, and other necessary tools, and ensuring their proper integration, can be challenging, posing obstacles for many in the PBPK modeling community. To address these issues and provide a user-friendly and consistent system for implementing, applying, and sharing PBPK models, we developed the R package MCSimMod. **Methods:** The MCSimMod package, which can be easily installed using a single R command, offers an object-oriented approach for handling PBPK and other ordinary differential equation (ODE) models. MCSimMod users can create model "objects" using human-readable text descriptions written in the MCSim model specification language. These model objects allow users to directly translate the MCSim model specification text into the C programming language,

which can then be compiled into machine code. This strategy greatly increases the speed of execution of model simulations. Utilizing intuitively named class methods (functions) like "updateParams" and "runModel," users can set and update model parameters and initial conditions, as well as perform simulations. Subsequently, they can analyze simulation results and carry out necessary post-simulation calculations within the R environment. **Results:** We have created a new version of the R package MCSimMod and have made it available in a repository on the U.S. Environmental Protection Agency's (EPA) GitHub institutional account. New features include a "verbose" output option that facilitates MCSim model debugging and the additional capability of working with multiple models within the same R session. As always, the package can be used without installing the MCSim software. The package's "vignettes" provide step-by-step instructions for basic modeling tasks and include examples involving a classical PK model and a more complex PBPK model. Since the first public release of MCSimMod in April 2025, the package has been downloaded more than 2800 times. Also, the U.S. EPA PBPK Model Template software has been updated to use MCSimMod, and U.S. EPA risk assessors have used it to implement PK and PBPK models for polychlorinated biphenyls (PCBs) and ethylene dibromide (EDB). **Conclusions:** The free and open-source R package MCSimMod provides a flexible and efficient means for implementing and applying PK and PBPK models. Users can easily install MCSimMod by downloading the source code and executing a single command in an R session. Additionally, the languages required for use, R and MCSim, are already well-known to many in the PBPK modeling community. Since the initial release of MCSimMod, we have added features to facilitate debugging during preliminary model development and to allow for working with multiple models simultaneously. MCSimMod has already been used for several modeling applications by researchers and risk assessors at U.S. EPA, and we expect that it will continue to gain traction as a popular tool for the development, application, and sharing of PBPK models.

PS 4582 Evaluation of a lung microphysiological system for measuring concentrations of anti-infective drugs in epithelial lining fluid

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Background and Purpose: Phase 1 intrapulmonary clinical studies in healthy subjects are recommended to assess anti-infective drug penetration into the alveolar epithelial lining fluid (ELF) for drugs with indications related to lung infections. While clinical intrapulmonary studies provide valuable data on lung penetration, the current practice is labor-intensive, invasive, and yields lung penetration results with high variability. New approach methodologies (NAMs), such as lung microphysiological systems (MPSs), offer a promising nonclinical alternative for modeling bronchopulmonary drug disposition by recapitulating key physiological features such as dynamic flow, cyclic stretching, and air-liquid interface culture. This study aims to evaluate whether an alveolar lung MPS can generate physiologically relevant drug concentration profiles and ultimately support or complement intrapulmonary clinical studies. **Methods:** A human lung alveolus MPS incorporating primary alveolar epithelial cells and microvascular endothelial cells was established and characterized. Model characterization included assessment of barrier integrity, alveolar type I/II cell markers, 3D morphology, surfactant activity, and barrier permeability using fluorescent tracers, along with ELF volume estimation via urea dilution method consistent with clinical bronchoalveolar lavage studies. Six anti-infective drugs with diverse physicochemical and pharmacokinetic properties (delafloxacin, azithromycin, lefamulin, omadacycline, ceftaroline, and levofloxacin) were selected for evaluation, and three compounds (delafloxacin, azithromycin, and lefamulin) have been tested to date. Following drug administration through the vascular channel, apical wash samples were collected for quantification via liquid chromatography-tandem mass spectrometry. **Results:** The alveolus MPS demonstrated structural and functional characteristics consistent with an alveolar barrier, including confluent epithelial and endothelial layers, expression of relevant markers (aquaporin 5 [AQP5] and surfactant protein B [SFTPB]) for alveolar type I/II cells, intact barrier properties determined by the expression of zonula occludens-1 (ZO-1) and vascular endothelial cadherin (VE-cadherin), and measurable surfactant activity (~484 ng/mL of SFTPB and 652 ng/mL of SFTPC). Preliminary drug studies with the first three compounds generated quantifiable concentrations in apical ELF-analog samples (1.63 µg/mL of delafloxacin at 1 hr, 0.45 µg/mL of azithromycin at 6 hr, and 0.42 µg/mL of lefamulin at 1 hr), which are broadly consistent with available clinical data. These findings support the feasibility of using the model to track drug movement across the alveolar barrier. **Conclusions:** The study demonstrates that a lung alveolus MPS can potentially evaluate bronchopulmonary drug penetration and generate ELF-like concentration data for anti-infective drugs. The model displays essential structural and functional characteristics of the human alveolar barrier and supports measurable drug transport. Continued testing with additional compounds and comparison with clinical intrapulmonary PK data will determine whether MPS-derived ELF concentrations can serve as supportive evidence or, in some cases, reduce the need for clinical intrapulmonary studies. Ongoing efforts are focused on improving experimental reproducibility, refining sampling conditions to enhance ELF-like recovery, and strengthening the robustness of drug penetration measurements. As the dataset expands, this work may help define how lung MPS approaches complement existing clinical methods and advance regulatory science initiatives aimed at integrating NAMs into drug development.

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Background and Purpose: Liver microtissues from human donor tissues have emerged as New Approach Methodologies (NAMs) for evaluating xenobiotic metabolism and toxicity potential in humans as they sustain hallmarks of hepatocellular function over weeks/months in culture. However, our understanding of their proficiency to emulate metabolite profiles and predict kinetic parameters is limited, particularly for low-turnover chemicals. Primary human hepatocytes (PHH) suspension cultures, the gold-standard method, have limited longevity (i.e., ~2 hours). In this study, we established 3D liver microtissues in both single- and pooled-spheroids, alongside suspension PHH, to investigate metabolic proficiency for moderate and low-turnover chemicals. We also investigated metabolism of per- and poly fluorinated alkyl substances (PFAS) like fluorotelomers present in aqueous film-forming foams (AFFF) including 6:2-FTS, 6:2-FtSiAoS, 6:2-FtTAoS, 6:2-FTNO, and 6:2-FtSaB using spheroids. **Methods:** Suspension, single spheroid, and pooled spheroid cultures were established in 96-well plates with CELLSTAR®, Akura™, and Corning® Eplasia®, respectively. Cryopreserved PHH (single donor) were purchased from Lonza (Durham, NC). After seeding PHH to ~2,000 or ~40,000 cells/well for single- or pooled-spheroid cultures, respectively, they were equilibrated for maturation over 7 days (37°C, 5% CO₂). For suspension PHH, cells were seeded at ~2,000 cells/well and incubated with test substances (1 µM tolbutamide, phenacetin, midazolam, 6:2-FTNO, and 6:2-FtSaB; 0.1% AFFF1) for 0 and 90 min with orbital shaking, while spheroids were incubated for 0 and 24 hrs. Cells were crashed with ice-cold methanol, followed by shaking and centrifugation. An aliquot of supernatant was transferred to 96-well plates (Corning® BioCoat®) for liquid chromatography-tandem mass spectrometry analysis for parent and/or metabolite concentration. Data were analyzed statistically with GraphPad Prism (Boston, MA). **Results:** Tolbutamide, phenacetin, and midazolam reference chemicals were depleted with suspension PHH to varying degrees. The turnover rate with suspension PHH was generally more extensive than spheroid cultures, though some baseline anomalies for time-zero were evident for pooled spheroids. Suspension PHH appeared to provide faster depletion than spheroids, which may be related to enhanced accessibility through cytosolic membranes and greater media exchange surface areas in suspension form. For metabolite formation, pooled spheroids produced higher metabolite formation to acetaminophen from phenacetin and 1-OH-midazolam from midazolam compared with single spheroids or suspension PHH. Fluorotelomers 6:2-FTNO was extensively depleted over 24-hrs with single- and pooled spheroids but not significantly in PHH suspensions over 90 minutes. AFFF1 incubations containing many PFAS fluorotelomer constituents revealed extensive 6:2-FTS and 6:2-FTNO depletion in single spheroids and suspension PHH with pooled spheroids again showing baseline anomalies relative to other time-zero samples. The maximum turnover rate was >60% for 6:2-FTNO (single spheroids). There was no evidence consistent with 6:2-FTS formation from 6:2-FTNO with human hepatocytes. Depletion trends for individual PFAS 6:2-FTNO, 6:2-FtSaB were similar to those observed in their complex mixture (i.e., AFFF1). Interestingly, depletion of 6:2-FtSaB was clear for suspension PHH but not significant in spheroid systems. Further experiments are ongoing. **Conclusions:** This study has clarified the potential for fluorotelomers found in AFFF products can be metabolized by patient-derived human liver cells and demonstrates for the first time that liver microtissues are proficient in biotransformation of PFAS fluorotelomers within complex PFAS surfactant mixtures. Investigations continue to expand experimental designs to provide additional context over time and dose to enhance our understanding of PFAS metabolism and bioaccumulation with 3D liver microphysiological systems (MPS).

PS 4584 HepaSH cells as a platform to evaluate the impact of genetic polymorphisms on cytochrome P450-mediated metabolic drug clearance

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Background and Purpose: Interindividual variability in metabolic drug clearance due to genetic polymorphisms in cytochrome P450 (P450) enzymes remains a major challenge for predicting human pharmacokinetics. The drug-metabolizing activity of primary human hepatocytes is influenced by donors' lifestyle factors, such as alcohol consumption, smoking, and diet, as well as by exposure to environmental chemicals, complicating the evaluation of the impact of P450 polymorphisms. In contrast, chimeric mice with humanized livers, maintained under strictly controlled conditions, are minimally affected by interindividual differences in lifestyle- and environmental factors, allowing their hepatic enzyme activities to more accurately reflect genetic polymorphisms. This study aimed to evaluate the utility of genotyped HepaSH cells for assessing the impact of P450 polymorphisms on metabolic drug clearance. **Methods:** Genetic polymorphisms of CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A5 were analyzed in three HepaSH strains (HepaSH-E, -L, and -AD) by direct DNA sequencing of the coding regions and relevant intronic/exonic variants. HepaSH cell suspensions (0.5 × 10⁶ cells/mL) were incubated with typical

P450 probe substrates. Aliquots were collected at 5, 10, 15, 20, 30, 45, 60, and 75 min and quenched in ice-cold acetonitrile. After centrifugation, drug concentrations in the supernatants were quantified by liquid chromatography-tandem mass spectrometry to calculate apparent intrinsic clearance (CL_{int}). The expression level of each P450 gene was quantified by reverse transcription-quantitative polymerase chain reaction using commercially available TaqMan assays. **Results:** CYP2D6 genotypes were *1/*1, *1/*9, and *1/*4 for HepaSH-E, -L, and -AD, respectively. CYP2D6*4 carries a splice site mutation (1846G>A) that causes abnormal mRNA splicing and produces a nonfunctional enzyme, while CYP2D6*9 is a decreased-function allele characterized by a 3-base pair deletion in exon 5 (2615-2617delAAG), resulting in the loss of one amino acid (Lys281) and reduced enzyme stability and catalytic efficiency. Accordingly, the CL_{int} of atomoxetine and propafenone, both CYP2D6 substrates, was higher in HepaSH-E than in HepaSH-L and -AD. CYP3A5 genotypes were *1/*1, *3/*3, and *3/*3 for HepaSH-E, -L, and -AD, respectively. CYP3A5*3 allele harbors a single nucleotide polymorphism in intron 3 (6986A>G) that causes a splicing defect and produces a truncated, nonfunctional protein. The CL_{int} of midazolam, a substrate for CYP3A4 and CYP3A5, was higher in HepaSH-L than in HepaSH-E and -AD. By contrast, the CL_{int} of nifedipine, a substrate primarily for CYP3A4 with minor CYP3A5 contribution, was higher in HepaSH-AD, indicating that nifedipine clearance depends mainly on CYP3A4 expression level. Thus, the individual HepaSH strains exhibited clear genotype-dependent differences in metabolic drug clearance, enabling prediction of the effects of P450 polymorphisms on human hepatic drug clearance. Analyses of CYP2B6, CYP2C9, and CYP2C19 are ongoing. **Conclusions:** Genotyped HepaSH cell strains provide a robust and practical platform for quantifying the effects of P450 genetic polymorphisms on metabolic drug clearance. These cell strains serve as a valuable tool for improving the prediction of interindividual variability in human hepatic clearance of drug candidates in drug development.

PS 4585 Single cell lung transcriptomics of low-level dietary and environmental cadmium exposure following early-life respiratory syncytial virus infection

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Background and Purpose: Respiratory syncytial virus (RSV) is a major cause of severe respiratory illnesses in young children and older adults. Epidemiological evidence suggests that RSV infection can leave long-term effects on lung function over time. Cadmium (Cd) is a heavy metal for which all individuals carry some exposure risk. High-level exposures occur in occupational settings and with tobacco smoke, but low-level exposures occur through numerous dietary sources. We previously found that early-life RSV infection (eRSV) exacerbates long-term effects of Cd in the lungs. We found that inflammation, mTORC1 activation and disruption of protein palmitoylation contribute to this effect, but effects on specific lung cell populations are largely unknown. To help unravel the mechanisms at play in this combined exposure paradigm, we conducted single cell lung transcriptomics analyses to identify key cell populations, pathways and functional networks at the intersection of eRSV and Cd exposure. **Methods:** To model eRSV, C57BL/6J mice were treated intranasally with saline (vehicle control) or RSV (Line 19F in saline; 4 × 10⁴ PFU/g body weight) at 14 days old. At 35 days old, mice were treated for 16 weeks with untreated drinking water (n = 3 for control; n = 2 for eRSV only) or 3.3 mg CdCl₂/L in water (n = 3 for eRSV+Cd; n = 2 for Cd only). After treatment, mice were euthanized, and segments of the medial side of all right lung lobes were collected, with representation of the hilum and right bronchus. Segments were snap frozen and later processed using the 10x Genomics Chromium Single Cell Gene Expression Flex protocol. Samples were sequenced on a NovaSeq S4 Flow Cell. Sequencing data were initially processed for quality control and data aggregation using Cell Ranger v8.0.1. Cells were further processed for batch effect correction, cell cycle scoring and cell filtering in Seurat v5. Cell annotations were performed using the LungMap CellRef mouse lung reference, with manual adjustment using marker gene expression. Compositional analysis with false discovery rate (FDR) set to 20% was performed using scCODA. Perturbation analysis was performed using Augur. Differential expression analysis was performed using DESeq2 in Seurat, with Bonferroni correction used to maintain the aggregate Type I error rate at 5%. Gene set enrichment analysis (GSEA) was performed using differentially expressed genes (DEG) labeled by gene ontology (GO) biological process terms, with FDR adjusted to 5%. **Results:** eRSV followed by oral Cd exposure resulted in widespread effects on cell composition and gene expression. eRSV+Cd lungs had more ciliated/deuterosomal cells, while eRSV and Cd treatments alone resulted in fewer ciliated/deuterosomal cells. Ciliated/deuterosomal cells also showed the largest perturbation, with an AUC of 0.697. Interestingly, the eRSV+Cd treatment resulted in the fewest DEGs in ciliated/deuterosomal cells, 33, while eRSV and Cd resulted in 54 and 82 DEGs, respectively. The alveolar fibroblast 1 (AF1) cell population contained the highest number of DEGs by eRSV+Cd treatment, 125 genes. Gene set enrichment analysis of these genes revealed enrichment of several GO biological process terms. These included expected terms, such as collagen metabolism, leukocyte activation involved in inflammatory response, and response to toxic substances. However, previously unexplored terms such as response to pH and hormone metabolic process were also upregulated. **Conclusions:** These results provide new understanding of mechanisms which may contribute to toxicologic synergy between common eRSV infection and low-level environmental Cd exposure. Results show that AF1 cells have widespread

changes to gene expression, including disrupted processes that have not previously been explored in eRSV with Cd exposure. Furthermore, the unique sensitivity of ciliated and deuterosomal cells to the combined exposure warrants detailed study of these cell types. These findings point to new avenues for intervention which could be pursued to improve lung health outcomes post RSV infection.

PS 4586 Palmitoyltransferase zDHHC9 Regulates Fibrotic Marker Col1A1 Expression Through a Post-Transcriptional Mechanism in Human Lung Fibroblasts

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Background and Purpose: Environmental exposure to cadmium (Cd), a toxic metal found in food and drinking water, is associated with profibrotic responses in the lungs. We previously showed that this toxicity is potentiated by early-life respiratory syncytial virus (RSV) infection using a mouse model. This combined insult re-programmed lung metabolism and induced profibrotic signaling that was dependent on protein S-palmitoylation, a post-translational modification catalyzed by zDHHC palmitoyltransferases. While our prior integrative multi-omics analysis determined the zDHHC11 transcript as a central hub, and pan-palmitoyltransferase inhibitor (2-bromopalmitate) blocked the Cd/RSV-induced fibrotic signaling, identification and verification of specific zDHHC enzymes responsible for regulating profibrotic signaling in human lung cells are needed. Here, using CRISPR-Cas9-facilitated genetic modifications, we investigated the role of individual zDHHC enzymes in regulating components of profibrotic signaling pathway. **Methods:** We used the CRISPR-Cas9 gene editing system to generate stable knockout (KO) cell lines from human lung fibroblasts (HFL1) targeting six different palmitoyltransferases: zDHHC3, zDHHC4, zDHHC5, zDHHC7, zDHHC9, and zDHHC13. A scrambled gRNA (SCR) line was generated as a negative control. Successful protein disruption in KO lines was confirmed by Western Blot. The expression levels of fibrotic markers Col1A1 and fibronectin were then assessed at both the transcriptional level (qPCR) and the protein level (Western Blot) and compared to the SCR negative control. **Results:** All generated KO cell lines showed effective disruption in baseline expression levels of their respective target zDHHC proteins. Analysis of fibrotic markers revealed significant, enzyme-specific effects on Col1A1, while fibronectin protein levels remained similar to the negative control across all lines. The zDHHC5 KO line showed a significant reduction in Col1A1 protein, to approximately 62% of the levels seen in the SCR control. Strikingly, the zDHHC9 KO line exhibited an almost complete loss of Col1A1 protein expression, with levels decreased to less than 10% of the SCR control. Other KO lines did not show significant changes in Col1A1. Interestingly, our assessment of Col1A1 mRNA levels via qPCR in both the zDHHC5 and zDHHC9 KO lines indicated similar expression levels to that of the negative control. **Conclusions:** These findings identify specific zDHHC enzymes as critical regulators of fibrotic marker expression in human lung fibroblasts. In particular, zDHHC9 emerges as a dominant enzyme governing Col1A1 protein level. The data demonstrating a disconnect between protein and mRNA levels strongly suggest that zDHHC9 regulates Col1A1 expression via a post-transcriptional mechanism, such as influencing protein translation or stability, rather than by altering gene transcription. These results identify zDHHC9 as a novel and specific therapeutic target for lung fibrosis driven by environmental toxicants and early-life viral insults.

PS 4587 Cell death mechanism of novel indium-containing terpyridine metal complexes for breast cancer cells

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Background and Purpose: Metal complexes have long been utilized as anticancer agents, such as cisplatin which is a platinum-based drug well-known and widely used for treating various solid tumors. A major challenge in anticancer drug development is achieving high selectivity to maximize cancer cell death while minimizing toxicity to normal cells. Terpyridine (tpy) metal complexes have been extensively investigated for decades due to their broad applications in functional materials, supramolecular chemistry, catalysis, and biomedical research, including anticancer, antibacterial, and antifungal studies. Previous work from Cheng's laboratory demonstrated that indium-based tpy complexes exhibit selective cytotoxicity toward either MCF-7 breast cancer cells or MDA-MB-468 triple-negative breast cancer cells. The present study further investigates the detailed cell death signaling mechanisms of the indium-tpy (In1 and In3) complexes. **Methods:** Protein level changes in cells treated with In1 and In3 for 72 hours at their IC50 concentrations were assessed using the Full Moon Biosystems Explorer Antibody Array, which contains 656 highly specific and well-characterized antibodies targeting multiple signaling pathways. Fold changes obtained from the array were analyzed using QIAGEN Ingenuity Pathway Analysis (IPA). Western blotting was performed to validate the expression of apoptosis-related proteins, including Bad, Bax, Bak, p53, Bcl-2, and Bcl-XL. **Results:** The array data showed that treatment of MCF-7 with In1 increased the expression of Bax, Bad, and p53 (fold changes: +1.153 to +1.204), while decreasing Bak, Bcl-2, and Bcl-XL (fold changes: -1.031 to -1.372). In MDA-MB-468, In1 increased Bax, Bad, Bcl-2, and p53 (fold changes: +1.006 to +1.308) but decreased Bak and Bcl-2 (fold changes: -1.000 to -1.304). Western blot analysis validated the IPA results, showing that In1 reduced

Bcl-2 expression by 58% in MCF-7 and 42% in MDA-MB-468, and decreased Bak and Bcl-2 in MCF-7 by 40% and 58%, respectively, as well as Bcl-XL in MDA-MB-468 by 8%. Similarly, treatment of MCF-7 with In3 increased Bax, Bad, Bcl-XL, and p53 (fold changes: +1.060 to +1.549) while decreasing Bak and Bcl-2 (fold changes: -1.016 to -1.196). In MDA-MB-468, In3 increased Bak, Bcl-2, and Bcl-XL (fold changes: +1.063 to +1.126) but decreased Bax, Bad, and p53 (fold changes: -1.024 to -1.068). Western blot analysis confirmed that In3 reduced Bak and Bcl-2 in MCF-7 by 40% and 43%, respectively. However, other validated proteins did not consistently reflect the IPA findings. **Conclusions:** Both In1 and In3 modulate the expression of key apoptotic regulators in MCF-7 and MDA-MB-468 cells, indicating their potential to induce cell death via the intrinsic mitochondrial pathway. In1 consistently reduced Bcl-2 and Bak in MCF-7 and Bcl-XL in MDA-MB-468, while In3 showed a similar pattern in MCF-7 but differential effects in MDA-MB-468, suggesting cell line-specific responses. The partial discrepancies between array and protein expression highlight the complexity of post-translational regulation and pathway crosstalk. Since indium is known to bind to transferrin and can be taken up via the transferrin receptor pathway, it may influence intracellular metal homeostasis and iron-related signaling. And given the involvement of Bcl-2 family proteins in both apoptosis and oxidative stress-related cell death, future studies should explore whether In1 and In3 also induce ferroptosis by evaluating markers such as GPX4, as well as performing time- and dose-dependent analyses to better understand their mechanisms and therapeutic potential across different breast cancer subtypes.

PS 4588 Optically filtered Monochromatic portable X-ray fluorescence (MXRF) quantifies lead in wild bird bone with ICP-MS validation and defined detection limits

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Background and Purpose: Lead (Pb) exposure threatens both wildlife and humans, causing neurological, cardiovascular, and renal damage. Conventional Pb analysis relies on costly wet-lab methods (e.g., Inductively Coupled Plasma-Mass Spectrometry, ICP-MS). Monochromatic X-ray fluorescence (MXRF) enables rapid, non-destructive, field-deployable Pb detection for environmental and biomedical use with lower detection limits than prior portable XRF systems. This study evaluated the feasibility, calibration, attenuation correction, and agreement of a doubly curved crystal (DCC) optically enhanced MXRF (X-ray Optical Systems (XOS), East Greenbush, NY) for real-time quantification of bone Pb of black vultures. **Methods:** We analyzed 38 bird bones *ex vivo* (n = 19 femur; n = 19 tarsometatarsus, TMT) with an HD Mobile MXRF (1-mm micro-spot; Mo anode; SDD, and 200s). Calibration used Pb-doped plaster phantoms (0-100 ppm) plus Lucite soft-tissue equivalents (0-5 mm). Spectra (10.0-13.6 keV) were fit with a Gaussian Pb peak on a smooth background; a Compton sideband normalized for mass/overlying tissue. Matched samples were quantified by ICP-MS. Agreement was assessed by OLS, Pearson r, Passing-Bablok, ICC (two-way, absolute), and Bland-Altman. Detection limits were derived from a blank-and-slope analytical MDL, and precision from 30 repeated measurements on two bones. **Results:** MXRF signal followed a quadratic attenuation with a restriction on measurement of bone under soft-tissue thickness less than ~3 mm, supporting reliable Pb quantification in femur, TMT, and phantoms (counts vs. ppm: R² = 0.996; phantom nominal vs. MXRF: R² = 0.994). MXRF tracked ICP-MS very closely (femur r = 0.959, ICC = 0.933; TMT r = 0.962, ICC = 0.953; combined OLS R² = 0.905). Bland-Altman mean biases were small (femur: -1.73 µg/g; TMT: -0.42 µg/g; overall: -1.07 µg/g) without proportional bias. Passing-Bablok slopes were near unity (~0.86-0.96) with small intercepts. Operational DLs were 1.79 µg/g (femur) and 0.80 µg/g (TMT); calibration-derived MDL was 0.03 µg/g at 200 seconds. **Conclusions:** A DCC-based, 1 mm-spot MXRF delivered rapid, non-destructive bone Pb estimates concordant with ICP-MS and low DLs. With attenuation correction and fixed geometry, it supports *ex vivo* Pb surveillance, carcass triage, and program evaluation in birds, advancing portable XRF for environmental health.

PS 4589 How Mercury Reshapes and Damages Neuronal Architecture: Insights from Cryo-Correlative Synchrotron-Fluorescence Imaging

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Background and Purpose: Mercury (Hg) is a highly neurotoxic environmental pollutant, ranked among the ten most hazardous chemicals by the World Health Organization (WHO). It has been implicated in both neurodevelopmental and neurodegenerative disorders through its capacity to disrupt redox homeostasis, interfere with intracellular trafficking, and damage organellar integrity. However, the subcellular distribution of Hg remains poorly understood, making the identification of its

intracellular targets and mechanisms of action a key challenge. Here, we aimed to determine how Hg alters the neuronal cytoskeleton architecture and organelle organization, using a novel approach of native cryo-correlative nano-imaging to directly relate metal localization to structural damages. **Methods:** We developed a cryogenic correlative imaging approach combining cryo-fluorescence light microscopy (cryo-FLM) and synchrotron-based cryo-nano X-ray fluorescence (cryo-SXRF), enabling high precision co-localization of metals with neuronal structures in native conditions. Primary rat hippocampal neurons were exposed for 24 hours to a sub-cytotoxic concentration of HgCl₂ (corresponding to Inhibitory Concentration 10%, IC₁₀). Cells were fluorescently labeled to visualize microtubules, the postsynaptic density (PSD95) and selected organelles (mitochondria and lysosomes). After rapid plunge-freezing in liquid ethane to preserve the native ultrastructure and elemental distribution, neurons were analyzed by cryo-FLM at -196 °C to map cytoskeletal architecture, postsynaptic compartments and organelles with a 350 nm spatial resolution. The same regions were subsequently examined using cryo-SXRF at the ID16A beamline (ESRF, European Synchrotron Radiation Facility), with an incident X-ray beam energy of 17 keV, achieving a spatial resolution down to 25 nm. The two datasets were correlated using the Icy software ec-CLEM plugin, allowing direct co-localization of Hg with neuronal structures at the subcellular level. These observations were supplemented by live-cell fluorescence microscopy of SiR (silicon rhodamine)-tubulin and immunofluorescence microscopy of monoclonal anti-β-tubulin and phalloidin (F-actin) in Hg-exposed and control neurons. **Results:** Live-cell fluorescence imaging revealed that Hg exposure induces progressive deformation of the microtubule network, forming bead-like tubulin varicosities along neurites. These structures were confirmed by quantitative immunofluorescence microscopy, which showed a statistically significant 1.5-fold increase ($p < 0.01$) in microtubule varicosities following Hg exposure ($n = 169$ neurons) vs controls ($n = 348$). Dual live imaging of mitochondria and lysosomes further revealed that these varicosities act as local traps for organelles, blocking their normal transport along axons and dendrites. This suggests a functional breakdown of cytoskeletal integrity and dendritic trafficking. Correlative cryo-FLM and cryo-SXRF imaging provided direct evidence that mercury accumulates within these microtubule varicosities, mainly within mitochondria. In addition, lower levels of Hg were detected all along microtubules and in postsynaptic compartments, where it appeared as small puncta (< 100 nm) associated with F-actin and PSD-95 labelling. Abnormal microtubule varicosities, disruption of mitochondrial activity and synaptic loss are common hallmarks of Alzheimer's disease (AD). **Conclusions:** Our findings demonstrate that Hg destabilizes the dendritic cytoskeleton, inducing microtubule varicosities that trap organelles and accumulate Hg mainly in the mitochondria. Hg is also present in lower content along undistorted dendrites and within postsynaptic compartments. This work demonstrates the power of cryo-correlative imaging to unveil spatial relationships between metal accumulation and neuronal structures. It provides direct evidence linking Hg-induced cytoskeletal disorganization with mitochondrial and synaptic sequestration, mechanisms that mimic AD-like neurodegeneration, suggesting that Hg exposure could be involved in the pathogenesis of AD.

PS 4590 NUPR1 Confers Protection Against Chromium Toxicity in Beas2B cells

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Background and Purpose: Chromium (VI) [Cr(VI)] is the product of various industrial processes and is classified as a group 1 carcinogen by IARC. Aside from the heightened risk of cancer, long term inhalation exposure to Cr(VI) decreased pulmonary function, perforation in the lung, and asthma. Earlier animal studies portend to developmental effects of chronic Cr(VI) including gross abnormalities and malignancies. In the cellular level, Cr(VI) promotes various signaling pathways and gene expression changes including increased expression of NUPR1, a stress response protein. Much of literature has described the protective role of NUPR1 in autophagy and apoptosis. However, NUPR1-mediated Cr(VI)-induced cell death has not been reported. The purpose of this study is to shed light on the role of NUPR1 in Cr(VI) induced autophagy and apoptosis in Beas2B cells, where in prolonged exposure, promotes tumorigenesis. **Methods:** Transient knockdown of NUPR1 was done using siRNA Beas2B cells and chromium-transformed Beas2B cells. Overexpression of NUPR1 was also performed with a pcDNA3.1 backbone. Beas2B cells were then exposed to 5uM of potassium chromate (K₂CrO₄) for 12 and 24 hours. Apoptosis was quantified by Annexin V FITC and PI and analyzed by flow cytometry. mRFP-GFP-LC3 puncta was also performed to analyze autophagic flux in B2B cells. Oxidative damage was assessed by using CellROX™ Green Reagent. Beas2b cells were exposed to 0.125 and 0.25uM Cr(VI) for 3 months. Then anchorage independent growth was quantified by soft agar. Chromium-transformed Beas2b cells were also subject to transient knockdown of NUPR1 and immunoblotting for autophagy and apoptosis markers. **Results:** Cr(VI) induces apoptosis and autophagy in a time dependent manner concomitant with NUPR1 upregulation in Beas2B. Endogenous NUPR1 levels were positively correlated with attenuated autophagy, apoptosis, and reactive oxygen species (ROS) mitigation in Cr(VI) exposure. NUPR1 expression is observed to be higher in chromium-transformed Beas2B cells. NUPR1 depleted Cr-T cells show elevated signs of apoptotic and autophagic activity. **Conclusions:** NUPR1, as a stress-response gene, is a master regulator of apoptosis and autophagy. Taken together, hexavalent chromium induces cell death pathways in Beas2b cells. Chromium activates autophagic and apoptotic pathways, and NUPR1 confers protection against the dysregulated activity in these pathways. Increased cell survival granted by NUPR1 expression

can promote sustained malignant transformation and tumorigenesis in prolonged exposure to Cr(VI). Further investigation of NUPR1 in Cr(VI)-induced cytotoxicity will provide insights into therapeutic approaches for chromium-associated lung cancer.

PS 4591 Correlation Between Urinary Metals and Chronic Diseases in Well-Water Users in New Mexico Border Counties

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Background and Purpose: In the US, around 15% of all residents, approximately 43 million individuals, rely exclusively on private or domestic wells for their drinking water. Oversight of domestic well water safety and quality lacks state or federal regulation, placing the responsibility on consumers to monitor these factors. Due to its unique geography and the presence of the metal production industry, New Mexico (NM), including Doña Ana County, exhibits high levels of metals, including arsenic, uranium, lead, cadmium and other metals sourced from anthropogenic and naturally occurring sources. This study aimed to examine the correlation between urinary metal levels [arsenic (As), lead (Pb), antimony (Sb), uranium (U), cadmium (Cd), iron (Fe), zinc (Zn), and selenium (Se)] and chronic health conditions, including high blood pressure (HBP), diabetes, chronic obstructive pulmonary disease (COPD) and cancers in well water users. **Methods:** Data, including questionnaires and specimens, were collected from adults aged 18 and older who used well water in border counties, NM. To collect urine samples, participants were provided with a wide cup. After receiving the urine samples, the study team divided them into two 15 mL conical tubes and labeled each tube with an arbitrary number corresponding to the participant. The urine samples collected were sent to the Center for Metals in Biology and Medicine (CMBM) laboratory at the University of New Mexico (UNM). The required temperatures for all specimens were maintained from the point of collection to laboratory analysis, ensuring proper conditions for accurate analysis of the biospecimens. The analysis of metals in urine samples was performed using inductively coupled plasma mass spectrometry (ICP-MS). The laboratory analyses conducted at the UNM were supported by Grant Number P20GM13042. The survey data and urinary analysis results were all analyzed using standard statistical techniques using R software version 4.2.2. The covariate analysis was done using standard chi-square analysis. The linear logit regression analysis was done using the absence/presence of a health condition as the response factor. The primary variate was one of the well-water metal concentrations, and the remaining covariates, all treated as categorical variables, were: gender, race, education, household income, ever consumed alcohol, ever smoked, current smoker, age category, and BMI category. The estimated regression coefficients and their 95% confidence levels were converted into odds ratios prior to being reported in the results sections. **Results:** A total of 80 participants were included in this study, and urinary metals were analyzed to assess their correlation with selected chronic diseases. Higher urinary Fe levels were significantly associated with lower odds of HBP (OR = 0.94, 95% CI: 0.88-0.98), suggesting a potential protective role. In contrast, higher urinary As levels were associated with a slight increase in the odds of HBP (OR = 1.02, 95% CI: 1.00-1.05). No significant associations were observed between HBP and urinary levels of Zn, Se, Cd, Sb, Pb, or U. Fe, As, and Se demonstrated notable correlations with diabetes. Increased urinary Fe was correlated with reduced odds of diabetes (OR = 0.93, 95% CI: 0.81-1.00), and Se also showed a protective effect (OR = 0.79, 95% CI: 0.65-0.90). In contrast, higher urinary As levels were correlated with increased odds of diabetes (OR = 1.01, 95% CI: 1.00-1.03). For COPD, higher urinary Fe levels were associated with increased odds of disease (OR = 1.10, 95% CI: 1.03-1.20). No statistically significant correlations were found between urinary metals and cancer outcomes, including lung, liver, bladder, skin, prostate, or breast cancer. **Conclusions:** This is an ongoing study, and further research with larger sample sizes is essential to validate these results and determine whether the correlations of metals and chronic diseases associations can be confirmed or refuted. Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under award number R16GM154680.

PS 4592 Analysis of potential drivers of tungsten-enhanced breast cancer metastasis: A longitudinal study of bone marrow adipocytes

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Background and Purpose: Tungsten (W) is classified as an emerging environmental toxicant due to increased human exposure and a lack of knowledge of the health risks. Epidemiological and *in vivo* studies demonstrate that exposure to W contributes to the carcinogenic process, but the molecular mechanisms are poorly defined. Due to a cohort of breast cancer patients accidentally exposed to W during intra-operative radiotherapy, our lab is investigating the effects of W on breast cancer progression and metastasis. W is known to accumulate in the bone, creating a site for long-term exposure and toxicity. Previous research in our lab has shown that in multiple triple-negative breast cancer orthotopic models, W enhances breast cancer metastasis to the bone niche. These findings suggest that W deposition in the bone

creates a favorable microenvironment to promote metastasis. Bone marrow adipocytes (BMA) play an important role in breast cancer metastasis to the bone through the secretion of adipokines/cytokines that drive tumor cells homing, colonization, and growth by changing the microenvironment. Understanding weekly changes throughout metastasis in BMA is critical to uncover adipokines/cytokines that could drive colonization and proliferation of tumor cells in the bone niche following W exposure. We aim to understand changes in BMA throughout the metastatic process to identify W mediated changes that could enhance breast cancer metastasis to the bone. **Methods:** 6-8 week old female BALB/c and C57BL/6 mice were exposed to 15ppm W in their drinking water or tap water for 4 weeks. After 4 weeks of exposure, mice underwent orthotopic injections of 4T1 (BALB/c) or E0771-Luc (C57BL/6) cells into the 4th mammary fat pad. Longitudinal time points were conducted to track changes after 4 weeks of exposure to W with no tumor cell injection (0 week; 0W), 2 weeks post-tumor cell injection (2 week; 2W), and 3 weeks post-tumor cell injection (3 week; 3W). At the end, bones were collected for multiple parameters. Femurs were used for immunohistochemistry staining of Perilipin-1+ (Plin1+) adipocytes in the bone marrow. Bone marrow from tibiae was cultured to select for mesenchymal stromal cells (MSCs), which were differentiated into BMA through media selection. Cell supernatant and RNA were collected from both MSCs and BMA to profile changes in adipokines/cytokines throughout the metastatic process. **Results:** While both models have increased metastasis to the bone following W exposure, 4T1 induced greater systemic inflammation compared to E0771-Luc. RANK L expression was significantly increased at 0W and trending at 3W in 4T1, but only a trending increase in E0771-Luc. This supports previous data showing increased osteolysis in 4T1 following W exposure at the final tumor endpoint whereas in E0771-Luc, W does not. Adiponectin levels remained unchanged in E0771-Luc, but significantly increased following W exposure at 2W and 3W in 4T1. CXCL2 expression was significantly increased following W exposure at 0W and 2W and trending at 3W in 4T1 and was significantly increased at 3W in E0771-Luc. Previous data from our research group also showed increased gene expression of CXCL2 at final tumor endpoint in 4T1. Plin-1+ staining revealed that E0771-Luc has more adipocytes compared to 4T1, regardless of exposure. **Conclusions:** Some analytes did not have changes either with W exposure or by week, suggesting that perhaps they are not the main drivers of metastasis. Analytes such as RANK L and Adiponectin showed changes following W exposure, but they were dependent upon the model used. These data suggest that there are inherent differences between models and W could be doing slightly different things in each model, including a systemic inflammation component that we observed more in the 4T1 compared to the E0771-Luc. CXCL2 was increased in both models, suggesting a common key player in enhanced metastasis to the bone. CXCL2 is known to play a role in tumorigenesis and immune escape of tumor cells. CXCL2 might be a key mediator that could be further studied in this context to provide insights. This study provides insights into adipokine/cytokines that might be critical players in W enhanced breast cancer metastasis to the bone.

PS 4593 Antibiotic Perturbation of the Gut Microbiome Increases Susceptibility to Arsenic-Induced Urothelial Cytotoxicity in the Murine Bladder

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Background and Purpose: Inorganic arsenic is a group 1 carcinogen and a well-known risk factor for human bladder cancer. Wild-type (WT) mice exposed to inorganic arsenic develop mild cytotoxic changes in the bladder urothelium when evaluated using scanning electron microscopy. Transgenic mice lacking normal inorganic arsenic metabolism (arsenite methyltransferase knockout, As3MT^{-/-}) develop more severe bladder cytotoxicity that increases over time compared to WT mice. The gut microbiome is an understudied modifier of arsenic toxicity, and our group demonstrated that gut bacteria protect against acute arsenic toxicity. For example, lethal toxicity in mice co-exposed to the broad-spectrum antibiotic, cefoperazone (CEF), and inorganic arsenic is four times greater than that observed in mice exposed to inorganic arsenic alone. The purpose of this study was to test the hypothesis that antibiotic perturbation of the gut microbiome accelerates and/or exacerbates arsenic-induced urothelial cytotoxicity in the murine bladder. **Methods:** Wild-type and transgenic (As3MT^{-/-}) C57BL/6 mice were exposed to one of the following treatments: 0.5 g/L CEF and 100 ppm inorganic arsenate (iAs^V); a heat-denatured CEF (0.5 g/L) and 100 ppm iAs^V (SHAM); 100 ppm iAs^V only; 0.5 g/L CEF only; and untreated (plain water) for 7 days. Following exposure, mice were put under anesthesia and the bladder was inflated with 10% buffered formalin. Following humane euthanasia, the bladder was removed and fixed with formalin for 48 hours. Following fixation, bladders were then cut in half exposing the internal urothelial lining. Bladders were then dried with increasing amounts of ethanol, critically point dried, and mounted for observation using Field Emission Scanning Electron Microscopy (FE-SEM). Micrographs from standardized anatomical regions were collected and blinded prior to histopathologic scoring by a trained pathologist. **Results:** Consistent with previous experiments, WT mice exposed to iAs^V alone showed only mild urothelial cytotoxic change and minimal to no hyperplasia. Also consistent with previous experiments, As3MT^{-/-} mice exposed to iAs^V alone exhibited increased urothelial cytotoxicity compared to WT counterparts. Both mice (WT and As3MT^{-/-}) treated with CEF+ iAs^V displayed increased urothelial cytotoxicity and frequent regions of hyperplasia compared to the same mice exposed to iAs^V alone. Notably, WT mice exposed to CEF+ iAs^V displayed urothelial necrosis and early hyperplastic changes. **Conclusions:** These results demonstrate

that antibiotic perturbation of the gut microbiome markedly increases susceptibility to arsenic-induced urothelial damage and accelerates the onset of early pathologic changes associated with bladder carcinogenesis. This is the first report of such histopathology in WT mice exposed to inorganic arsenic. Ongoing work includes additional blinded scoring of control/comparator groups (CEF alone and SHAM) and evaluations of dose- (10, 25, 50 ppm) and arsenic species-dependent effects (iAs^{III} vs. iAs^V). This study identifies the gut microbiome as a critical modifier of arsenic-induced bladder toxicity and highlights the need to consider antibiotic use and other types of microbiome perturbations in arsenic risk assessment.

PS 4594 A Lesson in Exposure Relevance: How Cr(VI) Concentrations Change Pathways of Toxicity in a C. elegans Model

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Background and Purpose: Hexavalent chromium [Cr(VI)] is a ubiquitous environmental pollutant and potent toxicant, currently ranked as the #1 chemical hazard in US public drinking water. Cr(VI) toxicity is well-characterized in many tissues, with *in vitro* and *in vivo* models generating substantial amounts of mechanistic data. While the literature contains a bevy of data describing Cr(VI) toxicity, there are few reports describing how pathways of toxicity change with exposure concentration. These data limitations impair our ability to extrapolate between studies and interpret discrepancies in the literature, as failure to assess toxicity across a wide range of concentrations may lead to relevant pathways of toxicity being overlooked. We recently established a model to investigate Cr(VI) neurotoxicity in *Caenorhabditis elegans*, identifying GABAergic neurons as the most vulnerable neuronal phenotype to Cr(VI). *C. elegans* is an outstanding model to study metals toxicity, given its high-throughput nature and 60 - 80% gene homology with humans. However, no groups have reported the effects of Cr(VI) on the *C. elegans* proteome. Here, we exposed worms to Cr(VI) and performed untargeted proteomics to investigate pathways of toxicity in *C. elegans* across a wide range of Cr(VI) concentrations. We hypothesized that Cr(VI) would primarily alter DNA damage repair pathways in *C. elegans*. Here, we report Cr(VI) induced distinct pathways of toxicity across a wide range of concentrations. **Methods:** Briefly, we synchronized Bristol N2 *C. elegans* and exposed L4 worms to sodium chromate (0, 5, 50, 500, 1000 mg/L) in 85 mmol sodium chloride with 0.001% Tween-20 for 24 h. After exposure, worms were collected, washed, and freeze cracked. Total RNA and protein were extracted with TRIzol. We performed untargeted proteomics with the University of Louisville Proteomics Technology Core to investigate pathways of Cr(VI) toxicity. **Results:** Cr(VI) induced proteotoxic stress across all concentrations (e.g., impaired protein translation, altered protein folding, impaired protein degradation); however, we also report that pathways of toxicity shifted across exposure groups. Exposure to 5 and 50 mg/L increased abundance of proteins related to cholinergic signaling. Exposure to 50, 500, and 1000 mg/L altered protein abundance in a manner consistent with metabolic dysfunction. Exposure to 500 and 1000 mg/L changed protein abundance in a manner consistent with impaired chromatin remodeling and inflammation. **Conclusions:** This is the first study to consider the effects of Cr(VI) on the *C. elegans* proteome, and we report concentration-dependent changes in pathways of Cr(VI) toxicity in a *C. elegans* model. The data presented here suggest that pathways of Cr(VI) toxicity in *C. elegans* are dependent on Cr accumulation, as we previously reported internal Cr levels increased with exposure concentration. Animal models suggest sinks for Cr accumulation change with exposure concentration, possibly explaining changes in toxicity reported here if tissue Cr accumulation in *C. elegans* changed with concentration. Here, we report Cr(VI) induced proteotoxic stress across all exposure groups, suggesting proteotoxicity is a critical factor for Cr(VI) toxicity in *C. elegans*. Importantly, the data presented here demonstrate that neurotoxicity may be a primary effect in worms exposed to low concentrations of Cr(VI). Exposure to 5 or 50 mg/L increased proteins related to cholinergic function - indicating activation of compensatory mechanisms in neurotransmission. At higher concentrations, we report broader toxicity. Exposure to 50, 500, and 1000 mg/L induced metabolic stress in *C. elegans*, possibly indicating mitotoxicity. Further, exposure to 500 and 1000 mg/L decreased expression of histone proteins and increased inflammation, suggesting Cr(VI) may induce DNA damage and significant systemic toxicity in *C. elegans* at these concentrations. While highlighting how Cr(VI) toxicity shifts with exposure concentration in a *C. elegans* model, these data also emphasize the critical nature for dose selection in toxicological studies. Future experiments will consider transcriptomic data and validate proteomics data. Support from SOT GIFT (STV), T32-ES011564 (STV), LRC Voucher Program (Q3-FY2025), R01-ES10563 (MA), and R21-ES033327 (JPW).

PS 4595 Nascent Proteome is the Main Target of Toxic Protein Damage by Cadmium in Human Cells

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Background and Purpose: Cadmium (Cd) is a biopersistent toxic and carcinogenic metal that poses health risks for the general population through dietary and other sources. Long-term exposures to Cd(II) causes damage to the lungs, kidney dysfunction

tion and neurotoxic effects. Cd(II) does not react with DNA but is known to bind to proteins via their SH-groups. The purpose of this work was to investigate what proteins are preferentially damaged by Cd(II) and whether global or protein-specific damage underlies its toxicity. **Methods:** Human lung and kidney cells treated with Cd(II) were analyzed for the formation of proteolytic K48-linked polyubiquitination of proteins, protein denaturation/misfolding and the appearance of cytoplasmic aggregates. Proteotoxic activities and cytotoxicity of Cd(II) were examined under normal conditions and in cells with inactivated ubiquitin-proteasome system, protein synthesis and integrated stress response. **Results:** We found that Cd(II) induced accumulation of K48-polyubiquitinated proteins primarily in the cytosol, and nuclear protein SUMOylation was important for the optimal expression of transcriptional targets of NRF2- and HSF1-driven proteostatic responses. Global protein damage was a major cause of Cd(II) toxicity, as inhibition of ubiquitination or proteasomal activity severely impaired survival of cells at otherwise minimally or nontoxic doses, indicating the requirement for the efficient disposal of abnormal proteins. We found that newly synthesized proteins were the main group of Cd(II)-damaged cellular proteins that were undergoing proteolytic K48-polyubiquitination or became denatured as a result of Cd(II)-induced injury. Proteins with high rates of continuing synthesis such as short-lived transcriptional factors or antiapoptotic proteins were especially sensitive to denaturation by Cd(II). Activation of the integrated stress response limiting protein translation rates diminished accumulation of damaged proteins and increased Cd(II) tolerance by cells. **Conclusions:** Our findings identified damage to nascent proteins as a major cause of global proteotoxicity and cytotoxicity by Cd(II), and highlighted a high vulnerability of short-lived proteins to this metal.

PS 4596 A New Model for Studying Arsenic-induced Skin Carcinogenesis

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Background and Purpose: HaCaT human keratinocytes malignantly transformed by continuous inorganic arsenic exposure (100 nM NaAsO₂, iAs), 28 weeks) serves as the gold standard model to study molecular mechanisms of iAs-induced skin carcinogenesis. However, the HaCaT cell line is inherently hypotetraploid and genomically unstable. Ker-CT cells are a non-tumorigenic near-diploid human keratinocyte cell line immortalized with hTERT and mouse CDK4 overexpression without Simian virus 40 T antigen expression. We hypothesize that chronic iAs exposure malignantly transforms Ker-CT cells, providing a genetically stable model to identify core drivers of iAs-induced skin carcinogenesis. **Methods:** To test our hypothesis, passage-matched, independent quadruplicate Ker-CT cell cultures were chronically exposed to sodium arsenite (iAs; 0 or 100 nM; 44 weeks). Longitudinal samples (DNA, RNA and whole cell lysates) were collected at 8-, 20-, and 44-week time points. Malignant transformation was evaluated through epithelial mesenchymal transition (EMT; immunoblotting), and anchorage independent growth. Longitudinal changes in iAs-induced gene expression was assessed by long-read RNA-seq (PacBio with full-length Kinnex; cutoff: $p < 0.05$, fold change $\geq \pm 30\%$). Ingenuity Pathway Analysis (IPA) was used to predict canonical pathway changes dysregulated by chronic iAs exposure. **Results:** After 44 weeks, iAs exposure concomitantly suppressed epithelial (E-cadherin) while inducing mesenchymal markers (N-cadherin and Slug), characteristic of EMT. Both iAs exposed and passage-matched unexposed cells (44 weeks) migrated to the top of soft agar, with iAs exposed cells exhibiting migration at an earlier timepoint compared to unexposed cells. Over 440 differentially expressed protein coding mRNAs were identified at each timepoint. IPA analysis revealed that 18, 33, and 48 pathways were dysregulated at 8-, 20-, 44-week timepoints, respectively. There was limited longitudinal overlap both for differentially expressed genes and dysregulated pathways highlighting the temporally dynamic nature of carcinogenesis in agreement with our published observations in the HaCaT cell line. Prominent cancer-associated pathways were dysregulated at each timepoint, elucidating the stepwise processes that promote iAs-induced skin carcinogenesis. **Conclusions:** Together, our findings support that Ker-CT cells provide a genetically stable model to study iAs-induced skin carcinogenesis.

PS 4597 Intestinal SLC30A10 suppresses manganese absorption in hereditary hemochromatosis

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Background and Purpose: Iron (Fe) and manganese (Mn) are essential trace elements that must be maintained within a narrow physiological range. The export protein SLC30A10 plays a critical role in Mn homeostasis by mediating its excretion from the liver into bile and from enterocytes into the intestinal lumen. Biallelic mutations in SLC30A10 cause a rare inherited Mn overload disorder characterized by dystonia, cirrhosis, and polycythemia. Studies using Slc30a10-deficient (Slc30a10^{-/-}) mice have demonstrated impaired gastrointestinal Mn excretion and systemic Mn excess. Our prior work revealed that dietary Fe supplementation attenuates disease phenotypes in these mice, suggesting that Fe and Mn compete for absorption through shared transporters such as DMT1 and ferroportin. Interestingly, despite impaired Mn excretion, Slc30a10^{-/-} mice also exhibit increased gastrointestinal Mn absorption, a phenomenon not observed in hereditary hemochromatosis, a disease characterized by hepcidin deficiency and enhanced Fe absorption. We

therefore hypothesized that intestinal SLC30A10 suppresses dietary Mn absorption, preventing Mn overload under conditions of hepcidin deficiency. **Methods:** To test this hypothesis, we employed mice deficient in hepcidin expression. We first generated and characterized Slc30a10^{-/-}/Hjv^{-/-} double-knockout mice to assess the combined effects of both deficiencies on Mn homeostasis. Mn absorption was quantified using gastric gavage of radiolabeled ⁵²Mn. To determine the specific contribution of intestinal SLC30A10, we generated Hjv^{-/-} mice with intestinal Slc30a10 deficiency by crossing Slc30a10^{fl/fl} mice with villin-Cre (Vil) transgenic mice. Mn levels in tissues (liver, brain, bone, pancreas) and bile were quantified using inductively coupled plasma-optical emission spectrometry (ICP-OES), and ⁵²Mn absorption assays were performed to measure intestinal uptake. **Results:** Slc30a10^{-/-}/Hjv^{-/-} double-knockout mice exhibited markedly increased Mn absorption compared to single-knockout or control mice. Similarly, intestinal-specific Slc30a10 deficiency in Hjv^{-/-} mice led to significantly elevated Mn accumulation across multiple tissues, including liver, brain, bone, and pancreas, along with increased biliary Mn levels. ⁵²Mn absorption assay confirmed that intestinal loss of Slc30a10 enhances Mn absorption in Hjv^{-/-} mice. These results demonstrate that intestinal SLC30A10 actively suppresses Mn absorption, even under conditions that favor Fe hyperabsorption due to hepcidin deficiency. **Conclusions:** This study reveals that intestinal SLC30A10 serves as a critical gatekeeper preventing Mn overload during states of low hepcidin, such as hereditary hemochromatosis. In diseases of Fe excess, reduced hepcidin levels lead to increased ferroportin and compensatory upregulation of DMT1, promoting excessive Fe absorption. However, Mn absorption does not concurrently increase, due to the protective action of intestinal SLC30A10. These findings provide a mechanistic explanation for why conditions that enhance Fe absorption do not result in Mn toxicity and establish intestinal SLC30A10 as a key regulator of Mn homeostasis in iron-loading disorders.

PS 4598 Leveraging a human induced pluripotent stem cell-derived cardiomyocyte system to study the effects of inorganic arsenic exposure during cardiac development

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Background and Purpose: Arsenic is a naturally occurring metalloid that has been mobilized in the environment via anthropogenic processes. Exposure to inorganic arsenic (iAs) during pregnancy is associated with increased risk for congenital heart defects (CHD). CHD affects 1 in 110 infants and accounts for approximately 40% of all birth defect mortality. Disruption of transcription factors that govern the differentiation of cardiac progenitor cells has been associated with the development of CHD. Prior work from our lab found that dams exposed to iAs during gestation had altered heart structure, which may impact embryonic development. Indeed, mouse embryos from exposed dams had larger hearts and increased left ventricular internal dimensions compared to non-exposed controls. Collectively, these findings suggest that gestational iAs exposure may impair cardiac differentiation and contribute to CHD development, but additional mechanistic studies are warranted. Our study aims to examine the impact of iAs on cardiomyocyte differentiation and maturation using a human induced pluripotent stem cell (hiPSC) model. **Methods:** To model cardiogenesis *in vitro*, hiPSCs were differentiated into cardiomyocytes using sequential Wnt activation and inhibition. hiPSCs were exposed to iAs starting at differentiation day (DD) 0. An MTT viability assay was performed on hiPSCs exposed to 0-100 ppb iAs on DD3. Doses of 0, 5, and 50 ppb iAs were selected, as they did not induce excess cell death compared to higher doses. hiPSCs were exposed to the selected doses starting at DD0. Cells were analyzed at DD3, DD5, DD9, DD12, and DD21 by testing the expression of differentiation and oxidative stress genes using qPCR. Beating frequency measurements (in bpm) were also quantified. **Results:** We found a significant decrease in cell viability, even at low doses of iAs after three days of exposure. Preliminary qPCR results show that markers of early cardiomyocyte development, *Tbx5*, *Gata4*, and *Nkx2.5*, were decreased in cardiac progenitor cells exposed to 5 ppb iAs at DD5, but these markers were not statistically different in cells exposed to 50 ppb iAs compared to non-exposed controls. Cardiomyocyte maturation markers, including *Tnni3*, *Myh6*, and *Actn2*, were upregulated at DD12 in hiPSC-cardiomyocytes exposed to 50 ppb iAs; *Myh6* was still upregulated in hiPSC-cardiomyocytes at DD21, compared to non-exposed controls. Markers of oxidative stress, *Sod1* and *Gpx1*, were increased in DD21 hiPSC-cardiomyocytes exposed to 5 ppb iAs compared to non-exposed controls, but expression levels were not statistically different in cells exposed to 50 ppb iAs. Finally, beating frequency showed a dose-dependent reduction in exposed cardiomyocytes at DD12 compared to non-exposed controls, indicating a potential delay in functional maturation associated with iAs exposure during cardiac development, but this decrease was no longer seen at DD21. **Conclusions:** Our work demonstrates that exposure to iAs at environmentally relevant concentrations leads to impaired differentiation of iPSCs into cardiomyocytes, thus leading to potential diminished cardiomyocyte functionality. Our findings suggest that iAs exposure may contribute to CHD development by impairing early cardiac differentiation and interfering with cardiomyocyte maturation at later timepoints. Due to the high prevalence of CHD in the population and the increased likelihood of iAs exposure in the environment, this study is critical for understanding how iAs exposure during development impacts cardiomyocyte differentiation.

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Background and Purpose: Inorganic arsenic (iAs) is a globally pervasive toxic metalloid, with more than 200 million individuals chronically exposed and no effective strategies available to prevent, detoxify, or eliminate it from the human body. Growing evidence indicates that the gut microbiome modulates host responses to arsenic, suggesting that targeted microbiome engineering may offer a novel detoxification approach. Although microbial arsenic metabolism has been described, it remains unclear which gut bacteria with meaningful detoxification functions can effectively reduce systemic arsenic burden *in vivo*. To address this gap, we integrated genome-scale screening with *in vitro* and *in vivo* models to identify native gut bacterial strains capable of mitigating arsenic burden. **Methods:** We conducted a genome-scale search for gut bacteria carrying key arsenic metabolism genes, including uptake genes (Pst, Pit, GlpF), the methylation gene (ArsM), and the efflux gene (ArsP). Protein sequences were queried against an in-house gut metagenome database. Isolates were cultured with 200 ppb sodium arsenite to quantify the capacity of strain-specific arsenic removal. High-performing isolates were then evaluated in human AS3MT germ-free mice and C57BL/6 hAs3mt mice exposed to 200 or 500 ppb arsenic via drinking water to determine effects on fecal elimination and systemic bioaccumulation. **Results:** *In vitro* screening revealed substantial variation across strains, with 27 isolates showing significant arsenic removal ranging from 6% to 45%. Mono-bacterial colonization markedly increased fecal arsenic elimination in both germ-free and wildtype hAs3mt mice. Importantly, single-strain supplementation in wild-type hAs3mt mice reduced arsenic accumulation in the liver and kidney, demonstrating bacterial detoxification efficacy even within a complex microbial community. **Conclusions:** Taken together, these findings establish mechanistic links between bacterial genomes, their detoxification capacity, and host arsenic burden, supporting mechanism-guided microbiome engineering as a promising strategy for mitigating risks associated with arsenic exposure.

PS 4600 Investigation into Immune Cell Dysregulation and Autoimmune Disease Mechanisms Following Exposure to Uranium Bearing Dust *in vivo*.

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Background and Purpose: The western United States consists of thousands of abandoned uranium (U) mines (AUMs), many of which are present in the 4 corners region of the Southwestern United States. These AUMs are in close proximity to residential areas, many of which exist on tribal lands. Improper cleanup procedures and lack of remediation have led to significant concerns of windblown, fugitive dust blowing into these communities contributing to chronic health disease, including autoimmune diseases. Investigation into these long-term chronic health effects following exposure is critical to understand human health risk. We hypothesize that chronic inhalation exposure to AUM particulate matter (PM) will enhance autoimmunity relative to controls through immune dysregulation and chronic inflammation. **Methods:** 9 - 11 week old female MRL/MpJ mice were exposed through oropharyngeal aspiration to AUM PM from the St. Anthony mine, a former U-mine near the Pueblo of Laguna in New Mexico (St. Anthony PM), and control PM from a former coal mine in Colorado containing low heavy metal content (Colorado Red PM). Energy-dispersive X-ray spectroscopy characterization of these dusts revealed that the St. Anthony PM is rich in silica and aluminum and heavy metals U and vanadium (V), while the Colorado Red PM has negligible levels of uranium and vanadium, but does contain silica (Si) and aluminum (Al). Mice were exposed four times in total at a concentration of 50µg/µL of PM in dispersion media across the course of two weeks. Endpoint analysis were evaluated at 2 weeks, 24-hours post the final exposure and 12 weeks, 10 weeks after final exposure. Primary endpoints methodology consisted of analysis of immune cell populations in the lungs and spleen, and T-cell subpopulations within the lymph nodes by flow cytometry, cytokine analysis in the bronchoalveolar lavage fluid (BALF) and plasma using multiplex cytokine arrays, Neutrophil elastase levels in the BALF and plasma by enzyme-linked immunosorbent assay (ELISA), metal deposition within the lungs and bones by inductively coupled plasma mass spectrometry (ICP-MS), and autoantibodies in the BALF and plasma. **Results:** Our findings illustrate significant alterations in immune cell populations in the lungs following exposure to St. Anthony PM at the 2-week timepoint. In particular, we found recruitment of neutrophils after exposure to the St. Anthony PM relative to both our vehicle control, dispersion media and the environmental dust control Colorado Red PM. In order to investigate neutrophil's involvement after observing their increased recruitment to lung, we ran neutrophil elastase ELISAs which revealed a significant increase in neutrophil elastase in the BALF and plasma in the St. Anthony PM group relative to the control groups. ICP-MS analysis revealed that in the St. Anthony PM group, there was a retention of heavy metals including U, V and Al in the lungs, that are not present in the other control groups, even at the 12 week timepoint, providing evidence of long-term metal retention in the lungs that could be contributing to immune dysregulation both in the lungs and systemically. Immune dysregulations revealed by flow cytometry, show alterations to T Cell populations in the spleen via an increase in PD-1+ T Cells

in the UBD test samples. **Conclusions:** Overall, we demonstrate that inhalation exposure to St. Anthony PM caused significant alterations to the immune cell populations of the lungs. Particularly, an increase in the levels of activated neutrophils secreting neutrophil elastase, leading towards production of neutrophil extracellular traps (NETs) that could drive immune dysregulation and the development of autoimmunity through the recognition of NET components, such as modified proteins and selfDNA, by T Cells. Retention of uranium and other heavy-metals from the St. Anthony PM is a unique factor across the conditions that we hypothesize is a leading driver of the effects seen after aspiration exposure to AUM dust.

PS 4601 Zinc and Nickel Inhibit GLP-1, GIP, and Glucagon Signaling in Insulin Secreting Beta Cells

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Background and Purpose: Zinc (Zn) and nickel (Ni) are reported to modulate several G-Protein Coupled Receptors (GPCRs) with resulting enhanced (β2-adrenergic) or reduced (serotonin 5-HT7, NMDA, D2 Dopamine) receptor ligand binding and activation. GLP-1, GIP, and glucagon receptors are crucial for the proper function of insulin secretion from beta cells -the cell type responsible for regulating glucose metabolism through controlled insulin secretion. Specifically, these receptors act to enhance insulin secretion in response to rising glucose. At the same time, insulin secreting beta cells within islets contain an exceptionally high concentration of the essential metal Zn -which is co-secreted with insulin into the islet milieu. They also contain a relatively high concentration of the non-essential metal Ni. Therefore, the actions of Zn and Ni on GLP-1, GIP and glucagon receptor activation and downstream signaling events were evaluated. GLP-1, GIP, and glucagon receptors are members of the B1 Class of GPCRs (B1GPCRs) that stimulate adenylyl cyclase, thereby increasing levels of the second messenger cAMP which in turn exerts multiple downstream effects, including potentiation of insulin production and secretion. **Methods:** To evaluate potential actions of Zn at B1GPCRs, we used the rat insulin secreting INS-1E cell line that expresses endogenous B1GPCRs, and that express the cAMP responsive FRET reporter H188 using an adenoviral vector. This allowed real-time kinetic measurements of cAMP production in response to GLP-1, GIP, or glucagon when cells were or were not treated with Zn (as ZnCl₂) or Ni (as NiCl₂). **Results:** A concentration dependent inhibitory action of Zn or Ni on all three B1GPCRs was found. This resulted in suppressed cAMP-elevating actions of all three hormones. This effect was observed at Zn concentrations that are expected to exist within the islet interstitial space. The IC50 for this inhibitory effect ranged from 12.0 to 15.3 µmol when testing these hormones. To test for an inhibition of GLP-1 downstream signaling, we examined changes of gene expression using a multimerized RIP1-CRE-LUC luciferase reporter that is cAMP responsive. Here, a significant and concentration dependent inhibition of GLP-1 induced luciferase expression by Zn was observed. Next, a fluorophore labeled GLP-1-[FAM-Trp31] was used to test if binding of GLP-1 to the GLP-1R is altered by Zn. In these experiments, a potent inhibition of GLP-1-[FAM-Trp31] binding by ZnCl₂ at 30 µmol/L was observed. This inhibition potentially reflects allosteric regulation of the GLP-1R by Zn. Finally, *ex vivo* assays of insulin secretion from islets isolated from C57BL/6N mice were performed. Here, a significant action by Zn to suppress the ability of GLP-1 (10 nM) to potentiate glucose-stimulated insulin secretion (GSIS) was observed. **Conclusions:** Excess Zn or Ni in insulin secreting beta cells may impair insulin secretion through inhibition of the B1GPCRs GLP-1, GIP, and glucagon.

PS 4602 Assessment of urinary metal exposure in sugarcane workers across a harvest season and their relationship to kidney health biomarkers

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Background and Purpose: Urinary metal co-exposure is hypothesized to contribute to chronic kidney disease progression in male sugarcane workers in the Pacific coastal region of Guatemala. **Methods:** Urine samples and kidney health biomarkers (estimated glomerular filtration rate [eGFR], albumin-to-creatinine ratio [ACR], and acute kidney injury estimation [AKI]) were collected across 4 time points during the 2021-2022 and 2022-2023 sugarcane harvest seasons in Guatemala. As, Cd, Co, Cu, Mn, Mo, Ni, Pb, Sb, U, W and Zn were quantified in 512 urine samples using inductively coupled plasma mass spectrometry (ICP-MS). Individual metal associations with kidney health biomarkers were evaluated, and possible synergistic effects of relevant metal mixtures were assessed using quantile g-computation. **Results:** Ni and Zn were negatively associated while Co and Mo (essential trace elements) were positively associated with eGFR. Mixture analysis showed a significant overall effect of all the metals, with Zn, Ni, and Cu contributing most to eGFR decrease, and Mo, Co, and Sb contributing most to eGFR increase. Mo was inversely associated with ACR, while As and Pb (well-known nephrotoxic metals) were positively associated with ACR both in the individual metal and mixture analysis. For AKI, Zn and Ni showed the strongest contributions, and a significant overall mixture effect was observed.

Conclusions: Overall, the findings indicate that urinary metal mixtures, particularly driven by Ni, Zn, and Mo, are associated with kidney function alterations among male sugarcane workers. Ni, found above normal urine metal reference values <4ppb, emerges as a metal of particular interest for future research in this population.

PS 4603 Uranium, Lead, and Other Toxic Metals Mapped in New Mexican Women's Triple-Negative Breast Cancer Samples

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Background and Purpose: Triple-negative breast cancer (TNBC), the most aggressive subtype with limited therapeutical options, is diagnosed at a younger age than other forms of breast cancer. Importantly, TNBC disproportionately affects women from minority communities, who also face higher environmental toxic metal exposures. However, the molecular pathways by which these heavy metals influence the tumor microenvironment and promote Cancer Hallmarks — like inflammation and increased phenotypic plasticity — remains unclear. This problem is especially urgent in communities with well-documented heavy metal exposures. In the Southwestern United States, several communities face legacy metal and metalloids burdens arising from several sources: arsenic contamination in ground/drinking water sources, nuclear weapons development and testing, as well as uranium mining activities. These toxic exposures position people from this region as a key population for understanding the mechanistic links between heavy metal exposure and TNBC etiology and progression. **Methods:** A 20 µm resolution laser ablation inductively coupled mass spectrometry (LA-ICP-MS) assay was developed to semi-quantitatively measure several heavy metals and metalloids. This assay was applied to archived FFPE TNBC samples from 16 patients surgically treated at the University of New Mexico Comprehensive Cancer Center. All samples were stored with broad consent for future research purposes. Uranium (U), lead (Pb), vanadium (V), tungsten (W), nickel (Ni), cadmium (Cd), arsenic (As) was measured for one of these samples thus far. Phosphorous (P) was measured as a surrogate for cell/tissue density. Porcine skin gelatin-based, tissue-mimicking standards were developed to build calibration curves, with spike-in metals concentrations ranging from 10 ppb to 10 ppm. Serial tissue sections were also assayed using the Xenium spatial transcriptomics platform with a breast cancer panel supplemented with 100 empirically derived metal-responsive genes, which include genes involved in immune activation, extracellular matrix & cell structure, cell cycle, metabolic, and stress response pathways. The xenium panel was used to annotate cell types and H&E staining was used for histological reference and confirmation of tumor margins. Pixel-level Spearman's rank correlations were calculated for each element pair. **Results:** U, Pb, V, W, Cd, Ni, and Cd, were detected in the TNBC sample, with concentrations ranging from 10 ppb to over 1 ppm for some metals. While there are noticeable edge bleeding artifacts of metals, likely arising from diffusion caused by solvents used in the FFPE process, substantial hotspots of U, Pb, and Cd were observed in the cancer epithelium. Single-case results show a positive pixel-level correlation between U and P ($p=0.44$, $p\text{-value} < 0.001$), as well as weak positive correlations between U and Pb ($p=0.21$, $p\text{-value} < 0.001$), Cd and P ($p=0.21$, $p\text{-value} < 0.001$), and Ni and V ($p=0.23$, $p\text{-value} < 0.001$). **Conclusions:** The significant positive correlation between uranium and phosphorus in this pilot case provides evidence supporting the accumulation of these metals within dense tumor regions, while inter-metal correlations suggest possible mixture effects. These data establish the beginnings of critical methodological foundations advancing environmental health toward the integrated spatial omics era. Our ongoing work is generating spatial metals maps of additional TNBC tissues and integrating these maps with spatially resolved transcriptomics to discover the specific molecular pathways by which these metals may alter the tumor microenvironment and drive TNBC progression.

PS 4604 Novel Application of the EPA All Ages Lead Model (AALM) to Determine an Acute (24-hour) Inhalation Threshold for Lead Protective of Neurotoxicity in Children

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Background and Purpose: Many state air toxics regulatory programs require inhalation reference criteria protective of acute (24-hour) exposures, which are challenging to determine for lead (Pb), as the most sensitive endpoint is neurotoxicity associated with increased blood Pb in children. One approach is to use the National Ambient Air Quality Standard (NAAQS) for Pb of 0.15 µg/m³, which was used by the Oregon Department of Environmental Quality. However, the NAAQS is intended for a 3-month average concentration, which is not well suited to characterize 24-hour exposures. An alternative approach to setting an acute threshold for Pb was developed, using the EPA's All Ages Lead Model (AALM) Version 3.1, which allows users to simulate single-day exposures (EPA 2025). Biokinetic modeling was performed to predict the air concentration for one day of exposure that would result in a blood Pb increase of 1 µg/dL. The California Office of Environmental Health Hazard Assessment (OEHHA) uses 1 µg/dL as the threshold for a source-specific incremental increase in blood lead levels (BLLs) for protection of school children and fetuses (OEHHA, 2007).

Methods: EPA's AALM was used to estimate BLLs for children each year for birth to 6 years because children are more sensitive than adults, and kinetic and exposure parameters change as children grow. First, baseline BLLs were modeled considering all exposure routes in the AALM (e.g., soil, indoor dust, food) except for air, using default model inputs (EPA, 2024). The upper tolerance limit for background Pb in Oregon soil of 79 µg/g was used for modeling soil and dust exposures. The AALM was subsequently run in an iterative process by changing the air concentration on a single day until the estimated BLLs corresponded to a BLL of 1 µg/dL above baseline. The single day of exposure (i.e., acute pulse) was simulated separately for each year from year 0 to year 6, such that there were seven different model simulations. To simulate an acute, 24-hour exposure scenario for one age group, the air concentration was assumed to be 0 µg/m³ except for the single day of exposure for the age group of interest. As shown by the magnitude of the results of the pulsed modeling, a default air concentration for Pb (0.01 µg/m³) would not contribute significantly to BLLs. The AALM uses masks to set exposures for specific repeating periods. In this case, an annual mask was set to 0 µg/m³ for days 2 through 365 of each year. Day 1 exposure was set to 0 µg/m³ for all age groups except for the specific age group with the acute 24-hour exposure. The year with acute/pulsed exposure had a non-zero air concentration on the first day. For example for year 1, the air concentrations on day 1 was modeled for three age groups: (1) age 0 at 0 µg/m³, (2) age 1 at the non-zero air concentration, and (3) age 2 and beyond at 0 µg/m³ through the end of the simulation (6.1 years). Default lung parameters from AALM 3.0 were selected (EPA 2024, 2025). These parameters represent lung kinetics for ultra-fine (approximately 0.1 µm in diameter) combustion aerosols, and relative bioavailability for inhalation exposure was assumed to be 1 (EPA, 2024). **Results:** Baseline BLLs ranged from 1.10-2.21 µg/dL. The day 1 air concentration that resulted in a predicted BLL closest to the target increased BLL ranged from 3.86 µg/m³ (year 0) to 7.64 µg/m³ (year 5). These values are all well above the NAAQS for Pb of 0.15 µg/m³. AALM predicted that the highest BLL following acute air exposures occurs two days after the 24-hour pulse exposure. The single-day air concentration for the most sensitive age group (newborn, 0-year age group) associated with a 1 µg/dL increase from the baseline BLL was 3.9 µg/m³ for the newborn age group. The estimated BLLs decreased to baseline BLLs within approximately 20 days, and the peak BLL was 3.21 µg/dL, which is lower than the CDC Pb reference level of 3.5 µg/dL. **Conclusions:** An acute 24-hour threshold for Pb in ambient air of 3.9 µg/m³ would be appropriate and protective for all ages, specifically children. This guideline is complementary to the NAAQS because a single pulsed 24-hour concentration of 3.9 µg/m³ would not exceed the NAAQS when averaged with background Pb in air concentrations over three months.

PS 4605 Scrambling the Genome: Mechanisms of Cr(VI)-induced Chromosome Instability in Lung Carcinogenesis

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Background and Purpose: Hexavalent chromium [Cr(VI)] is a well-established human lung carcinogen, but the mechanisms behind how Cr(VI) induces lung cancer are poorly understood. Chromosome instability (CIN), a hallmark of lung cancer, is considered a major driving factor in Cr(VI)-induced lung cancer. Studies in cultured human lung cells show prolonged Cr(VI) exposure induces a DNA-damage repair deficiency leading to chromosome instability and neoplastic transformation. How Cr(VI) impacts the genomic sequence is not well understood. Determining the impact of Cr(VI) on the whole genome can provide insight into how chromosome instability arises to drive lung cancer. **Methods:** A clonal human lung cell line was exposed for 24 or 120 h to two particulate Cr(VI) compounds, lead chromate and zinc chromate. Following treatment, 5 subclones for each exposure were isolated. We performed karyotype analysis and compared results to the whole genome sequencing for each clone. Genomic lesions were characterized using strict thresholds. **Results:** Our karyotype analysis revealed minimal changes above control with both particulate Cr(VI) compounds after 24 h treatments, consistent with previous data. A pronounced increase in chromosome instability was observed after 120 h exposure to zinc chromate. The genomic analysis revealed both Cr(VI) compounds caused a significant number of large structural variants, including, from most abundant to least: inversions, translocations, deletions, and duplications at both time points. **Conclusions:** This study demonstrates Cr(VI) is a potent genotoxic agent that causes many genomic rearrangements not observed through traditional karyotypic analysis. The many small indels detected in the genomic analysis are predicted to cause transcription factor binding site ablation, frameshift variants, in-frame deletions, and in-frame insertions in surviving cells. These smaller genomic rearrangements can then lead to the permanent karyotypic changes observed that drive tumor development and carcinogenesis. These data can better inform risk assessment to determine safe exposure limits and provide insights for future mechanistic studies on Cr(VI)-carcinogenesis. This work was supported by NIEHS grant R35ES032876 to JPW.

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Background and Purpose: Drinking water contaminants often co-occur and are encountered as co-exposures, but there is limited research on potential toxicological interactions between drinking water contaminants. In a previous study of federally unregulated metal contaminants in US public drinking water systems surveyed over years 2013-2015 [see doi: 10.3390/ijerph18157884], geometric means were estimated for each measured metal for each drinking water system separately, and then those geometric means were compared across measured metals. The geometric means of two measures of chromium (total [Cr] and hexavalent [Cr(VI)]) were highly correlated, and both chromium geometric means were associated with vanadium (V) geometric means. Thus, there is likely to be co-exposure to chromium and vanadium in public water supplies in the United States. While the toxicity of these compounds from ingestion exposure pathway is a topic of ongoing research, inhaled Cr(VI) has been associated with lung, nasal, and sinus cancers in occupational studies, and studies of inhaled exposure to vanadium pentoxide have also suggested possible respiratory toxicity. Given the potential for both joint exposure and similar physiological impacts, this environmental genomics study sought to examine patterns of documented gene-environment interactions for chromium and vanadium to characterize potentially shared biology between these drinking water contaminant metals. **Methods:** We queried the Comparative Toxicogenomics Database (CTD) on October 17, 2025 for genes that have been previously shown to have gene-environment interactions with "chromium", "hexavalent chromium ion", or "vanadium". We then reviewed all cited studies corresponding to each gene-environment interaction indexed in CTD and returned by that query. Genes in the initial candidate list from CTD were excluded from our bioinformatic analysis if they were not human genes, if the metal of interest was not the actual exposure in the original study, if the studies were retracted, if the study could not be accessed, or if the study details were incongruent with what was reported in CTD. This resulted in a reduced gene list of confirmed-as-relevant *Homo sapiens* genes for analysis. We considered pairwise overlap of genes interacting with each metal. We then performed signaling pathway enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database on the Database for Annotation, Visualization, and Integrated Discovery (DAVID) platform for the confirmed-as-relevant *Homo sapiens* gene list. **Results:** This scan identified 60 genes that have been documented as having interactions with Cr, 1296 with Cr(VI), and 157 with V. The gene lists for Cr and Cr(VI) had 42 overlapping genes, Cr and V had 9 overlapping genes, and Cr(VI) and V had 25 overlapping genes. In total, 1259 unique genes were identified as having at least one documented interaction and were included in the pathway enrichment analysis. The DAVID/KEGG analysis found that 176 pathways were statistically significantly enriched (53 Bonferroni significant, 150 Benjamini-Hochberg (BH) significant, and 158 False Discovery Rate (FDR) significant). The top 10 gene-enriched KEGG pathways were cell cycle, lipid and atherosclerosis, pathways in cancer, AGE-RAGE signaling pathway in diabetic complications, apoptosis, human T-cell leukemia virus 1 infection, colorectal cancer, hepatitis B, microRNAs in cancer, and FoxO signaling pathway. **Conclusions:** Bioinformatic analysis suggests that there may be overlapping biological pathways between these metal exposures, including for genotoxic and replication stress pathways; oxidative stress, inflammatory, and immune-like signaling; and cancer pathways. There is some overlap in these KEGG gene pathways but the bioinformatic analysis suggests potential for multiple mechanisms of toxicological interaction. Given the potential for joint exposure from drinking water among other exposure pathways, toxicological studies administering doses of combined exposure to evaluate joint toxicity may be warranted.

PS 4607 Weight of Evidence Assessment for Toxicities from Retained Lead Pellet

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Background and Purpose: Retained bullet (RB) fragments represent a recognized but often underappreciated source of systemic lead exposure. Historically, RBs embedded in soft tissue were considered relatively benign due to presumed tissue encapsulation. However, accumulating evidence suggests that these fragments can leach lead over time, resulting in elevated blood lead levels (BLLs) and potential systemic toxicities. To address this concern, we conducted a weight-of-evidence (WOE) assessment to evaluate whether RB fragments contribute to clinically significant lead exposure and toxicity. This assessment aimed to synthesize available data on lead leaching, BLL elevation, and associated health outcomes, while critically examining the strength and limitations of the evidence base. **Methods:** A comprehensive review of human epidemiology studies and case reports was conducted, including an assessment of methodological strengths and weaknesses. Searches were performed in PubMed using free text terms: ("gunshot wound" OR gunshot* OR shot* OR bullet* OR missile* OR pellet* OR projectile* OR "retained fragment*" OR "retained lead") AND ("lead poisoning" OR "lead toxicity" OR "lead exposure" OR "blood lead" OR "serum lead"). Filters were applied to include only human studies published in English. Eligible studies were limited to epidemiology research and case reports involving adults that reported blood or serum lead levels; studies lacking

these measurements were excluded. Data were evaluated with respect to Bradford-Hill guidelines for the presence or absence of cause-effect relationship between RBs and risk of elevated BLLs and lead toxicity, including strength of association, temporality and dose-response of the relationship, replication of findings, biological plausibility, alternative explanations, specificity of the association, and consistency with other knowledge. **Results:** The search identified 342 articles published between 1946 and 2025, including 30 reviews, 1 meta-analysis, 65 case reports, 4 case-control studies, 5 cross-sectional studies, 3 prospective cohorts, and no randomized controlled trials. Epidemiology studies have shown that individuals with RB fragments exhibit elevated blood lead levels (BLLs). Anatomical location significantly influences BLLs, with fragments near bone fractures associated with higher concentrations. Increased fragment count correlates with greater BLL elevation. Numerous case reports also document delayed onset of lead toxicity symptoms, sometimes occurring years after the initial injury. Despite these findings, the evidence base is dominated by case reports and small observational studies, with few well-designed epidemiological investigations. This reliance on lower-confidence sources limits the statistical strength and generalizability of conclusions. **Conclusions:** The available evidence supports an association between retained lead fragments and elevated BLLs, with toxicity risk influenced by fragment location, number, and time since injury. However, the absence of robust epidemiological studies suggests that current assessments depend heavily on case reports and other lower-confidence sources, constraining the overall strength of inference and certainty of risk characterization. Additional high-quality, longitudinal research is needed to clarify exposure dynamics and inform clinical management guidelines.

PS 4607a Prediction of Obeticholic Acid (OCA) Liver Injury Liability in Patients with Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) Using Quantitative Systems Toxicology

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Background and Purpose: Obeticholic acid (OCA), a synthetic bile acid analog of chenodeoxycholic acid (CDCA), showed some promise for the treatment of metabolic dysfunction-associated steatotic liver disease (MASLD) but did not receive FDA approval for this indication, in part because of concerns about liver safety. OCA was granted accelerated approval in 2016 for treatment of primary biliary cholangitis but was recently withdrawn from the US market, also related to liver injury concerns. In clinical trials, elevated liver injury biomarkers were observed in both healthy subjects at supratherapeutic doses (100-250 mg) and in some MASLD patients at therapeutic doses (10-25 mg). MASLD patients exhibited higher plasma and predicted liver concentrations of OCA and its metabolites (glyco-OCA and tauro-OCA), particularly in advanced disease stages. We have previously used DILIsym, a quantitative systems toxicology model, to recapitulate OCA-associated liver injury in healthy subjects at supratherapeutic OCA doses. This project extended the developed DILIsym model using a virtual MASLD population to determine whether liver safety liability could have been predicted in this patient population prior to the MASLD clinical trials. We also used the model to explore mechanisms underlying the toxicity observed in this population. **Methods:** Exposure to OCA and OCA metabolites was simulated in healthy subjects using a previously developed physiologically based pharmacokinetic (PBPK) model. Concentrations of OCA and metabolites in plasma, liver, and sinusoidal compartments were increased 2-, 5-, or 10-fold at each dose level to assess the impact of increased exposure in the MASLD population. DILIsym version 8A with an extended bile acid representation (similar to version 11, commercially available since 2025) incorporated bile acid transporter inhibition (uptake, biliary and basolateral efflux) using half-maximal inhibitory concentrations. In addition, the DILIsym model incorporated mitochondrial dysfunction parameters assuming CDCA-like mitochondrial uncoupling. Liver injury biomarkers that were investigated included plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Predicted liver injury was reported as the percentage of virtual patients with biomarker elevations at least 2-fold from individual patient baseline values. **Results:** In the virtual MASLD population, dose-dependent increases in ALT and AST were observed at supratherapeutic OCA doses. However, susceptibility to liver injury was not increased in the virtual MASLD patients compared to healthy subjects at OCA and metabolite exposure levels typical of healthy subjects. A 2-fold increase in OCA and metabolite exposure led to ALT and AST elevations following daily OCA doses greater than 50 mg; a 5-fold increase in OCA and metabolite exposure triggered ALT and AST elevations at daily OCA doses greater than 25 mg; and a 10-fold increase in OCA and metabolite exposure predicted ALT and AST elevations at all OCA doses. At daily OCA doses of 25 mg, 5-fold and 10-fold increases in OCA and metabolite exposure resulted in ALT/AST elevations in 3.8%/4.2% and 21.3%/20.9% of virtual MASLD subjects, respectively. Mechanistic analysis revealed that bile acid transporter inhibition alone reproduced most biomarker elevations, with minimal contribution from mitochondrial uncoupling. Liver bile acid concentrations were elevated in virtual patients with increased liver injury biomarkers. **Conclusions:** Simulations in virtual MASLD patients predicted dose-dependent increases in ALT and AST at supratherapeutic OCA doses. At the clinically relevant daily OCA doses of 10 and 25 mg, biomarker elevations were observed after 10- and 5-fold increases in OCA and metabolite exposure, respectively. Bile acid transporter inhibition accounted for

most of the predicted increases in ALT and AST. Our findings indicate that DILLsym modeling would have predicted the elevations in liver injury biomarkers observed in the MASLD clinical trials. *Funded by NIH grant R35 GM122576*

PS 4608 Comparative 28-day mouse study of topically applied aryl hydrocarbon receptor ligands: microbial-derived indoles, therapeutic tapinarof, pollutants 2,3,7,8-tetrachlorodibenzo-p-dioxin and diesel exhaust particles

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Background and Purpose: The aryl hydrocarbon receptor (AHR) regulates skin responses to microbial, therapeutic, and pollutant ligands in chronic inflammatory psoriasis, atopic dermatitis (AD), and chloracne diseases. While AHR activation has been linked to both cause and treatment of skin diseases, the comparative effects of distinct ligand classes remain unclear. Here, we evaluated systemic and cutaneous outcomes of topical exposure to different AHR ligands. **Methods:** Male and female C57BL/6J mice were topically treated with microbial-derived indole-3-pyruvic acid (1% IPY), indole-3-aldehyde (0.1% IAlD), therapeutic, tapinarof (1% TAP), or pollutant 2,3,7,8-tetrachlorodibenzo-p-dioxin (3E-06% or 3E-07% TCDD) or diesel exhaust particle (0.1% DEP), for 5 day/week for four consecutive weeks. Systemic effects, skin condition and atopic dermatitis- and chloracne-like outcomes were assessed. **Results:** Weight gain was largely unaffected. High-dose TCDD showed signs of limited focal hepatic changes, suggesting early hepatocellular injury, and DEP elevated serum bile acids. No consistent effects were observed on kidney histology or other serum analytes. Trans epidermal water loss, ear thickness, scratching and grooming were unchanged. DEP worsened the overall skin condition index. Both TAP and TCDD strongly induced *Cyp1a1* and *Cyp1b1*, while other treatments produced transient effects. TAP and high-dose TCDD elevated desquamation and presented with a chloracne-like pathology characterized by sebaceous gland involution, reduced numbers of sebocytes, hyperkeratosis, and cyst-formation. While these chloracne-like effects appeared to be similar, quantitative image analysis demonstrated that TCDD caused epidermal atrophy, whereas TAP resulted in epidermal hyperplasia with thickening extending into the infundibulum, along with hyperkeratosis. RNA analysis revealed that TAP selectively elevated levels of the *Nrf2* target genes, *Epgn*, *Sprr2d*, and *Slpi*, previously reported to drive the chloracne response to transgenic epidermal overexpression of *Nrf2* in mice. **Conclusions:** Overall, ligand effectiveness appears to drive compound-specific effects. Equally effective doses of TAP and TCDD elicited related effects of a chloracne-like response, yet selective differences in the modulation of *Nrf2* appear to impart unique, TAP-specific, phenotypes. These findings highlight AHR ligand-specific skin effects, in part, associated with differences in selective ligand activation of modifying signaling pathways.

PS 4609 Single-cell analysis of aryl hydrocarbon receptor-dependent effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on regional and cell specific changes in the pilosebaceous unit of murine skin

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Background and Purpose: The aryl hydrocarbon receptor (AHR) is a transcription factor that is activated by 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD), a persistent environmental pollutant. Human exposure to dioxin causes chloracne, characterized by acanthosis, hyperkeratosis, sebaceous gland involution, and hamartomas. While mice were believed to be refractory to chloracne, recent studies report similar chloracne-like phenotypes in mice. One study showed that *Lrig1*+ progenitor cells colocalized with TCDD-mediated CYP1A1 induction, indicating that TCDD may target specific cells to cause sebaceous gland (SG) involution. To better understand the effects of TCDD on the skin *in vivo*, we analyzed single cell mRNA expression in wild-type and *Ahr*-null mice. **Methods:** Wild-type (C57BL/6J) and *Ahr*-null (B6.129-*Ahr*^{tm1Bra}/J) mice were used in accordance with approved IACUC protocols. In the developmental study, dams were treated by oral gavage with vehicle (corn oil) or TCDD (5µg/kg body weight) on E12, and pups were exposed *in utero* and through lactation until P21. In the short-term dose-response study, C57BL/6J mice were treated topically once per day for 3 days with either vehicle (DMSO:Acetone, 1:9) or TCDD (3E-07%, 3E-06%, or 3E-05%), and tissues were collected 24 hours after the last treatment. Single cell analysis was performed using a RNAscope multiplex *in situ* hybridization protocol. **Results:** Histological analysis of the developmental P21 skin demonstrated that TCDD-mediated sebaceous gland atrophy was prominent, and *Ahr*-dependent. Regional analysis of *Cyp1a1* and *Cyp1b1* showed that TCDD markedly increased *Cyp1a1* in the lower part of infundibulum near the junctional zone where *Lrig1*+ stem cells reside. This increase was *Ahr*-dependent and consistent with reports of protein induction. Compared to *Cyp1a1*, expression of *Cyp1b1* was lower in this region, but significantly elevated. A second prominent region of *Cyp1a1* was present in the lower cells of the SG. Here, *Cyp1a1* was markedly induced in both sebocytes and the basal

epithelial cells of the SG, and expression was *Ahr*-dependent. Together these observations are suggestive of TCDD affecting both gland morphogenesis and sebocyte differentiation. To explore these regional effects a 3-day dose-response study was performed. The response to TCDD appeared slightly more dose-sensitive in the SG compared to the infundibulum. Within the SG, dose-dependent increases in *Cyp1a1* expression were observed in the more differentiated cells. To determine whether TCDD affected cellular differentiation in the SG, the expression of *Scd1*, a marker of sebocyte differentiation, was measured. High-dose TCDD increased the expression of *Scd1* by an area of 25%, indicating that TCDD increased cellular differentiation. This was further demonstrated by Oil Red O staining, that showed an increase in lipid levels in the epidermis. To explore SG morphogenic mechanisms, we analyzed *Lrig1*, *Myc*, and *Blimp1*. *Blimp1* is a transcriptional repressor responsible for regulating sebaceous gland size, and *Myc* is a transcription factor believed to both mark and drive SG epithelial morphogenesis and the formation of sebocytes. *Blimp1* was induced by TCDD in the cells of the lower infundibulum. Results from the P21 study demonstrated that this increase was *Ahr*-dependent. Interestingly, the epithelial cells of the junctional zone expressing *Lrig1* and *Blimp1* were apical to the cells expressing *Blimp1* and *Myc*, which were located above the cells expressing only *Myc*. In the less differentiated abapical sebocytes both *Myc* and *Blimp1* were expressed, while the terminally differentiated sebocytes expressed only *Blimp1*. TCDD appeared to increase the number of *Myc* positive cells and the levels of *Blimp1* in terminally differentiated cells. **Conclusions:** Either systemic or topical exposure to TCDD affects the sebaceous gland and infundibulum. Regional and single cell analysis of SG involution indicates an initial increase of sebocyte differentiation as an early event. Expression of *Blimp1* appears to act as an intermediary modulator of cell transitions from SG stems cells to SG epithelial cells to sebocytes. Overall, these findings provide insights into the effects of TCDD on the sebaceous gland.

PS 4610 An Investigational Study Aimed at Establishing the Respiratory Minute Volume of the Göttingen Minipig

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Background and Purpose: An appropriate estimation of respiratory minute volume (RMV) is essential for pre-clinical inhalation toxicology research as it defines inhaled dose and enables interspecies extrapolation. Göttingen Minipigs are increasingly used as a non-rodent model due to their anatomical and physiological similarity to humans and ethical advantages over dogs and non-human primates. However, limited reference data exist for their RMV, and current equations, such as Alexander et al. 2008, and Bide et al. (2000), were developed from other species. **Methods:** Respiratory minute volumes of Göttingen Minipigs were measured and compared with predictions from established allometric equations used in inhalation toxicology. Eight naïve Göttingen Minipigs were acclimated for 14 days to the respiratory restraint system before measurement. Respiratory rate (RR), tidal volume (TV), and minute volume (MV) were recorded during the 14-day acclimation phase and then weekly over a 13-week period. No test or control items were administered. Body weight measurements were taken weekly and minute volumes were compared to the current inhalation equations. **Results:** Respiratory parameters remained consistent throughout, with growth-related shifts in tidal volume and respiratory rate producing stable minute volumes. Existing predictive equations underestimated measured RMVs, although the AIT equation aligned more closely with observed data. A short acclimation period provided reliable results, confirming the Göttingen Minipig as a suitable and stable model for inhalation studies. **Conclusions:** The difference in measured vs calculated RMV was comparable across species, including minipigs. The equation proposed by Alexander et al. resulted in smaller % differences compared to the formula proposed by Bide et al. The Alexander et al. formula is widely accepted in the industry despite differences relative to the actual/measured RMV values. As such, RMV in Minipigs can also be estimated using the Alexander et al equation.

PS 4611 Polyvinyl Chloride Nanoparticles Induce Mitochondrial Dysfunction in Human-Induced Cardiomyocytes

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Background and Purpose: Polyvinyl chloride (PVC) is a highly toxic plastic that continuously releases harmful compounds throughout its lifecycle, which can bioaccumulate and pose serious cardiovascular risks. PVC plastics have been detected in multiple human organs, including the heart, liver, and lungs, as well as in carotid plaques, with their abundance positively correlating with increased mortality in patients with cardiovascular disease. These micro-plastics (MPs) and nano-plastics (NPs) can enter the body via ingestion, inhalation, or dermal exposure, accumulate in tissues, and disrupt cellular function. Experimental studies show that PVC particles damage cardiac mitochondria, inducing oxidative stress, abnormal gene expression, and apoptosis. However, the precise cellular and molecular mechanisms of PVC-NPs-induced cardiotoxicity remain unclear. This study aims to investigate these mechanisms, focusing on mitochondrial dysfunction and autophagy/mitophagy dysregulation, to provide novel insights into cardiovascular risks posed by environmental PVC particles and inform strategies for prevention and health risk

assessment. **Methods:** Cardiomyocytes derived from human stem cells were used as an alternative *in vitro* model to investigate the toxic effects of PVC-NPs. They were exposed to PVC-NPs of different sizes and concentrations to assess cell viability, intracellular ROS levels, and types of cell death (by flow cytometry). Contractile function was evaluated using live-cell imaging and calcium handling properties were assessed by Fluo-4 calcium imaging. Mitochondrial function and morphology were analyzed via Seahorse XF assays and immunofluorescence staining. Mitophagy was further assessed by p62 colocalization (immunofluorescence) and the expression of autophagy-related proteins (LC3-I/II and p62) by western blot. **Results:** PVC-NPs exhibited size- and concentration-dependent cytotoxicity in cardiomyocytes, and they caused impaired cardiac functions, such as reduced contractility, and irregular beat frequency. Moreover, PVC-NPs induced remarkable mitochondrial dysfunction and fragmentation. The PVC-NP-treated cardiomyocytes exhibited altered LC3-II/LC3-I ratios and increased p62 expression, suggesting disrupted autophagy/mitophagy. **Conclusions:** PVC-NPs significantly impair human-derived cardiomyocyte function, mitochondrial integrity, and autophagic activity. These findings provide important insights into the cardiovascular hazards posed by environmental plastic particles and offer a foundation for refined risk assessment, public health decision making, and the development of strategies to mitigate their adverse impacts on human health.

PS 4612 In The Zone: A Transcriptomics Approach To Understanding Zone-Selective Fatty Liver Disease

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Background and Purpose: Metabolic dysfunction-associated steatotic liver disease (MASLD), also known as non-alcoholic fatty liver disease, is a growing public health concern across the United States. In clinical cases and rodent models, MASLD has been shown to develop in a zone-selective manner relating to the centrilobular and periportal regions of the liver lobule. While the underlying mechanisms behind this zone-selectiveness are unknown, it is influenced by both toxicants and dietary factors. Based on previous research, it's suggested that centrilobular steatosis disrupts the hepatic packaging and transport of lipids, which is characteristic of disruption of the peroxisomal β -oxidation pathway and activation of the PPAR α receptor. It has also been shown that portal steatosis disrupts the metabolism of lipids in the liver, which corresponds to disruption of the one-carbon metabolism (OCM) pathway. This study tests our hypothesis that zone-selective steatosis is driven by distinct mechanisms either disrupting peroxisomal β -oxidation pathway or repression of rate limiting enzymes in the OCM pathway. **Methods:** To investigate the mechanisms of zone-selective steatosis, Bulk RNA sequencing (RNAseq) was performed on livers from two toxicant and two diet-induced steatosis models. Male C57BL/6J mice were treated with 0.3 or 1 mg/kg/day PFOS in drinking water (0.5% Tween-20 vehicle) or fed a high fat (GAN) diet to model centrilobular steatosis. To model periportal steatosis, mice were gavaged with 3 or 10 μ g/kg 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) every four days or fed a methionine-choline deficient (MCD) diet. **Results:** Clinical chemistry data demonstrated that serum glucose decreased significantly in the MCD and high dose TCDD periportal models. Furthermore, all three portal models significantly increased serum alanine aminotransferase (ALT) suggesting more severe hepatic injury compared to the central models. Gene expression analysis found 844 unique differentially expressed genes (DEGs) common to both periportal steatosis models, and 43 unique DEGs common to both centrilobular steatosis models. Gene set enrichment analysis (GSEA) found induction of β -oxidation pathway genes in the centrilobular models, while the portal models showed overall repression. In addition, genes *Acaa1b* and *Inmt* both showed induction in central steatosis models and repression in the portal models representing putative markers of zone-selective steatosis. While we did not see a significant overall change to OCM, portal repression of genes reliant on OCM like *Inmt*, *Bhmt*, and *Gnmt* suggests downstream metabolic disruption. **Conclusions:** Our findings suggest differences in disruption of the β -oxidation pathway between models of periportal and centrilobular steatosis, and repression of metabolic pathways reliant on OCM, reflect a putative distinguishing signature for zone-selective steatosis induced by either toxicants or dietary factors.

PS 4613 Mapping Heterogeneity of Hepatic Zonation in Genetically Diverse Mice Using Single-Nuclei Transcriptomics

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Background and Purpose: The liver plays a central role in maintaining physiological homeostasis through nutrient metabolism, detoxification, and energy balance. It is a principal site of xenobiotic metabolism, and a primary target of drug and chemical induced toxicity. The liver's functions are compartmentalized across the hepatic lobule, known as zonation, which is the spatial metabolic and functional organization along periportal and pericentral regions shaped by nutrient and signaling gradients. Hepatic lipids can accumulate in zonal patterns, and previous studies indicate that

genetics plays a significant role in the development of steatotic liver disease (SLD); however, limited work has examined how genetic variation influences global portal-to-central gene-expression patterns. We hypothesized that genetic variation modulates spatial cellular heterogeneity in the liver, thereby influencing the zonation of metabolic pathways, and the susceptibility to toxicant-induced steatosis. **Methods:** To investigate the role of genetic diversity on hepatic zonation we used single-nucleus RNA sequencing (snRNA-seq) to characterize zonal expression in hepatocytes. Four genetically diverse mouse strains were used: two from the Collaborative Cross (CC) mouse diversity panel (CC005 and CC042), and two founder strains for the CC mice (A/J, C57BL/6J). Untreated mice, following 15 days acclimation with free access to water and standard chow, were used to establish zonal differences using snRNAseq. A second cohort was used to evaluate the susceptibility to toxicant-induced hepatic steatosis using the same CC strains treated with environmental contaminant and steatogenic agent perfluorooctane sulfonate (PFOS; 1 mg/kg/day) for 42 days. **Results:** A total of 10,830 cells were captured across all mice, and annotation identified most major hepatic cell types as expected primarily consisting of hepatocytes (70-80%). Differential expression analysis of hepatocytes identified 1,346 differentially expressed genes (DEGs) in A/J, 1,050 in CC005, and 1,068 in CC042 mice compared to C57BL/6J mice (lfold-change ≥ 1.5 , padj ≤ 0.05). Gene set enrichment analysis identified enrichment genes from the lipid metabolism, immune signaling, and nucleic acid processing pathways. Pseudospacial/trajectory analysis identified strain-dependent portal-to-central gradients, with genes involving immune regulation (*Tox*), extracellular matrix and adhesion (*Reln*), lipid metabolism (*Acly*), cytoskeletal and membrane architecture (*Sgcz*), and protease inhibition and the acute-phase response (*Serpina1e*). Moreover, compared to A/J and C57BL/6J, CC042 and CC005 exhibited attenuated periportal expression of *Pdzrn3* and increased pericentral expression of the regulatory lncRNA *Meg3*, both involved in *Wnt* signaling. In PFOS exposed cohort, CC042 mice showed evidence of greater liver injury compared to CC005 with increased serum ALT and hepatic lipid accumulation. **Conclusions:** Collectively, snRNAseq analysis demonstrates that genetically diverse mouse models show differences in zonal expression and PFOS injury. This heterogeneity may contribute to variation in the development and progression of hepatic steatosis.

PS 4614 Developmental TBBPA exposure exacerbated western diet-induced metabolic inflammation

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Background and Purpose: Metabolic liver diseases are increasing in global prevalence and have traditionally been linked to nutritional imbalances, such as a high-fat western diet (HFD). However, the pathogenesis of complex metabolic disorders is rarely caused by only one risk factor. Early life exposure to environmental stressors is an emerging contributor to the delayed onset of metabolic diseases later in life. Tetrabromobisphenol A (TBBPA) is currently the most widely used brominated flame retardant worldwide. Exposure to TBBPA is associated with altered carbohydrate and lipid metabolism, and immune modulation, which are hallmarks of metabolic fatty liver diseases. The pregnane X receptor (PXR) and constitutive androstane receptor (CAR) are important liver-enriched nuclear receptors predicted to be activated by TBBPA and are involved in energy and xenobiotic metabolism through key targets, such as cytochrome P450 3A4 (CYP3A4). Little is known regarding the contribution of TBBPA in altering metabolic and immunological dysfunction in the liver. We hypothesized that early life TBBPA exposure exacerbates HFD-induced metabolic dysregulation. **Methods:** Female humanized (h) PXR/hCAR/CYP3A4/CYP3A7 (a fetal isoform of CYP3A4) dams, from 2 days before mating, were dosed with corn oil (vehicle, 10 ml/kg) or TBBPA (0.2 mg/kg) once daily until pups were weaned at postnatal day (PND) 21. Pups were placed on either a control diet or HFD until 6 months of age. Phenotypic outcomes, including body and liver weight, serum lipids, triglycerides, circulating glucose, and glucose tolerance, were measured. Single-cell RNA sequencing (scRNA-seq) of male pups was conducted to profile cell type-specific responses from early life TBBPA exposure following HFD. **Results:** TBBPA exposure exacerbated HFD-induced metabolic alterations, particularly in males, increasing body weight, liver-to-body weight ratios, and circulating cholesterol levels. ScRNA-seq showed that early life TBBPA exposure upregulated PXR transcripts in hepatocytes. Early life TBBPA exposure dysregulated intermediary metabolism and immune response in liver cell types, with a shift towards a more proinflammatory state after HFD. For example, in hepatocytes, early life TBBPA exposure upregulated genes involved in amino acid metabolism and downregulated genes related to reactive oxygen species response. Upon HFD, early life TBBPA exposure upregulated genes involved in oxidative stress and microbial influx and downregulated genes involved in drug and amino acid metabolism. Moreover, early life TBBPA exposure and upregulated genes involved in tumor necrosis factor (TNF) production, antigen presentation, and angiogenesis in non-parenchymal cells, such as Kupffer cells, monocyte-derived macrophages, endothelial cells, and dendritic cells. **Conclusions:** In summary, early life TBBPA exposure reprogrammed the hepatic immune-metabolic network in a cell type-specific manner associated with worsened HFD-induced metabolic dysfunction. Our findings highlight the metabolic toxicity of environmental toxicants, which may act as environmental contributors of complex metabolic diseases. The mechanistic insight from our results can help develop mitigation and therapeutic strategies to decrease the internal burden of environmental toxicant exposures at critical developmental timepoints.

PS 4615 Muscle-Protective Effects of *Cryptotaenia japonica* Extract Against Glucocorticoid-Induced Sarcopenia in Cellular and Animal Models

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Background and Purpose: Sarcopenia, a progressive loss of skeletal muscle mass and function, is frequently exacerbated by chronic glucocorticoid exposure. Although *Cryptotaenia japonica* has been traditionally used for its antioxidant, anti-inflammatory, and metabolic regulatory properties, its muscle-protective potential remains largely unexplored. Despite growing interest in botanical agents that modulate muscle homeostasis, studies evaluating *C. japonica* in the context of sarcopenia are limited. This study aimed to investigate the myoprotective effects of *C. japonica* using both *in vitro* and *in vivo* experimental models. **Methods:** Aerial parts extract of *C. japonica* (CJE) was applied to C2C12 myotubes at 10–100 µg/mL with or without dexamethasone (Dex, 10 µM). Expression of mTOR, MuRF-1, Atrogin-1 (FBX32), and FOXO1 was assessed by Western blotting and confocal microscopy. *In vivo*, Dex-induced muscle atrophy was established in ICR mice, which received oral CJE at 200 mg/kg/day. In a separate non-atrophic model, male Sprague-Dawley rats were administered CJE at 50 mg/kg/day for 5 consecutive days without Dex treatment. In both models, atrophy-related proteins including FOXO3a, MuRF-1, and Atrogin-1 were evaluated specifically in the calf muscle. Planned analyses also include histological assessment of calf muscle fiber cross-sectional area (H&E) and serum measurements of IGF-1 and GDF-8. **Results:** CJE preserved C2C12 myotube morphology and significantly attenuated Dex-induced upregulation of MuRF-1 and Atrogin-1. CJE also inhibited nuclear translocation of FOXO1, indicating suppression of FOXO-mediated catabolic signaling. *In vivo*, CJE reduced Dex-induced increases in FOXO3a, MuRF-1, and Atrogin-1 in the calf muscle, demonstrating inhibition of proteolytic pathways. Histological findings indicate that CJE contributes to the preservation of muscle fiber architecture, and serum analyses confirmed enhanced IGF-1-related anabolic activity together with reduced GDF-8 levels. **Conclusions:** The aerial parts extract of *Cryptotaenia japonica* effectively mitigates glucocorticoid-induced muscle atrophy by modulating the mTOR-FOXO signaling axis and suppressing ubiquitin-proteasome-mediated protein degradation in the calf muscle. These results support CJE as a promising natural therapeutic candidate for preventing sarcopenia and muscle-wasting conditions.

PS 4616 Comparing Models of Zone-Selective Steatosis Using Spatial Transcriptomics

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Background and Purpose: Environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and perfluorooctane sulfonate (PFOS) are known to induce metabolic dysfunction-associated steatotic liver disease (MASLD), characterized by lipid droplet accumulation in hepatocytes (steatosis). The liver exhibits functional and metabolic zonation along the periportal-to-centrilobular axis, and the development of steatosis shows zone selectivity dependent on the model. However, the mechanisms and consequences of this zonal development remain unclear. With increasing evidence that toxicants and dietary factors drive zone-selective disease, and the existence of multiple MASLD subtypes, we hypothesized that toxicant-induced spatially resolved gene expression patterns drives zonal selectivity of steatosis and may correspond to distinct subtypes. **Methods:** Male C57BL/6J mice were treated with PFOS (1 mg/kg/day) in 0.5% Tween-20 water, orally gavaged with TCDD (3 µg/kg) in sesame oil (every 4 days) for 84 days or fed either the Gubra Amylin NASH (GAN; 12 weeks) or control diet to represent models of zone-selective steatosis. Spatial transcriptomics was performed on liver cryosections, and sequencing data were analyzed using Python and R tools. The Robust Cell Type Decomposition (RCTD) R package was used to deconvolute cell types and annotate each spatial transcriptomics spot using a scRNAseq reference dataset. Moran's I statistic was used to assess global spatial autocorrelation and identify spatially variable genes (SVGs). STAGATE, a graph attention autoencoder framework, was used to define spatial domains based on neighbourhood similarity among spots. **Results:** An average of 120,073 on-tissue 10 µm spots were detected per tile, representing 13,345 average genes. RCTD identified 11 expected cell types, dominated by periportal (~50.7%) and centrilobular (~19.6%) hepatocytes. The proportion of central hepatocytes increased, and portal hepatocytes declined for all treatments. Moran's I identified canonical zonal marker genes for portal (*Cyp2f2*, *Ass1*, *Arg1*) and central (*Glu1*, *Cyp2e1*, *Oat*) hepatocytes as the most significant SVGs, followed by *Saa1*, *Gstm3*, and *Aldh3a2*. Comparison of pseudo-bulk differentially expressed genes (DEGs), bulk RNA-seq DEGs, and Moran's I SVGs revealed 26, 16, and 21 concordant genes for GAN, PFOS, and TCDD, respectively, reflecting spatially resolved expression of genes altered by treatment. Differential expression in STAGATE-defined central and portal neighbourhoods revealed zone-specific and treatment-dependent gene regulation including *Cyp2c50* induced in portal regions but repressed in central regions by the GAN diet, *Anxa2* with opposing central-portal patterns between PFOS and

GAN, and *Rac2* upregulated in central but downregulated in portal regions by TCDD. **Conclusions:** Altogether, these findings support the hypothesis that zone-selective steatosis results in spatially distinct changes in gene expression patterns. These zone-specific molecular signatures likely represent distinct mechanisms of disease progression, contributing to the molecular heterogeneity that may underlie MASLD subtypes.

PS 4617 Sex-Stratified Transcriptomic Analysis Identifies Cholesterol Metabolism Dysregulation in Alzheimer's Disease

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Background and Purpose: Alzheimer's disease (AD) exhibits clear sex-specific differences in prevalence, progression, and metabolic dysfunction. Despite this distinctive pattern, clinical trials have not systematically considered sex as a confounding factor influencing therapeutic outcomes. Studies have shown that there are sex-specific lipid alterations in AD. Women with AD show greater reductions in unsaturated lipids and differential disruption of cell membrane lipids and bioactive lipids, such as phosphatidylcholine and sphingolipids. The brain contains 25% of total body cholesterol, and alterations in cholesterol homeostasis are associated with increased tau hyperphosphorylation and amyloid beta production. Therefore, cholesterol metabolism is considered a critical pathway in AD pathogenesis. The sex-specific differences in lipid and cholesterol metabolism are not fully understood and need further investigation. This study hypothesizes that AD pathogenesis involves sex-specific disturbances in lipid and cholesterol metabolism and requires different therapeutic targets for males and females to achieve optimal treatment outcomes. **Methods:** In this study, we leveraged post-mortem brain transcriptomic data from the temporal cortex comprising 148 samples (80 AD patients (49 females, 31 males) and 68 controls (33 females, 35 males)). The data was acquired from the AD Knowledge Portal. We performed differential gene expression analysis using the Limma package in R. We conducted three separate analyses: (1) AD vs Control in females, (2) AD vs Control in males, and (3) a sex-by-diagnosis interaction analysis to identify sex-specific disease effects. We stratified all differentially expressed metabolic genes ($\log_2FC > 0.2$, $FDR < 0.1$) into three categories: (1) sex-specific genes only altered in one sex; (2) sex-neutral genes altered in both sexes with no statistical difference; and (3) sex-differential genes altered in both sexes, with statistical difference. Gene-set enrichment analysis of sex-specific genes was performed using the ShinyGO tool. **Results:** Transcriptomic analysis identified 5383 and 4883 differentially expressed genes in females and males, respectively. Of these, 515 metabolic genes were significant in females, and 438 in males. Based on our defined categories, 117 metabolic genes were female-specific, and 51 were male-specific. Enrichment analysis of these sex-specific genes identified lipid metabolism enriched in both males and females, including cholesterol metabolism and steroid biosynthesis. Cholesterol metabolism genes exhibited sex-specific expression patterns: *CYP46A1* (downregulated, female-specific), *CYP27A1* (upregulated, female-specific), while *CYP7B1* and *HMGS1* were male-specific (downregulated). Efavirenz, an FDA-approved drug for HIV treatment, targets *CYP46A1*. Studies have shown that low dose of efavirenz could be used as disease-modifying therapeutic option for AD. Our transcriptomic analysis, combined with drug target identification, suggests that efavirenz can be repositioned for therapeutic intervention in female AD patients. **Conclusions:** Our results show that females exhibit greater metabolic dysregulation than males, with lipid and cholesterol metabolism identified as sex-distinguishing pathways in AD. Using a systems toxicology approach, by integrating transcriptomics and metabolic pathway analysis, we identified *CYP46A1* as a female-specific target with mechanistic implications for cholesterol regulation. The identification of efavirenz, a *CYP46A1* activator, demonstrates the application of systems toxicology in translating molecular insights into therapeutic strategies for drug repurposing and designing sex-specific treatment options in AD.

PS 4618 Single-Cell Profiling of Normal Development and ATRA-induced Teratogenicity in Rat Whole Embryo Culture

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Background and Purpose: The rat post-implantation whole embryo culture (WEC) model enables direct assessment of environmental chemical exposures on embryogenesis during the critical windows of neurulation and early organogenesis. Gestational day (GD) 10 rat embryos are cultured *ex vivo* for 48h and evaluated for growth parameters, developmental progression, and specific anomalies. Recent advances in single-cell transcriptomic profiling now provide unprecedented resolution in defining how teratogens affect key cell populations, developmental trajectories, and underlying gene expression. **Methods:** In this study, we characterized the *ex vivo* development of rat embryos via morphological analysis and bulk and single-cell RNA sequencing under control and teratogenic conditions, using all-trans retinoic acid (ATRA) as a prototypical toxicant. **Results:** Embryos cultured under normal conditions exhibited dynamic morphological changes, recapitulating *in vivo* development, successfully completing neural tube formation and early development of multiple organs, including ear, eye, heart, and limb. These morphological changes were accompanied by global transcriptomic changes, with 731 differentially

expressed (DE) genes identified over 0-48h (561 upregulated, 170 downregulated; $p < 0.0001$, absolute fold change >1.25), enriched for nervous system development, tube formation, organ morphogenesis, embryonic patterning, and circulatory system development. At the single-cell level, embryos profiled at 0, 4, 12, 24 and 48h ($n=4$ embryos per timepoint), revealed 36 distinct cell populations representing all three germ layers. Significant shifts in cell types were evident across the time course. Prior to neural tube closure (0-12h), primitive neuroectodermal cells—neuroectoderm (NE), neuromesodermal progenitors (NMPs), rostral and caudal NE, and floor plate cells—were more abundant. By 24-48h, these progenitors transitioned toward more differentiated populations, including neural progenitor cells, post-mitotic CNS neurons, peripheral neurons/neural crest derivatives, craniofacial ectoderm and non-neural epithelium, with parallel temporal differentiation patterns observed in mesodermal and endodermal lineages. To model teratogenic perturbation, embryos were exposed to $1.7\mu\text{M}$ ATRA ($\sim 20\%$ reduction in TMS) or 0.1% DMSO and analyzed at 4 and 48h. ATRA exposure caused cell-identity shifts as early as 4h, including depletion of craniofacial ectoderm, NE and rostral NE, and expansion of neural crest, NMPs and craniofacial mesoderm. By 48h these effects intensified, with marked loss of NMPs, fore/mid/hindbrain NE, and somite populations, and increases in PNS neurons, lateral plate mesoderm, and cardiomyocytes. At the gene-expression level, ATRA altered expression of patterning and hindbrain regulators, neuroectodermal markers, and mesodermal differentiation genes, along with enzymes critical for ATRA metabolism, in a cell type-specific manner. **Conclusions:** The results identify neuroectodermal, fore/mid/hindbrain neural, and mesodermal populations as particularly vulnerable to ATRA-induced dysregulation. These findings align with ATRA's known ability to disrupt embryo patterning, neural tube and CNS morphogenesis, and craniofacial and mesodermal development, and highlight the power of single-cell approaches to pinpoint specific cell types and gene networks driving teratogenic outcomes.

PS 4619 Alcohol impairs TFEB-mediated autophagy while exacerbating inflammatory response in the aged liver

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Background and Purpose: Alcohol-associated liver disease (AALD) is a major cause of chronic liver disease. Cells protect themselves by activating protective mechanisms like autophagy. Aging is a well-known risk factor associated with AALD. Alcohol metabolism and cellular aging induce hepatic inflammation. Inflammatory signaling can be stunted by autophagic processes. Though TFEB autophagy is impaired by alcohol consumption, the role of autophagy and aging in the development of AALD is not completely understood. This study investigates changes in TFEB-regulated autophagy and its role in mitigating inflammation in alcohol-induced liver injury in aged mice. **Methods:** Young (3-month-old) and aged (22-month-old) C57BL/6N mice were subjected to chronic feeding plus acute ethanol binge model (Gao-binge model). Unbiased liver tissue RNAseq and metabolomics were performed. Tail-vein injection of adenovirus-TFEB was performed to overexpress hepatic TFEB in aged mouse livers with Gao-binge alcohol feeding. Liver injury and steatosis were determined. Western blot analysis and qPCR were performed to assess the mRNA and protein levels of autophagic machinery and inflammatory mediators. **Results:** PCA analysis of RNAseq and metabolomics showed distinct separation of four experimental groups, suggesting both aging and alcohol have specific effects on hepatic gene expression and metabolic changes. Hepatic triglycerides increased in ethanol-fed aged mice compared with the young mice fed control diet. Hepatic TFEB decreased but levels of autophagic proteins such as p62 and LC3-II increased in ethanol-fed aged mice. Protein expression of LAMP1 and ATP6v1B2 decreased in ethanol-fed aged mice compared to young, control diet-fed mice. Several inflammatory pathways and cGAS-STING genes were upregulated in aged, alcohol-fed mice. TFEB overexpression in aged, alcohol-fed mice led to decreased ALT, increased lysosomal proteins, and decreased cGAS signaling. **Conclusions:** Aging was found to exacerbate alcohol-induced hepatic steatosis despite promoting increases in liver injury markers of ALT. Additionally, aging led to further impairment of autophagy and promoted inflammatory signaling induced by alcohol consumption.

PS 4620 High-Throughput, Extraction-Free miRNA Profiling for Toxicological Applications

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Background and Purpose: MicroRNAs (miRNAs) are post-transcriptional regulators of gene expression, and circulating miRNAs offer a non-invasive means to monitor tissue-specific injury. Because distinct miRNA expression patterns often reflect the tissue of origin and the nature of toxic insults, they provide valuable mechanistic insights into cellular processes such as apoptosis, inflammation, and oxidative stress. These properties make miRNAs powerful tools for monitoring safety risks in preclinical and clinical studies. Integrating miRNA expression profiles with other omics data further enhances understanding of toxicity pathways and supports the identification of potential therapeutic targets. Although small RNA-seq is used for miRNA profiling, it requires RNA purification, larger sample inputs, and incurs higher cost than TempO-Seq platform-based assays, which are target-based and do not require RNA purification. The goal of this study was to develop a targeted

miRNA assay that eliminates the need for RNA extraction, accommodates flexible sample formats, and enables rapid, cost-effective profiling of miRNAs. The simplified sample processing, combined with reduced data complexity, enables more reliable detection of cell type- or disease-specific miRNA signatures. **Methods:** The TempO-Seq Whole miRNome assay, built on established TempO-Seq technology, is a targeted platform designed to profile 2,112 human miRNA targets derived from miRBase v22.1. Because of the short length of miRNA targets, optimized probe design strategies and specialized enzymatic reactions were implemented to ensure robust performance. Initial validation was performed using purified RNA reference materials cited in benchmarking studies comparing miRNA profiling platforms. The assay was then adapted to an extraction-free workflow compatible with a wide range of sample types, including cell and FFPE lysates, plasma, and dried blood spots (DBS). To demonstrate biological and translational relevance, miRNA expression changes were characterized in two breast cancer cell lines, MCF7 and MDA-MB-231, following rapamycin treatment ($0.1\mu\text{M}$, $1\mu\text{M}$, $10\mu\text{M}$) for 24 hours, in parallel with TempO-Seq whole transcriptome analyses. Furthermore, the miRNA assay was performed on DBS samples from individuals with Alzheimer's disease and matched controls. **Results:** The TempO-Seq Whole miRNome panel is the most comprehensive set in any targeted miRNA profiling platform to date. Performance was first benchmarked using Universal Human miRNA Reference RNA, human brain RNA, and defined mixtures of the two. When compared with published data from other commercial assays (EdgeSeq, TruSeq), the TempO-Seq miRNA assay demonstrated excellent reproducibility among replicates ($R^2=0.97$) and comparable accuracy (titration response) while requiring substantially less input. The extraction-free workflow showed robust gene detection and high reproducibility across diverse and challenging sample types, including plasma, FFPE, and DBS, simplifying handling and reducing overall cost. Differential expression analysis of MCF7 and MDA-MB-231 cells treated with rapamycin revealed dose-dependent miRNA and mRNA changes consistent with mTOR pathway modulation. MCF7 cells exhibited stronger transcriptional and miRNA-level responses than MDA-MB-231 cells, aligning with known differences in rapamycin sensitivity. Finally, miRNA profiles obtained from DBS samples of individuals with Alzheimer's disease and controls demonstrated visible separation by unsupervised clustering, indicating the assay's potential for distinguishing disease states using minimally invasive specimens. **Conclusions:** The TempO-Seq miRNA assay provides a robust, extraction-free solution for high-throughput miRNA profiling across multiple sample types. The assay achieves accuracy and reproducibility comparable to established platforms (EdgeSeq, TruSeq) while requiring lower input and offering more cost-effective workflows. Combined miRNA and mRNA analyses in rapamycin-treated breast cancer cell lines confirmed expected pathway-level responses, with greater compound sensitivity observed in MCF7 cells. The distinct clustering of Alzheimer's disease DBS samples from controls further highlights its translational and diagnostic potential. Overall, this targeted miRNA assay expands the utility of TempO-Seq technology, enabling mechanistic and biomarker studies in both toxicological and disease contexts.

PS 4621 Your clues are in the proteins: enhancing toxicology insights with proteomics

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Background and Purpose: Investigating proteome-wide changes upon treatment with therapeutic candidate(s) may provide mechanistic insights to understand adverse events (AEs). Thus, creating a proteomics workflow that captures a broad spectrum of proteins is needed. Focusing on Wistar Han rats (a predominant species used in early toxicity testing) and the liver (a key organ in drug metabolism), we developed a robust platform that generates informative proteomic data for toxicology studies. In-depth proteome-wide characterization, as well as gender differences in key pathways and enriched proteins are presented. **Methods:** For spectral library building, male and female Wistar Han (WH) rat livers ($n=3$ per gender) were prepared using S-Trap columns. Digested peptides were pooled by gender for offline fractionation to increase peptide coverage with greater sensitivity and accuracy. Data-dependent acquisition (DDA) for each fraction was performed on Bruker timsTOF and Thermo Vanquish Neo. DDA files were processed using Spectronaut to generate rat liver spectral libraries for each gender. For data-independent acquisition (DIA) method development, additional rat livers ($n=2$ per gender) were prepared using S-Trap columns. DIA methods were optimized using py_diaID to boost proteome coverage, and samples were run on the same instruments. DIA files were processed using Spectronaut for downstream protein analysis. In the case study example, female WH rats ($n=4$ per group) were dosed daily with aripiprazole (10mpk), rosiglitazone (20mpk), or vehicle (1% hydroxypropyl methylcellulose) for 28 days. Glucose tolerance test (GTT) was performed on day 27 after fasting. Livers were collected post-GTT for proteomics. **Results:** The spectral library offered in-depth comprehensive coverage for the rat liver proteome, featuring 11,388 proteins (265,024 peptides) for male and 10,144 proteins (199,846 peptides) for female. Proteins were identified across various subcellular components, including 27% membrane, 42% intracellular, 26% nuclear, and 6% extracellular proteins. Tailored DIA methods boosted protein identification, averaging over 100,000 peptides and 8,000 proteins with only 250ng of total peptides per run. Building a DDA-based spectral library is labor intensive and resource heavy. Under these circumstances, *in-silico* libraries are valuable alternatives. Our results showed that *in-silico* methods have a strong 0.9 Pearson correlation with the library-based DIA analysis, thus validating their utility when facing a pressing timeline for issue resolution. To further assess our proteomics workflow,

label-free quantification from naïve male and female rat livers identified differentially expressed proteins. Downstream pathway analysis offered insights into biological processes that may be enriched in one gender versus the other. For example, "ABC Transporters" was captured in female samples and featured four ATP-binding cassette (ABC) transporters that are liver-enriched and important for bile acid formation and detoxification [Abcb4, Abcg5, Abcc6, Abcc3]. On the opposite end, pathways enriched in males included "Retinol metabolism", which may be due to Cyp2a2 and Cyp3a18 which are known to be strongly expressed in males and are important for liver drug metabolism. This baseline analysis requires additional experimentation (such as hormonal manipulation or metabolic challenges) to further reveal potential male-female differences. As a final example on how our workflow can be applicable in safety, female Wistar Han rats were treated with rosiglitazone or aripiprazole to assess the liabilities of glucose intolerance and weight gain. Pathway enrichment analysis confirmed rosiglitazone's mechanism of action (upregulation in "PPAR Signaling Pathway"). Rats treated with aripiprazole exhibited impaired glucose tolerance, and proteomics captured downregulation of "Glycolysis/Gluconeogenesis". These insights validated the sensitivity and potential for proteomics to elucidate drug mechanisms and pathways. **Conclusions:** Our proteomics platform - from spectral library building to deep proteome profiling - achieved comprehensive coverage with high reproducibility and accuracy. Its application successfully identified differentially expressed proteins and enriched pathways, thus supporting mechanistic insights into drug effects. Ultimately, this approach offers a powerful tool to improve drug safety evaluation and hypothesis generation.

PS 4622 The Development of Gene Expression Modules from Rat Drug Response Studies Across Heart, Kidney, Liver, and Muscle

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Background and Purpose: RNA expression biomarkers can be leveraged to evaluate the potential safety liabilities of preclinical candidates and to understand the molecular mechanisms of toxicities observed during development. This *toxicogenomic* approach has traditionally relied on a small, pre-defined subset of genes associated with known biological pathways. However, the rapidly falling cost of sequencing and the increasing pressure to reduce animal studies has put a premium on extracting more value from each *in vivo* sample. In this light, full transcriptome RNA sequencing has become commonplace in recent years, with modern analysis methods needed to derive comprehensive mechanistic conclusions from this data. **Methods:** Here, we use the *Weighted Gene Correlation Network Analysis* (WGCNA) framework to derive co-regulated gene expression modules in RNAseq data from heart, kidney, liver, and muscle tissues of rats treated with over 300 compounds in short term toxicity studies. Data was processed using standardized workflows and the newest version of the rat genome (GRCh8), quantifying expression changes for each gene in over 1,400 comparisons. The total number of usable genes in each tissue was filtered by applying minimum expression cutoffs. WGCNA was then applied using the eponymous R package in order to define separate sets of gene expression modules for each tissue. **Results:** >3,000 genes in each tissue were classified into one of 40-80 distinct expression modules. Comparison of the liver modules defined in this study via RNAseq with previous reports using publicly available microarray data in liver demonstrated the constancy of the module biology across platforms and laboratories. While certain modules recapitulated known toxicogenomic biomarkers (e.g., Podtelezhnikov et al. 2020), other modules are currently being annotated via a combination of pathway analysis, deconvolution of cell population changes, and association with ancillary study endpoints such as clinical chemistry and histopathology. **Conclusions:** The resultant biologically annotated expression modules will provide additional mechanistic insights into the molecular mechanisms of toxicities observed in preclinical studies. Furthermore, this analysis workflow can be applied to other species such as dogs, monkeys, or mice as standardized databases become available. Lastly, module scores based on eigengene expression in each sample may be a useful way to reduce dimensionality when using toxicogenomic data as features in machine learning models.

PS 4623 Evaluation of 2,2'-Dimorpholinodiethyl Ether (DMDEE) in Pathology, Perinatal, and Genotoxicity Studies

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Background and Purpose: 2,2'-Dimorpholinodiethyl ether (DMDEE) is a tertiary amine catalyst used in the production of flexible and molded polyurethane foams. Human exposure occurs occupationally through inhalation of DMDEE vapors during the production process. In rat models, the fetus may be exposed through maternal transfer during gestation. DMDEE consists of two morpholine rings and may interact with nitrates to form carcinogenic products. Studies were conducted to assess subacute toxicity, genetic toxicity, and perinatal toxicity following exposure to DMDEE in rodent and *in vitro* models. **Methods:** Male and female B6C3F1/N mice (5-6 weeks old) were exposed to DMDEE (0, 62.5, 125, 250, 500, 1,000 mg DMDEE/kg) in deionized water (10 ml/kg) by oral gavage daily for 4 weeks. At necropsy, blood was collected

for hematology and for analysis of micronuclei; selected tissues were stained and analyzed for histopathology. DMDEE (500-6000 µg/tube) was also evaluated for mutagenicity using *Salmonella typhimurium* (TA98, TA100) or *Escherichia coli* (WP2 uvrA pKM101) tester strains with and without S9 activation. To evaluate reproductive toxicity, time-mated female Sprague Dawley rats (12-14 weeks old) were exposed to DMDEE (0, 31.25, 62.5, 125, 250, 500 mg DMDEE/kg) in deionized water (5 ml/kg) daily from gestation day (GD) 6 through post-natal day (PND) 27 by oral gavage. The pups were dosed by gavage at the same dose level as their dams from PND12 through PND27. Blood and tissue were collected from the dams on GD18, and from dams and pups on PND 4 and 28 for hematology, clinical chemistry and micronucleus evaluation. **Results:** No test article related effects were observed on maintenance of pregnancy or litter parameters in Sprague Dawley rats. The micronucleus test was negative in rats and B6C3F1/N mice. DMDEE was also negative in bacterial reverse mutation tests. After 4 weeks of exposure at 1000 mg /kg, DMDEE induced consistent findings of cytoplasmic vacuolization in the reproductive organs of male mice, and in the brain and kidney of 100% of male and female mice. Vacuolization of the brain choroid plexus epithelial cells in mice was accompanied by circling, repetitive head lifting, and abnormal gait in 90% of female mice and 10-30% of male mice at the highest dose. Female mice also displayed lower brain weights. Female mice treated with 500-1000 mg DMDEE/kg, and male mice treated with 1000 mg/kg, for 4 weeks exhibited vacuolization of cortical renal tubular epithelial cells. Kidney weights were higher in female B6C3F1/N mice and F0 maternal female rats at the upper dose levels. DMDEE induced modulation of clinical chemistry markers associated with kidney function (globulin, aspartate aminotransferase, albumin/globulin ratio, urea nitrogen, and creatine) in F0 female rats and in F1 pups exposed to 250 mg DMDEE/kg. Vacuolization in the epithelial cells of the coagulating gland, prostate, and epididymis, and decreased testis weight, were observed in male mice treated with 1000 mg DMDEE/kg. Hematocrit, hemoglobin, and erythrocyte counts were higher in F1 female rat pups exposed to 250 mg/DMDEE/kg but were lower in male and female B6C3F1/N mice at 1000 mg DMDEE/kg, along with a decrease in mean cell hemoglobin and mean cell volume. Lymphocytes, white blood cells, and monocytes were dysregulated in both mice and F0 and F1 rats at high doses. **Conclusions:** Mild alterations of cell populations in the blood and hemoglobin metrics in DMDEE-exposed B6C3F1/N mice and Sprague Dawley rats may be early indicators of anemia and altered immune function. The cytoplasmic vacuolization observed in mice following 4 weeks of exposure to DMDEE and the supportive behavioral observations, along with the clinical biomarkers in F0 and F1 rats exposed to DMDEE during gestation and lactation suggest potential deficits in the cellular structure and function of neurological, renal, and male reproductive tissues at higher doses. Collectively, these data suggest caution in the handling and industrial use of DMDEE in manufacturing processes.

PS 4624 Oral Toxicity of Hexanitrohexaazaisowurtzitane (CL-20) in Rats (*Rattus norvegicus*)

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Background and Purpose: Hexanitrohexaazaisowurtzitane (CL-20) is a polycyclic nitroamine energetic that is currently being evaluated by the U.S. military for use in numerous weapons systems. CL-20 has demonstrated favorable performance characteristics, delivering 20% more power than benchmark energetics such as hexagen (RDX) or octogen (HMX). Toxicity of CL-20 is a potential concern to the warfighter and other Department of War-affiliated personnel who may handle the raw material or be exposed during training, combat, or as a result of environmental contamination. U.S. military interest in CL-20 warrants a complete toxicological test series consisting of acute (one-dose), 14-day repeated dose, and 90-day repeated dose *in vivo* exposure studies to assess toxicity. **Methods:** Oral toxicity testing was conducted in Sprague-Dawley rats via oral gavage, followed by euthanasia, via carbon dioxide, and necropsy. For the acute study, CL-20 was delivered at a limit dose of 2000 milligrams/kilogram in a methylcellulose vehicle to six male and female rats. For the subacute study, 6 rats of each sex were administered doses of 62.5, 125, 250, 500, 1000, 1500, and 2000 milligrams/kilogram daily for 14 days. For the subchronic study, 10 rats of each sex were administered doses of 0, 2.22, 6.66, 20, and 60 milligrams/kilogram daily for 90 days. All testing was performed in accordance with an approved animal use protocol in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. **Results:** The medial lethal dose (LD₅₀) was >2000 milligrams/kilogram based on 100% survival 14 days post-dosing with no significant body weight loss or clinical signs. For the 14-day study, noted clinical signs of morbidity included hunching, squinting, lethargy, severe weight loss, and swollen face. Nearly 100% mortality was observed among rats receiving 250 milligrams/kilogram-day (10 of 12), 500 milligrams/kilogram-day (11 of 12), and all rats receiving ≥1000 milligrams/kilogram-day. Male rats in the 125-2000 milligrams/kilogram-day dose groups experienced either decreased weight gain compared to controls or weight loss. The percent of micronucleated reticulocytes increased 3-fold and 2.5-fold in the 62.5 and 125 milligrams/kilogram-day dose groups, respectively, indicating a genotoxic effect. For the 90-day study, mortality was observed in the highest dose group, with 5 out of 10 male animals dying after an average of 75 days on the study, and 3 out of 10 female animals dying after an average of 85 days on the study. Reduced body weight was significant in female rats but also trended downward in male rats. Clinical pathology in both the subacute and subchronic studies indicated anemia and immunotoxicity. Prevalent gross pathological signs included

enlarged heart, decreased thymus, swollen or discolored lymph nodes, pale or discolored organs, dark red stomach contents, and fluid in the chest cavity. These signs, combined with indicators of anemia in the clinical pathology results, corroborate anemia as a strong toxicological effect of CL-20. Neurotoxic measurements were insignificant, indicating little or no neurotoxicity of CL-20, which is a departure from other currently-fielded energetic materials such as RDX and 1,1-diamino-2,2-dinitroethene (FOX-7), both of which cause epileptic effects. **Conclusions:** Despite low acute toxicity, CL-20 caused significant toxicological effects, including genotoxicity, after subacute and subchronic dosing. Current toxicity data for CL-20 do not demonstrate neurotoxicity. As such, given the concerns of RDX as a neurotoxin, toxicity thresholds identified in this study indicate that CL-20 has lower toxicity than that of RDX. However, identification of a critical effect and subsequent benchmark dose analysis are pending. Therefore, CL-20 exhibits mixed toxicological results that will inform its ongoing development and use in a military context for the purposes of replacing legacy energetics.

PS 4625 Assessment of the reproductive toxicity and neurotoxicity of 1,2,4-trimethylbenzene in rats following gestational and subchronic whole-body inhalation exposure

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Background and Purpose: Alkylbenzenes are a chemical class produced during petroleum refining for a variety of uses including gasoline mixing and blending, industrial coatings, and combustion processes. The most abundant alkylbenzene, 1,2,4-trimethylbenzene (20-45% weight naphtha fraction), is an environmental contaminant with concerns for both developmental/reproductive toxicity and neurotoxicity. In the 2016 EPA IRIS report, confidence in the *in vivo* data to support these concerns was considered low-medium as based on study design and reporting criteria. This subchronic study was designed to investigate the toxicity of 1,2,4-trimethylbenzene (TMB) with special emphasis on assessments of reproductive and nervous system toxicity. **Methods:** Whole-body inhalation exposure of Sprague Dawley rats (0, 30, 100, 300, 600, and 1000 ppm TMB for 6 hours/day) occurred from gestation day 6-21 for the assessment of effects on maternal and fetal toxicity as well as pregnancy outcomes. Dams and offspring were monitored for toxicity on postnatal day (PND) 1-21 (without TMB exposure) and weaned offspring (n=22/sex/group) continued exposure under the same conditions as their dams for 3 months (beginning post-natal day 21). Offspring were monitored for signs of general toxicity as well as pubertal delay by time via assessment of vaginal opening (VO) and balanopreputal separation (BPS). Motor activity and auditory startle response were examined in juvenile offspring for all concentrations and again as adults for doses $\leq$ 300 ppm with additional assessment of learning and memory. Brain weight, size, and standard histopathology were assessed in a separate interim group (n=10/sex/group). Following subchronic exposure, male and female non-littermate exposed pairs were mated to assess TMB exposure effects on fertility. **Results:** Survival was not affected by TMB exposure. Gestational exposure to 1000 ppm TMB was associated with lower body weight compared to controls in dams (12% lower) and offspring (10% lower in both sexes) in the perinatal period which persisted into adulthood for both male and female rats. BPS was delayed 4-5 days in males exposed to $\geq$ 300 ppm TMB and VO was delayed 4 days in females exposed to $\geq$ 100 ppm TMB (with and without adjusting body weight at weaning). Although TMB exposure did not affect F1 pregnancy outcomes (e.g., litter size, weight, etc), mating of F1 (non-litter pairs) after subchronic exposure showed a marginal decrease in number of females mated based on sperm positivity/vaginal plug (81% 1000 ppm vs 91% in controls), but no effects on pregnancy rates were observed. Exposure-related decreases (10-24%) in both F1 corpora lutea and implantations/female was observed in TMB exposure groups $\geq$ 300 ppm. Furthermore, the number of live fetuses/litter were decreased in groups exposed to $\geq$ 300 ppm and we observed positive trends in early resorptions/litter, total resorptions/litter, and calculated post-implantation loss. Evaluation of offspring as juvenile and adults showed diminished motor activity and rearing in the 1000 ppm males and $>$ 600 ppm females. No effects were observed at either age in auditory startle response and habituation, pre-pulse startle inhibition, and in adults for Morris Water Maze learning and memory. TMB exposure did not alter brain development (e.g., weight, size) or histopathology. **Conclusions:** This study demonstrates that subchronic whole-body inhalation exposure to TMB can delay pubertal development in males ($\geq$ 300 ppm) and females ($\geq$ 100 ppm) and may reduce fertility in rats at concentrations as low as $\geq$ 300 ppm. Exposure levels $\geq$ 600 ppm suggested diminished motor function in juveniles. Lower exposure levels were not found to alter motor or sensory function or learning and memory in adults. These results build confidence in the database used to support our understanding of the human health effects associated with TMB inhalation exposure.

PS 4626 Hazard characterization of 1,2,4-trimethylbenzene in rats and mice following whole-body inhalation exposure

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Background and Purpose: The naphtha component of crude oil contains aromatic alkylbenzenes, including trimethylbenzenes. Alkylbenzenes are used in a variety of industrial and consumer applications. Understanding the adverse effects caused by exposure to these chemicals both occupationally and within the general population is essential to human health and safety. This 3-month exposure study investigated the toxicological hazards of the most prominent isomer, 1,2,4-trimethylbenzene (TMB), for which there have been mixed findings in both rodent studies and anecdotal reports from occupational settings. **Methods:** Male and female Hsd:Sprague Dawley rats and B6C3F1/N mice were exposed to 0, 30, 100, 300, 600, and 1000 (rat only) or 1200 (mouse only) ppm TMB via whole-body inhalation. Adult mice (n = 10/sex/group) were exposed 6 hr/day for 3 months and evaluated for exposure-related effects on survival, body and organ weights, clinical pathology, and histopathology. For rats, exposure was perinatal and initiated in pregnant dams from gestation day (GD) 6-21 to assess maternal and fetal toxicity and pregnancy outcomes. Toxicity was monitored in the dams and offspring during lactation (no TMB exposure) at postnatal day (PND) 1-21, and the weaned offspring (n=10/sex/group) were then exposed to TMB under the same conditions as their dams for 3 months to assess similar toxicity endpoints as in mice. **Results:** In mice, 1200 ppm TMB was overtly toxic. 9 males and all 10 females were found dead on the second day of exposure; the remaining male was euthanized moribund. Clinical signs following the first and prior to the second exposure included ataxia, slow/shallow or labored breathing, abnormal gait, hunched posture, hyperactivity, lethargy, or tremors. No exposure-related mortality/moribundity or adverse clinical signs were observed in mice in the remaining exposure groups throughout the study. Exposure to 600 ppm TMB was minimally toxic to males and females, and there was nominal to no exposure-related effects on clinical observations, body weights, hematology, or gross findings at necropsy. TMB exposure-related effects on organ weights were observed, but these changes were not associated with any histological findings in mice. In males, absolute and relative liver weights were increased (11 and 9%, respectively) at 600 ppm, and absolute and relative kidney weights were decreased (up to 16%) at 300 and 600 ppm. Relative liver weights were increased (9%) at 600 ppm in females. In rats, gestational exposure to 1000 ppm TMB was associated with lower body weights in the dams (decreased 12% at GD21) and in both sexes of offspring during lactation (decreased 16% at PND1 and 10% at PND21). There was no exposure-related mortality/moribundity in the dams. Pregnancy outcomes (litter size, sex ratio, and survival) were similar across all the exposure groups. Post-weaning, males exposed to $\geq$ 600 ppm TMB exhibited lower body weight gains throughout the 3-month exposure period, and terminal body weights were 12% and 10% lower in the 600 and 1000 ppm groups, respectively (at PND119). Female body weight changes were not significantly different than the controls in any of the exposure groups. There was no exposure-related mortality/moribundity in the males or females. Relative liver and kidney weights were increased 16-27% and up to 22%, respectively, at 600 and 1000 ppm in males and females. There were no hematologic changes in males or females, but there were exposure-related increases in liver enzymes and bile acids (up to 44% and 346%, respectively), and mild decreases in globulins (up to 11%). There was nominal to no exposure-related effects on histopathology. Plasma TMB concentrations measured at the end of the study correlated with increasing exposure concentrations with no apparent sex differences. **Conclusions:** This study provides valuable insight into the health hazards of TMB, which will help bridge critical data gaps needed to better assess the human health risks associated with exposure to this ubiquitous environmental chemical. These data will be interpreted in the context of a parallel study investigating the reproductive toxicity and neurotoxicity of TMB following inhalation exposure.

PS 4627 Single-Crystal Structure and Protonophoric Potential of N-(3,5-dichloro-4-hydroxyphenyl)acetamide: Implications for Mitotoxicity of Chlorinated Acetaminophen

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Background and Purpose: Under neutral and mildly oxidative conditions, HOCl/OCl⁻ (pK_a $\approx$ 7.53) chlorination of acetaminophen forms N-(3-chloro-4-hydroxyphenyl)acetamide, N-(3,5-dichloro-4-hydroxyphenyl)acetamide (DCHPA), and other transformation products in chlorine-treated waters. Although formation via the myeloperoxidase-H₂O₂-Cl⁻ pathway is likely minor, DCHPA is mechanistically important as a biomarker of chlorination and a toxicological concern. Empirically, each *ortho*-Cl reduces the phenolic pK_a by $\sim$ 1.1-1.2 units; thus, the two Cl substituents shift the pK_a of acetaminophen from $\sim$ 9.5 to $\sim$ 7.2-7.3, within the "uncoupler window" (pK_a 4.0-8.0). At physiological pH (7.4), $\sim$ 60% of DCHPA is phenoxide, with the remainder being the neutral and membrane-permeant phenol form; together these favor protonophoric cycling and potential dissipation of the mitochondrial protonmotive force. To ground these mechanistic hypotheses in definitive structure, we determined the single-crystal X-ray structure of DCHPA. **Methods:** DCHPA was synthe-

sized by acetylation of 4-amino-2,6-dichlorophenol with slightly molar excess (1.1–1.2-fold) acetic anhydride in glacial acetic acid solvent at room temperature (24–48 h) under stirring. The product was dried under vacuum and recrystallized once from water. Single crystals (colorless plates/needles) suitable for X-ray diffraction were obtained by slow cooling and evaporation of a near-saturated aqueous solution at room temperature. Diffraction data were collected at 100 K on a Bruker D8 Venture DUO equipped with a Photon III C14 detector using Cu K α radiation (λ = 1.54178 Å); a multi-scan (SADABS) absorption correction was applied. The structure was solved with SHELXT and refined on F² with SHELXL. Intermolecular interactions and graph-set motifs were analyzed using PLATON and Mercury. pK_a was estimated empirically from the *ortho* effect (~1.1–1.2 units decrease per *ortho*-Cl) rather than from *meta/para* Hammett constants. **Results:** DCHPA, C₈H₇Cl₂NO₂, crystallizes in triclinic space group P-1 with three independent molecules in the asymmetric unit. Two of them are essentially planar, with mean deviations of 13 atoms 0.029 and 0.030 Å and are nearly parallel, forming a dihedral angle of 7.5°. They differ only in the conformation of the OH hydrogen atom. The third molecule is quite nonplanar, with the CCNO acetamide plane forming a dihedral angle of 67.6° with the remainder of the molecule. C-Cl distances are in the range 1.7197(17)–1.7381(18) Å with a mean value of 1.7311 Å. C-OH distances are in the range 1.351(2)–1.359(2) Å with a mean value of 1.354 Å. The two planar molecules form antiparallel hydrogen-bonded chains through N-H...O interactions of the acetamide substituents. The NH group of the nonplanar molecule donates a bifurcated hydrogen bond to the OH and a Cl substituent of one of the planar molecules. The OH groups of all molecules form intermolecular hydrogen bonds to other OH or carbonyl oxygen atoms. N...O distances in the chains are in the range 2.913(2)–2.920(2) Å and O...O distances are in the range 2.6776(18)–2.9546(18) Å. **Conclusions:** The definitive solid-state structure (Z' = 3) and cohesive hydrogen-bond/halogen-bond network provide a robust molecular reference for DCHPA, a chlorination product of acetaminophen. The structural features (conjugation, hydrogen-bond topology, and halogen contacts) are consistent with enhanced phenoxide stabilization and the empirically expected $\Delta pK_a \approx 2.2$ –2.3 for two *ortho* Cl substituents, yielding pK_a ~7.2–7.3. These properties place DCHPA within the protonophore-active range, supporting its potential to uncouple oxidative phosphorylation and its value as a biomarker of HOCl/-OCl exposure in environmental and biological settings.

PS 4628 Single Crystal Structure of 4-Acetamido-3-chlorophenyl Acetate: An O-Acetylated Proxy for a Putative *In Vivo* N-Acetyl-1,4-benzoquinoneimine-Chloride Ion Biomarker Precursor

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Background and Purpose: With about 25 billion doses sold annually in the United States, acetaminophen (Tylenol) is among the most widely used analgesic and antipyretic agents. Its metabolism is dominated by sulfation, glucuronidation, and, to a lesser extent, CYP2E1 oxidation; however, nonenzymatic transformations mediated by cellular oxidants, such as, peroxytrinitrate-CO₂ and the myeloperoxidase-H₂O₂-Cl⁻ system that generates HOCl/-OCl (pK_a ≈ 7.53), also warrant consideration. While CYP2E1-mediated reactions are implicated in overdose-level formation of N-acetyl-1,4-benzoquinoneimine (NBQI), nonenzymatic biotransformations involving peroxytrinitrate-CO₂ and HOCl/-OCl may operate continuously, producing low-level *in vivo* formation of NBQI in acetaminophen users. Our focus, therefore, centers not only on overdose but also on normal exposure conditions and the identification of practical biomarkers. Given the high abundance of Cl⁻ in the cellular milieu (≈ 150 mM), we propose N-(4-hydroxy-2-chlorophenyl)acetamide (the 2-chloro isomer) as a direct, LC-MS/MS-amenable readout of endogenous NBQI-Cl⁻ chemistry. Accordingly, recognizing that O-acetylation can facilitate cellular uptake, we synthesized 4-acetamido-3-chlorophenyl acetate, the O-acetylated derivative of N-(4-hydroxy-2-chlorophenyl)acetamide, and examined its structure. **Methods:** 4-Acetamido-3-chlorophenyl acetate was synthesized by acetylation of 4-amino-3-chlorophenol with acetic anhydride following Naik *et al.* (Arkivoc, 2004, 55-63) with minor modifications. Briefly, 4-amino-3-chlorophenol hydrochloride (1.80 g, 10 mmol) was dissolved in ~50–75 mL water and adjusted to pH 1.5–1.7 with 1 N HCl. The cooled solution (ice bath) was treated with acetic anhydride (1.21 mL, 12 mmol), then NaHCO₃ (2.16–3.00 g, 25–35 mmol) was added with continuous stirring, maintaining the reaction pH between 5.5 and 6.5. The off-white precipitate was collected by filtration. Crystals were grown from a hot, near-saturated ethanolic aqueous solution by slow cooling and evaporation and subjected to single-crystal X-ray diffraction. Data were collected at 100 K on a Bruker D8 Venture DUO diffractometer with a Photon III C14 detector. Structure solution and refinement were carried out using SHELXT and SHELXL2019. Bond lengths, hydrogen-bonding patterns, and dihedral angles were analyzed to assess molecular conformation and supramolecular organization. **Results:** 4-Acetamido-3-chlorophenyl acetate, C₁₀H₉ClNO₃ (Mr = 227.64) crystallizes in monoclinic space group I 2/a and can be characterized by three planes, the phenyl plane, the acetamide plane, and the acetate plane. The phenyl and acetamide planes make a dihedral angle of 40.7°, the phenyl and acetate planes are nearly orthogonal, with a dihedral angle of 88.1°, and the acetamide and acetate planes make a dihedral angle of 51.4°. The C-Cl distance is 1.7378 (15) Å, the C(Ph)-N distance is 1.4129 (17) Å, and the C(Ph)-O distance is 1.4033 (17) Å. Molecules are linked by intermolecular hydrogen bonds involving the acetamide group, with NH as donor and carbonyl O as acceptor, forming chains along the [010] direction. The N...O distance

is 2.8335 (17) Å, and the angle about H is 157.5 (17)°. **Conclusions:** Single crystal data establish the solid-state geometry and packing of 4-acetamido-3-chlorophenyl acetate, an O-acetylated scaffold that masks the phenolic oxygen. As an acetate ester, it is expected to be more lipophilic than the free phenol and may facilitate cellular entry. It can therefore serve as a proxy for delivery and subsequent unmasking of N-(4-hydroxy-2-chlorophenyl)acetamide, the proposed readout of endogenous NBQI-Cl⁻ chemistry. This study does not demonstrate biological uptake or biomarker performance. Next steps will quantify esterase mediated O-deacetylation and formation of the amide, establish targeted LC-MS/MS detection in cells and biofluids using authentic standards, and assess stability and conversion kinetics. Finally, the proxy ester approach may provide a practical route to probe downstream signaling: after intracellular deacetylation, the liberated phenolic amide could enter conjugation pathways that generate anandamide like derivatives, enabling systematic evaluation of nociception and toxicity in cell-based and animal models.

PS 4629 Ezetimibe-Glucuronide as an exogenous biomarker probe identifies MASH

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Background and Purpose: Metabolic dysfunction-associated steatohepatitis (MASH), the progressive form of metabolic dysfunction-associated steatotic liver disease (MASLD), remains underdiagnosed due to the invasiveness of liver biopsy and limited accuracy of non-invasive tests. MASH progression involves altered hepatic transporter processes that reduce biliary efflux and increase systemic retention of transporter substrates. This study aimed to determine whether ezetimibe glucuronide (EZEG), a selective substrate of three transporter pathways dysregulated in MASH, could serve as a non-invasive diagnostic biomarker. **Methods:** Biopsy-verified patients with NAFLD activity scores (NAS) ranging from 0–5 received an oral 1 mg sub-therapeutic microdose of ezetimibe (EZE). Plasma and urine EZEG levels were quantified post-dose to assess transporter-dependent disposition. Diagnostic performance was evaluated using receiver operating characteristic (ROC) analysis. **Results:** Plasma EZEG concentrations were significantly elevated in patients with MASH (NAS ≥5), showing excellent discrimination (AUROC = 0.99; 95% CI: 0.98–1.00) using the 60-minute post-dose measurement. MASH patients exhibited approximately six-fold higher plasma EZEG levels than non-MASH individuals (NAS 0–4), consistent with impaired hepatic uptake and efflux. Urinary EZEG excretion over six hours was increased but showed weaker group differentiation, likely reflecting reduced renal clearance from diminished glomerular filtration and tubular secretion. **Conclusions:** Altered hepatic transporter function in MASH leads to systemic accumulation of EZEG, providing a sensitive, mechanism-based biomarker capable of distinguishing MASH (NAS=5) from non-MASH cases. This exogenous probe drug strategy offers a non-invasive, reasonably likely surrogate endpoint to identify early-stage MASH and support disease stratification, therapeutic monitoring, and patient selection in clinical studies.

PS 4630 Copper Isotope Ratios as Biomarkers of Combined Dietary and Environmental Stressors

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Background and Purpose: Vinyl chloride (VC), an environmental pollutant and chemical of concern following the East Palestine, Ohio train derailment, is a known hepatotoxicant that directly causes liver injury at high exposure levels. The impact of lower concentrations of VC on progression of underlying liver disease remains less clear. Given the high prevalence of metabolic dysfunction-associated steatotic liver disease, (MASLD, formerly known as NAFLD) in the U.S., the potential for overlap between diet-induced liver injury and VC exposure is an important concern. Recent studies suggest that Western diet can modify VC-associated liver injury, raising the need for improved biomarkers. Isotope metallomics, an emerging field examining stable isotope distributions of essential metals, offers a novel approach to investigate such interactions. Copper (Cu) isotopes are of particular interest due to copper's role in liver metabolism and redox regulation. The objective of this study was to investigate the role of dietary background on modifying hepatic Cu isotope ratios in response to VC exposure, with the overall goal of assessing their potential as biomarkers of diet-pollutant interactions. **Methods:** Male C57/BL6 mice were fed either a control diet (CD; nutrient-balanced) or a Western diet (WD; high-fat, high-sugar) and exposed to VC (–0.85 ± 0.1 ppm) or air for 6 hours per day, 5 days per week for 12 weeks. Experimental groups included CD, CD+VC, WD, and WD+VC (n=5/group). Banked liver tissue was collected and analyzed for Cu isotope ratios (⁶⁵Cu/⁶³Cu) using multi-collector inductively coupled plasma mass spectrometry (Neptune Plus MC-ICP-MS). Results are reported in delta notation (δ⁶⁵Cu), calculated using the NIST 3114 standard, and a final correction is made to report δ⁶⁵Cu data versus NIST 976. A concentration-matched standard-sample bracketing

approach with gallium doping was used for internal normalization. Reproducibility was confirmed using an internal Cu standard (ESPI) at $n = 16$ measurements relative to the NIST 3114 standard. Yielding $\delta^{65}\text{Cu}$ relative to NIST 976 of $-0.02 \pm 0.031\%$ (2SE). **Results:** Group mean hepatic $\delta^{65}\text{Cu}$ values ($\%$) $\pm$ SEM were $\text{CD} = -0.06 \pm 0.11$, $\text{CD+VC} = -0.19 \pm 0.08$, $\text{WD} = -0.60 \pm 0.03$, and $\text{WD+VC} = -0.49 \pm 0.09$. Analysis revealed significant differences between dietary exposure groups (CD and WD, $p = 0.0040$), as well as between CD and WD+VC ($p = 0.0152$). VC exposure alone (CD vs. CD+VC) produced a modest isotopic shift that did not reach significance, and WD vs. WD+VC were not significantly different. These results suggest that diet has a dominant effect on hepatic Cu isotope ratios. **Conclusions:** This study demonstrates that hepatic Cu isotope ratios are sensitive to dietary background, with Western diet significantly shifting $\delta^{65}\text{Cu}$ toward lighter values compared to control diet. These findings support the potential of $\delta^{65}\text{Cu}$ as an innovative biomarker of nutritional status. While VC alone did not significantly alter isotopic composition in this study, the combined effects of diet and exposure underscore the importance of considering co-exposures in biomarker development. Ongoing studies will assess mechanistic endpoints, including Cu transport and oxidative stress, to better define pathways underlying isotopic fractionation.

PS 4631 Therapy-Induced Rewiring of Synovial Sarcoma Metabolism

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Background and Purpose: Synovial sarcoma (SS) is one of the most common soft tissue cancers in pediatric, adolescent, and young adult patients in the world. Despite this, present treatment options are limited due to lack of targeted therapies for SS tumors. Current standard-of-care treatment depends on surgery, radiation, and cytotoxic chemotherapeutic agents such as doxorubicin. Metabolic rewiring in SS in response to treatment is poorly characterized and there are currently no metabolism-targeted interventions available for SS. Therefore, discovering treatment-induced metabolic vulnerabilities in SS tumors will guide the development of new treatment strategies for these patients. **Methods:** In this study, we aimed to characterize the metabolome changes of human synovial sarcoma cells caused by treatment with radiation and doxorubicin. We utilized liquid chromatography-mass spectrometry (LC-MS) to conduct broad targeted analysis on water-soluble metabolites in extracts from HSSYII and SYO1 cells before and after treatment with clinically relevant dosages of radiation and/or doxorubicin. Metabolomics analysis was performed to assess both their individual and combined effects in the SS cell line models. Additionally, cell proliferation was measured by an SRB assay to understand how alterations in metabolite levels correlated with growth rate. **Results:** LC-MS metabolomics quantified levels of central carbon metabolites, including amino acids, TCA cycle intermediates, and redox-related metabolites. Significant perturbations in metabolite levels were observed post-treatment within 24 hours when compared to non-treated cells. Of particular interest are alterations in levels of redox metabolites such as glutathione and its precursors following radiation treatment, indicating a metabolic response to the treatment-induced oxidative stress and potentially modulating radiosensitivity. **Conclusions:** Investigation of the efficacy of therapies targeting the unique metabolism of synovial sarcoma is ongoing and may include both dietary and pharmacological interventions to selectively disrupt tumor nutrient availability, such as for synthesis of the major antioxidant glutathione. We also aim to expand our model to better capture clinical conditions through providing multiple doses of radiation to mimic fractionated radiation therapy and expand our analysis to include measuring metabolic pathway fluxes through the use of stable isotope tracers. Continued research is crucial in translating our findings into viable treatments that can help improve outcomes for patients.

PS 4632 Combining immunocapture and microflow liquid chromatography-tandem mass spectrometry to advance bioanalysis of protein biomarkers in toxicological studies

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Background and Purpose: Biomarkers play a critical role throughout the entire drug-development lifecycle, contributing to both safety and efficacy assessments. As industrial report suggested, from 2011 to 2020, drugs developed with biomarker guidance led to 2 times higher success rate in Phase II trial and 3 times higher overall approval than those developed without. Proteins, as the functional end products of the central dogma, their expression profiles closely reflect the physiological activity, making them particularly suitable as biomarkers. So far, the Early Detection Research Network (EDRN) catalogs 627 protein biomarkers across ten organs (45% of the total entries). Nearly all clinical biomarkers approved by FDA are protein-based. Protein biomarkers are commonly measured by ligand-binding assays such as ELISA and electrochemiluminescence assay. These platforms however, have relatively limited assay ranges and rely upon specific antibody accessibility. By contrast, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has emerged as an important alternative due to high specificity and methodological robustness. LC-MS/MS methods are also inherently high-throughput and can quantify multiple biomarkers in a single assay, thereby shortening laboratory turnaround time, and lowering cost.

The conventional LC-MS/MS platforms however, generally achieve lower limits of quantification (LLOQ) for proteins/peptides in the order of 1-10 ng/mL, which is insufficient for many endogenous protein biomarkers (expressed at pg/mL to fg/mL). Improving LC-MS/MS sensitivity is therefore the key to further advance protein biomarker bioanalysis. In this work, we streamlined an analytical workflow integrating immunocapture and microflow liquid chromatography (microLC), aiming to push further the protein quantitation limit, while maintaining a wide dynamic range. The established methods were validated and applied in real-world preclinical toxicology studies. **Methods:** The serum samples were incubated at room temperature overnight, with in-house prepared anti-biomarker beads based on biotin-streptavidin interaction. After repeatedly washing the beads with PBST buffer, the captured proteins were eluted with water containing 0.1% v/v TFA. Following centrifugation, dithiothreitol and iodoacetamide were added sequentially for reduction and alkylation. The proteins were digested with trypsin at 37 °C for 3 h before injection. Chromatographic separations were running at 12 $\mu\text{L}/\text{min}$ on an ACQUITY M-Class System equipped with a BEH C18 column (130Å, 1.7 μm , 300 μm * 100 mm). The chromatograms were acquired in MRM mode on a SCIEX 7500 system, with transitions of the signature peptides preselected by empirical validation accessing public databases. The endogenous protein biomarker was quantitated with external standard method using surrogate matrices. **Results:** Systematic optimization was implemented regarding immunocapture recovery, digestion efficiency, chromatographic resolution and MS detectability. Among all parameters, beads-to-protein ratio, enzyme-to-substrate ratio, signature peptide selection, and chromatographic flowrate were most impactful for sensitivity improvement, while surrogate matrices selection was critical to assure quantification precision and accuracy. The applicability and figure of merits of the developed workflow were showcased with bioanalysis of TL1A in NHP serum in general toxicology studies, to monitor the immunomodulation of DR3-TL1A pathway upon dosage. The LLOQ was achieved at 50 pg/mL with linearity across three orders of magnitude. The precision, accuracy as well as method robustness have been validated fully adhering GLP regulatory guidance. **Conclusions:** A streamlined bioanalytical workflow has been established combining immunocapture and microLC-MS/MS towards highly sensitive protein biomarker quantitation in biological matrices, with significantly reduced analytical turnaround time and reagent costs as well as improved method robustness. The applicability and advantages have been demonstrated in real-world general toxicological studies with full GLP compliance. It also suggests great potential in multiplexing, to simultaneously quantify a larger biomarker panel in "single-shot", providing in-depth physiological information towards preclinical development.

PS 4633 Arsenic & Zinc Exposure: Correlation with Diabetes and Hypertension in Doña Ana County Well-Water Users

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Background and Purpose: Arsenic is a toxic chemical that occurs naturally as a mineral in the earth's crust. Drinking water in New Mexico (NM) contains some of the highest levels of naturally occurring arsenic in the U.S. In NM, approximately 400,000 residents (20%) rely on private wells for their water supply. Among all counties in NM, Doña Ana has the highest average arsenic concentration at 17.3 $\mu\text{g}/\text{L}$, with 50.3% of samples exceeding the Environmental Protection Agency (EPA) Maximum Contaminant Level (MCL) of 10 $\mu\text{g}/\text{L}$. Zinc, an essential micronutrient, plays a key role in many biological processes, including immune function and protection against toxic chemicals through its antioxidant and anti-inflammatory properties. Areas with high arsenic exposure from groundwater often overlap with regions at risk of zinc deficiency. Both arsenic exposure and zinc deficiency share similar features such as increased oxidative stress and inflammation and have been linked to chronic conditions like cardiovascular disease, diabetes, and kidney disease. The biomarker 8-hydroxy-2'-deoxyguanosine (8-OHdG) is a recognized indicator of oxidative damage. The purpose of this study is to assess households with private wells and examine the presence of arsenic and zinc, as well as their correlation with diabetes and hypertension, focusing on the interaction between arsenic and zinc in Doña Ana County, NM. **Methods:** The research team recruited well-water users in Doña Ana County through online well-water data, household visits, social media, and health fairs. After obtaining informed consent, participants completed a questionnaire capturing demographics, disease history, and water-related information. Urine samples were collected in wide cups and split into two 15 mL conical tubes. One tube was stored on dry ice at -20°C for 8-OHdG analysis, while the second tube, along with a 15 mL well-water sample, was kept on ice for metal analysis. Adults (≥ 18 years) using well water in Doña Ana County, NM, were included. Urinary and well-water As and Zn were measured using inductively coupled plasma mass spectrometry (ICP-MS), and urinary 8-OHdG was analyzed using a commercial ELISA kit. Laboratory analyses were supported by P20GM130422. For statistical analysis, logistic regression was employed to examine the associations between the concentrations of these biomarkers and the prevalence of hypertension and diabetes, adjusting for relevant covariates such as age, gender, ethnicity, education, income, alcohol use, smoking status, and BMI. Statistical significance was defined as a P-value < 0.10. All statistical analyses were conducted using R software. **Results:** A total of 111 participants were analyzed, 62% of whom were women. Hypertension prevalence was 35% among women and 40% among men, and diabetes was reported in 16% of women

and 24% of men. Significant differences were observed in urinary As, Zn, well-water As, and urinary 8-OHdG levels by hypertension and diabetes status. Hypertensive smokers showed higher urinary Zn (OR = 3.15, 95% CI: 1.09-16.05), and hypertensive non-smokers had slightly elevated Zn levels (OR = 1.06, 95% CI: 1.01-1.15). Among women, hypertension was inversely associated with urinary As (OR = 0.93, 95% CI: 0.85-1.00). Hypertensive individuals with normal/low BMI were less likely to have elevated urinary 8-OHdG (OR = 0.44). Diabetic Hispanic participants had lower urinary Zn (OR = 0.56, 95% CI: 0.25-0.92). Diabetics aged ≥ 64 had higher odds of elevated well-water As exposure (OR = 1.32, 95% CI: 1.02-1.90). Diabetic males had more than twice the urinary 8-OHdG levels of non-diabetic males (OR = 2.16, 95% CI: 1.12-5.16). Diabetic college graduates also showed higher urinary 8-OHdG (OR = 1.23, 95% CI: 0.99-1.61). **Conclusions:** This cross-sectional study limits the ability to establish causal relationships between arsenic-zinc interactions and hypertension or diabetes. A larger sample size is needed to improve statistical power, and further research is warranted to confirm these findings. This project was supported by a subaward from the Mountain West Clinical and Translational Research Infrastructure Network (MW CTR-IN), funded by the National Institute of General Medical Sciences (NIGMS), NIH, under award number U54GM104944 (funded 10/27/2022).

PS 4634 Associations Between Serum PFAS Profiles and Glaucoma: Evidence for a Branched Amide PFAS Effect in U.S. Adults

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are a diverse class of persistent chemicals with variable chain lengths, functional groups, and branching patterns that influence bioaccumulation and cellular uptake. Longer-chain PFAS (PFDeA, PFNA) exhibit higher lipophilicity, suggesting greater accumulation in lipid-rich retinal tissues. Sulfonic acid-terminated PFAS (PFOS, PFHxS) display higher plasma protein binding, potentially extending systemic exposure. Based on structure-activity relationships reported for PFAS, we hypothesize that Me-PFOA-AcOH, a branched amide PFAS, may exhibit enhanced membrane permeability and increased uptake in retinal ganglion cell (RGC)-relevant tissues. We focused on PFAS structural features to hypothesize how these chemicals may interact with ocular tissues and then evaluated their associations with glaucoma using epidemiologic data from a U.S. adult cohort. Specifically, six PFAS (PFOA, PFOS, PFHxS, Me-PFOA-AcOH, PFDeA, and PFNA) were examined. **Methods:** PFAS structural properties were compared with known toxicokinetic and bioactivity profiles from the literature, including lipophilicity, plasma protein binding, membrane permeability, and oxidative stress potential. Epidemiologic data from 1,837 adults in NHANES (2005-2007) were analyzed to identify PFAS with higher serum levels among participants with glaucoma, and weighted quantile sum (WQS) regression determined the relative contribution of each PFAS to glaucoma. **Results:** Epidemiologic analyses showed that Me-PFOA-AcOH and PFOS levels were higher in participants with glaucoma. Me-PFOA-AcOH had the strongest association with glaucoma (adjusted OR 1.67, 95% CI 1.34-2.09, $P < 0.001$) and contributed 41.4% to the overall PFAS mixture effect, while PFHxS contributed 20%. Cubic spline modeling revealed a consistent dose-dependent increase the odds of glaucoma with Me-PFOA-AcOH exposure. **Conclusions:** Branched amide PFAS such as Me-PFOA-AcOH appear to pose the greatest association, consistent with both structural predictions and observed epidemiologic associations. Our theory posits that structural features, including chain length, functional group, and branching, correlate with potential oxidative stress, mitochondrial impairment, and neuroinflammatory signaling in RGCs. These structure-activity relationships provide a framework for understanding PFAS-induced ocular toxicity and prioritizing compounds for further mechanistic studies.

PS 4635 Early GDF15 Elevation as a Predictive Biomarker in a Long-Latency Model of Doxorubicin-Induced Cardiomyopathy

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Background and Purpose: Doxorubicin (Dox), an anthracycline chemotherapeutic, is widely used in the treatment of pediatric and adult cancers. Dox is known to cause dose-dependent cardiotoxicity that can progress to heart failure. Early detection of cardiac injury remains a major clinical problem. Growth differentiation factor-15 (GDF15) is emerging as a promising biomarker in this context. GDF15 is a stress-responsive cytokine of the TGF- β superfamily that is upregulated in the heart under oxidative stress and injury conditions. We investigated circulating and myocardial GDF15 as a predictive biomarker of Dox-induced cardiotoxicity in male and female rats across acute and chronic time-course models and examined its relationship with echocardiographic measures of cardiac function (fractional shortening, FS%). **Methods:** Male and female Sprague-Dawley rats (5-7 weeks old) were administered doxorubicin (3 mg/kg, or saline, intrajugular vein injection, 2 injections 2 weeks apart). An acute cohort was euthanized 24hr, 48hr and 72hr after doxorubicin treatment and plasma frozen. The chronic cohort (capturing the longer-latency to cardiomyopathy- up to 12 months) underwent echocardiography (Vevo 3100) until FS declined to 30% or less (50% of control values). Plasma was collected for ELISA-

based quantification of the GDF15 assay. At the time of euthanasia for our earlier pilot studies, fractional shortening data were correlated with GDF15 levels to determine biomarker sensitivity to cardiac dysfunction. Due to the pandemic, 25 rats were evaluated at euthanasia, approximately 6 months post treatment. For correlation of the last echo FS% to the last plasma GDF15, data were expressed as mean $\pm$ SD and analysed by one-way ANOVA and Pearson correlation. In a second rat study with monthly serial analysis of 54 rats (22 saline and 32 doxorubicin-treated) (done in 3 separate cohorts), blood was sampled monthly and correlated with echocardiography data. Specific data were used for predictive values from data collected from months 1 and 2 post chemotherapy using the following characteristics: early FS% decline, early GDF15 increase, sex, and weight values at the last doxorubicin treatment. Using this data logistic regression model was compared to Random Forest and XGBoost classifiers in predicting the 7-month HF outcome. Model performance was evaluated by area under the ROC curve (AUC), Brier score, calibration plots, decision curve analysis (DCA), and DeLong tests for AUC differences. Feature importance was examined via permutation testing, SHAP values, and accumulated local effects (ALE) plots. **Results:** In the first chronic cohort study, doxorubicin-treated rats exhibited a marked reduction in FS% compared to saline controls (mean FS: $\approx$ 35% vs. $\approx$ 57%, $p < 0.001$). Correspondingly, plasma GDF15 levels measured at euthanasia were significantly elevated in Dox groups, showing an inverse correlation with FS ($R^2 = 0.71$). Protein and RNA studies revealed that myocardial levels of GDF15 were higher in Dox treated rats. Ingenuity Pathway Analysis of RNA sequencing revealed activation of TP53 and other stress-response genes linked to GDF15 upregulation. In a second chronic multiple cohort study, blood collection and echocardiography were performed monthly starting one month after the last injection of doxorubicin. GDF15 increases (one month 200 pg/mL) preceded visible cardiac dysfunction, suggesting predictive potential in early sampling. Elevated GDF15 concentrations were also observed within 24hrs, 48hrs and 72hrs of treatment (800- 1000pg/mL), indicating an acute stress response. For the machine learning models, key predictors included early elevation in GDF15, early decline in FS (Δ FS), body weight, and sex. An interaction between Δ FS and GDF15 was noted, whereby rats with both an early FS drop and high GDF15 were at markedly elevated HF risk. Random Forest model showed the highest AUC at 97. **Conclusions:** In this doxorubicin cardiotoxicity animal model with a long latency to heart failure, machine learning substantially improved the early prediction of HF, highlighting the complementary value of stress biomarkers (GDF15) and functional imaging (FS) for risk stratification. Continued validation of GDF15 across sexes and timepoints will strengthen its utility for cardiotoxicity risk stratification and preclinical drug safety screening within the broader context of toxicological evaluation.

PS 4636 Red Blood Cell-Derived Extra Cellular Vesicles Serve as Biomarkers of α -Synuclein Pathology and Neuroinflammation in Neurotoxicant-Linked Parkinson's Disease

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Background and Purpose: Environmental toxicants are known to induce protein misfolding, oxidative stress, and mitochondrial dysfunction, with certain herbicides strongly associated with an increased risk of Parkinson's disease (PD). Red blood cells (RBCs) are a major peripheral reservoir of α -synuclein (α Syn), can release extracellular vehicles (EVs) carrying bioactive cargo that may reflect systemic stress responses. We hypothesized that exposure to Agent Orange (AO), a dioxin-containing herbicide historically associated with Parkinsonian outcomes, promotes the release of α Syn-enriched RBC-derived EVs with enhanced aggregation propensity and proinflammatory activity. Thus, this study investigates AO-mediated alterations in RBC-EVs, evaluates their α Syn seeding capacity, and examines their contribution to microglial inflammation. Given their stability and accessibility in circulation, RBC-EVs represent a promising minimally invasive biomarker for early detecting α Syn pathology, monitoring disease-relevant processes, and elucidating mechanistic links between environmental exposures to neurodegenerative disease risk. **Methods:** RBCs from healthy individuals were obtained from GRIFOL bio supplies and exposed to acute dose of 100 μ M AO for 24 h, and EVs were isolated using differential ultracentrifugation or EXODUS nanofiltration. EV purity and concentration were verified via nanoparticle tracking analysis (NTA) and nanoflow cytometry from EV-specific dye. Total and aggregated α Syn content was quantified using Meso Scale Discovery assay and AlphaLISA assays. α Syn aggregation and seeding kinetics were assessed by Thioflavin T fluorescence assays as described in our publications. Nano-flow cytometry and super-resolution microscopy were used to characterize aggregated α Syn (α Synagg)-positive EVs. RBC-EVs isolated from AO-exposed veterans and age-matched healthy controls were analyzed in a blinded manner to quantify α Synagg-positive EV subsets and assess seeding activity. Microglial inflammatory responses to AO-exposed RBC-EVs were measured by quantifying TNF- α , IL-6, and IL-1 β levels *in vitro*. **Results:** AO exposure markedly increased the release of RBC-EVs enriched in both total and aggregated α Syn. Nano-flow cytometry and super-resolution imaging revealed elevated α Synagg-positive EVs and higher EV density in AO-treated samples. Seeding activity assays demonstrated accelerated α Syn fibrillization and potent seeding activity in EVs derived from AO-exposed RBCs, whereas control EVs failed to induce detectable aggregation. EXODUS-purified EVs exhibited stronger seeding activity than ultracentrifugation-derived EVs, indicating improved recovery of bioactive vesicles and reduced heme contamination. Blinded analyses of clinical samples revealed that $\sim$ 70% of AO-exposed individu-

als displayed an expanded α Synagg-positive EV subset (~15-22% of total EVs) and enhanced seeding activity relative to healthy controls. *In vitro*, human C20 microglia treated with AO-exposed RBC-EVs showed significant upregulation of TNF- α , IL-6, and IL-1 β , confirming that toxicant-exposed RBs release EVs capable of mediating robust proinflammatory activation. **Conclusions:** AO exposure enhances the release of RBC-EVs enriched in aggregation-competent α Syn capable of seeding pathological fibril formation and inducing microglial inflammation. EXODUS-based ultra-purification improves detection of these bioactive vesicles. Collectively, these findings identify RBC-EVs as a mechanistic link between environmental toxicant exposure and α Syn pathology. It also highlights their potential as blood-based biomarkers for early detection and monitoring of exposure-driven neurodegenerative processes.

PS 4637 Stable Copper Isotope Ratios as a Biomarker of Aflatoxin-Induced Hepatocellular Carcinoma

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Background and Purpose: This project applies isotope metallomics, the blend of geochemistry and biomedicine, to investigate Cu fractionation in the context of hepatocellular oxidative stress resulting from exposure to the known hepatocarcinogen, aflatoxin B₁ (AFB₁). Cu is transported to hepatocytes by metalloproteins (e.g., ATP7b) which are upregulated during oxidative stress. We explore differences in transport and accumulation of ⁶⁵Cu and ⁶³Cu and present results using delta notation ($\delta^{65}\text{Cu}$ (‰)) in reference to a certified Cu isotopic standard (NIST SRM 976). $\delta^{65}\text{Cu}$ values in hepatic tissue and serum may shift in response to different levels of oxidative stress, and therefore act as a prognostic biomarker of liver health. Establishing sensitive biomarkers for hepatocellular carcinoma (HCC) is crucial for early detection against a world leader in cancer mortality. **Methods:** We leveraged existing liver and serum samples from rats ranging from 43-90 weeks old, which were part of a lifetime cancer bioassay. There were two groups in this sample set; group one (AFB₁) were orally dosed with aflatoxin and a vehicle solution. Group two (AFB₁ + CDDO-Im) were dosed with aflatoxin and a potent Nrf2 activator, CDDO-Im, as a chemoprotective agent. A high percentage of rats in the AFB₁ group (22/23 rats) developed HCCs, 96% incidence. CDDO-Im provided complete protection (20/20 rats) against liver cancer. Hepatic tissue (tumor and non-tumor) and sera were digested, and Cu was purified using AG-MP1 anion exchange resin in a class 1000 clean lab. Cu isotope ratios of all samples were measured by MC-ICP-MS in triplicates, and reproducibility was additionally verified with a secondary internal standard (ESPI, $\delta^{65}\text{Cu}_{\text{NIST976}} = +0.100 \pm 0.007\text{‰}$ (2SE, n = 36)). All standards and samples were doped with a certified ⁶⁹Ga/⁷¹Ga standard (NIST SRM 994) to correct for internal fractionation. **Results:** Comparison of $\delta^{65}\text{Cu}$ values between healthy hepatic tissue samples from the CDDO-Im group (n=9) and tumorous tissue samples from the AFB₁ group (n=17) showed an enrichment of ⁶⁵Cu in tumorous tissue. We find a significant pairwise distinction between healthy tissue (avg. $\delta^{65}\text{Cu}_{\text{NIST976}} = -0.58\text{‰}$) and tumorous tissue (avg. $\delta^{65}\text{Cu}_{\text{NIST976}} = -0.33\text{‰}$) at $p = 0.021$. We additionally measured tumor-adjacent, healthy tissue from individuals with HCC and found no significant trend. **Conclusions:** Our preliminary results suggest preferential transport/accumulation of ⁶⁵Cu in the site of hepatocellular oxidative stress resulting from aflatoxin exposure. Cu fractionation may be due to redox reactions as metalloproteins use Cu to catalyze the stabilization of reactive oxygen species. We anticipate reporting the results of serum samples (work ongoing) to reflect a mass-balanced depletion of ⁶⁵Cu when individuals have HCC. This work demonstrates interdisciplinary collaboration between geochemistry, toxicology, and biomedicine and may yield a powerful new biomarker for HCC.

PS 4638 Implication of route of blood collection on rodent clinical pathology and plasma drug level output

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Background and Purpose: Blood collection in rodents is a common practice employed throughout preclinical safety and efficacy research to monitor health status and as a typical biochemical endpoint indicating systemic and organ specific changes. Historic control values of routine biochemical parameters are maintained in the laboratory as standard ranges for comparison. As a part of this study we compared the key parameters of clinical pathology, hematology and clinical chemistry, and pharmacokinetics evaluation when samples were withdrawn via retrobulbar plexus (RO) and sublingual (SL) routes (with and without clearing for saliva) blood collection. **Methods:** Standard methods for sample collection were utilized in Sprague Dawley Rats for RO blood collection under CO₂/O₂ anesthesia (followed by propacaine administration for pain management). Blood was also withdrawn from the sublingual vein without the need for anesthesia. In a separate group of rats sublingual blood collection was performed following drying of the oral cavity using a cotton swab prior to each sampling for clinical pathology parameters. For the effect on pharmacokinetics, blood was withdrawn via both routes for plasma drug level comparison at three timepoints. **Results:** Absolute and percent leucocytes, absolute lymphocyte count, hemoglobin, WBC and MCV values were significantly different between RO and SL bleeds without swabbing. However, following swabs for

saliva reduction, absolute and percent basophils and eosinophils and MCHC values were significantly ($p < 0.05$) different between RO and SL. RBC values showed statistically significant difference ($p < 0.05$) between RO and SL with or without swabbing. Glucose, total bilirubin and AST values showed statistically significant difference ($p < 0.05$) between RO bleeding and SL with and without swabbing. Albumin, globulin and total protein showed significant difference from RO levels when SL was used without swabbing. Plasma levels of the test drug (A) in samples collected from sublingual route was consistently lower in all animals tested at all timepoints (0.25, 1 and 8 hrs. post dose) and up to a 21% lower was noted following SL blood collection at the earliest time point. These samples were not collected following oral swabbing. **Conclusions:** Oral swabbing made the SL data more comparable to the RO data for both hematology and clinical chemistry. Samples collected through SL appeared with mild to moderate levels of hemolysis at most instances. AST and total bilirubin were both analyzed using assays that are impacted by hemolysis and the levels may have been falsely elevated following SL bleeds due to hemolysis. More samples will be run for comparison between the routes to choose the parameters most likely to be acceptable through SL route. The SL route was a more efficient and less invasive technique and did not require the use of anesthesia for routine analysis.

PS 4639 Analytical method performance evaluation and its application to breath analysis

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Background and Purpose: Confident identification of human breath biomarkers lacks consistency across research laboratories, which may be due to methodological differences between research groups. This difference may be due to consistency in collection procedures, collection containers, storage container and temperature as well as lack of agreement on selection of breath reference standards for analytical methods used. Therefore, an approach was developed to ensure data from disparate laboratories conducting breath research can be reliably compared and validate the chemicals being identified or missed. **Methods:** This study developed a novel framework for assessing and comparing the Gas Chromatography-Mass Spectrometry (GC-MS) detectable chemical space by integrating Abraham solubility parameters of different stationary phases. **Results:** Retention and response prediction models were established to probe the breath chemicals for a detectable space and establish method comparison and performance parameters. A breath chemical space consisting of 1164 breath related volatile organic compounds (VOC's) has been established based on an in-depth literature search. From this chemical space VOC reference standards were carefully selected to represent over 95% of the functional groups present in the breath chemical space based on cumulative representation. We have developed a DB-624 and a PEG stationary phase model which allows for detection of additional compounds using analytical methods. **Conclusions:** Our established stationary phase models may aid in establishing method performance parameters for expanding detectability of specific regions within the breath VOC space.

PS 4640 Associations Between Occupational and Environmental Metal Exposure, Genotoxic Effects, and Haematological Alterations Among Nigerian Bronze Casters-Biomarker Patterns and Health Outcomes

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Background and Purpose: Nigerian bronze casters are routinely exposed to hazardous metals through traditional casting practices, particularly in underregulated environments, posing potential environmental and public health risk. While toxic metal exposure is widely recognized, its link to genotoxicity, immune suppression and haematological alterations in this population remains underexplored. This study investigates toxic metal exposure, associated biomarker changes, and potential health risks in Nigerian bronze casters. **Methods:** A cross-sectional study was conducted with 200 participants from Benin City, Nigeria, comprising 100 Benin Bronze Casters (BBC), 40 environmentally exposed individuals (EC), and 60 unexposed participants (UP). Blood samples were analyzed for toxic metal levels using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). An Enzyme-Linked Immunosorbent Assay (ELISA) was employed to assess biomarkers such as mutant p53 (m-p53), 8-hydroxy-2'-deoxyguanosine (8-OHdG), DNA methyltransferase-1 (DNMT-1), and Siglec-3 (CD33), while haematological indices were analyzed using a 3-part differential analyzer. **Results:** Bronze casters exhibited significantly elevated blood levels of cadmium, lead, mercury, and arsenic compared to the control group. Genotoxic biomarker m-p53 was upregulated in BBC and EC, while DNA methyltransferase-1 (DNMT-1) activity was reduced. Elevated oxidative DNA damage marker 8-OHdG and altered haematological indices suggested potential haematotoxicity. Siglec-3 (CD33) expression was notably higher in BBC (340.20 ± 19.35 pg/mL) compared to UP (233.00 ± 14.63 pg/mL), alongside significantly reduced WBC and lymphocyte counts. **Conclusions:** These findings reveal a heightened burden

of toxic metals in Nigerian bronze casters, with evidence of genotoxic, epigenotoxic, and hematological risks. Of particular note is the elevated CD33 levels, which potentially indicate an increased risk of inflammatory stress and immune dysfunction. This study underscores the need for enhanced environmental and occupational safety measures, biomonitoring protocols, and public health interventions to mitigate these risks. This may enhance the sustainability and economic viability of the historic bronze casting vocation in Nigeria.

PS 4641 Identification of transcriptional biomarkers for major molecular initiating events in rat liver carcinogenesis

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Background and Purpose: The two-year rodent bioassay has long served as the gold standard of chemical carcinogenicity testing. However, calls are growing for reevaluation of the effectiveness of the two-year bioassay based on numerous scientific, ethical, and practical concerns. There is also mounting pressure to move away from the use of animals in research in line with the Replacement, Reduction, and Refinement (3Rs) principles and increased support for New Approach Methodologies (NAMs). Consistent with these efforts, the Health and Environmental Sciences Institute (HESI) eSTAR Carcinogenomics Workgroup has adopted an AOP framework of key hepatic molecular initiating events (MIEs) and is developing biomarkers designed to detect MIE modulation in short-term rat studies to inform on tumorigenic risk. To this end, this study focuses on identifying specific gene expression changes after activation of critical nuclear receptors in the liver for each MIE, including Aryl Hydrocarbon Receptor (AhR), Constitutive Androstane Receptor (CAR), Pregnane X Receptor (PXR), Peroxisome Proliferator-Activated Receptor Alpha (PPARα), Estrogen Receptor Alpha (ERα), and tumor suppressor protein p53 using wild type (WT) and knockout (KO) rats on a Sprague-Dawley background. **Methods:** The study included male WT and KO rats corresponding to each MIE, except for ERα, which also included female rats. Three known activators were chosen for each MIE and administered at high doses known to exceed tumorigenic activation levels. Compounds were suspended in carboxymethylcellulose sodium (CMC Na) and administered to rats (n=5 per group) by oral gavage once daily for five days. Controls were only treated with vehicle. Rats were euthanized and samples collected on the sixth day. RNA-seq was performed on total RNA extracted from liver tissue, and differentially expressed genes (DEGs) between agonist and corresponding vehicle samples were assessed. In addition, Gene Set Enrichment Analysis (GSEA) was conducted to evaluate associated pathway changes. **Results:** Comparative analyses of DEGs induced by the three activators of each MIE were conducted to identify genes consistently modulated across treatments. In addition, comparison between WT and KO treated rats identified the respective transcription factor-dependent DEGs for each MIE. GSEA further identified common pathways and leading-edge (LE) genes associated with each of the compounds in an MIE-dependent manner. **Conclusions:** For each MIE, several genes were identified as regulated by all three activators in WT but not in KO rats, suggesting these genes could serve as MIE-dependent biomarkers. The sizes and membership of these gene sets were consistent with known MIE biology, which was supported by GSEA analysis. Collectively, these analyses identify candidate genes that can be used to support the development and validation of MIE-specific biomarkers to facilitate earlier detection of liver tumorigenic risk in short-term rat studies.

PS 4642 Persistent transcriptomic changes following repeated exposure to wildfire smoke in non-human primate airway epithelial cells

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Background and Purpose: Exposure to wildfire smoke poses significant public health concerns due to its association with adverse respiratory effects. Inhalation of wildfire smoke particulate matter can lead to airway inflammation, oxidative stress-induced lung injury, impaired mucociliary clearance, and exacerbation of respiratory conditions such as asthma and chronic obstructive pulmonary disease (COPD). Despite these well-documented health risks, the molecular mechanisms underlying the respiratory effects of wildfire smoke exposure remain incompletely understood. In this study, we utilized transcriptomic and epigenomic analyses to investigate how exposure to simulated wildfire smoke influences the expression patterns of gene networks in differentiated rhesus tracheobronchial epithelial cells. **Methods:** Primary tracheobronchial epithelial cells from four adult female animals (ages 3-14yrs) were cultured under air-liquid interface conditions. They were exposed to lab-simulated wildfire smoke (WS) or filtered air (FA) 6 hours per day, either for 1 day, for 5 consecutive days, or for 5 consecutive days followed by a one-week recovery period. The PM concentration was 242.8 µg/m³ for the single-day exposure and averaged 166.66 µg/m³ over 5-day exposure. RNA sequencing was performed at all three timepoints to characterize transcriptomic alterations and differentially expressed genes (DEGs) between WS- vs FA-exposed cells were identified using

DESeq2. Gene expression data were also analyzed using WGCNA (Weighted Gene Co-expression Network Analysis, using WGCNA package in R) to identify modules of co-expressed genes and relate these modules to WS exposure. Whole-genome bisulfite sequencing was performed on the cells with 5-day exposure and differentially methylated regions (DMRs) between WS- vs FA-exposed cells were identified using DMRichR. Correlations between gene expression and DNA methylation were performed. Representation of lung disease-associated transcriptomic signatures among exposure-induced changes were evaluated. Ingenuity Pathway analysis was used to identify enriched pathways among DEGs, genes annotated to DMRs, and gene modules associated with exposures. **Results:** Our analysis identified 39, 615 and 680 WS associated DEGs at three time points, respectively. IPA revealed significant enrichment for Aryl Hydrocarbon Receptor (AHR) Signaling at the first two time points, and pulmonary healing signaling, TGF-β signaling, and regulation of the EMT by growth factors at the recovery time point. We also found three modules with significant correlations with WS exposure, with the most significantly correlated module enriched for genes in epithelial remodeling, inflammation/immune signaling, epigenetic regulation, and apoptosis/cell cycle control. 8,045 differentially methylated regions (DMRs) were detected, located proximal to genes with significant enrichment for xenobiotic metabolism, cytokine signaling, neuroinflammation signaling, pulmonary healing signaling, and regulation of the EMT by growth factors, which highlights potential regulatory mechanisms underlying the molecular response to WS exposure. While global correlations between DNA methylation and gene expression were limited, subsets of genes involved in transcriptional regulation and respiratory disease showed significant associations. Lastly, we found that exposure-induced transcriptomic signatures overlapped with those of chronic respiratory diseases, particularly COPD and idiopathic pulmonary fibrosis. **Conclusions:** Our study demonstrates that repeated wildfire smoke exposure provokes persistent transcriptional and epigenetic reprogramming in the airway epithelium involved in the disruption of pathways central to inflammation, repair, and remodeling. These findings extended our previous work on early-life exposures and highlighted the complexity of airway epithelial responses to repeated smoke inhalation. Together, these data underscored the potential for WS to leave a lasting molecular imprint on the airway epithelium, with important implications for respiratory health in populations exposed to wildfire events.

PS 4643 Ancestral Bisphenol A Exposure Resulted in Sexually Dimorphic Liver Disease, Mitochondrial Complex Suppression, and Gene-Body CpG Island Methylation

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Background and Purpose: Environmental stressors can produce heritable phenotypes through germline-mediated epigenetic alterations, yet the underlying mechanisms through which these changes drive transgenerational disease are not well defined. Bisphenol A (BPA), a widespread endocrine disruptor, is linked to metabolic liver disease and offers a relevant model for studying inherited risks without direct exposure in descendants. Using medaka, a vertebrate model with established BPA transgenerational responses, this study examined the unexposed F2 offspring derived from BPA-exposed F0 ancestors to assess sex-specific liver disease outcomes. F2 medaka developed sexually dimorphic NAFLD/MAFLD phenotype characterized by more severe macrovesicular steatosis in females and milder microvesicular changes in males. The objective was to mechanistically define these sex-specific disease manifestations by profiling hepatic transcriptomes and epigenomes of the F2 livers, thereby linking ancestral exposure to divergent molecular programs in female versus male livers. **Methods:** Hepatic transcriptomes from F2 females and males were analyzed by RNA-seq after QC with fastp, STAR alignment to the medaka reference genome, and DESeq2 differential expression (FDR < 0.05), followed by KEGG pathway and gene-set enrichment, and comparison to a published 25-gene advanced NAFLD signature. Additionally, whole-genome bisulfite libraries were prepared with NEBNext Ultra II FS and processed via nf-core/methylseq (Bismark) to obtain CpG-level coverage and methylation profiles. Each group comprised three biological replicates from the BPA lineage and matched F2 controls derived from F0 unexposed ancestors. **Results:** In the unexposed F2 generation medaka, ancestral BPA exposure resulted in a strong female-biased liver transcriptomic response, with 5,376 DEGs in females versus 306 in males, and only female DEGs enriched for human NAFLD, including concordant changes in the markers *HSD17B14*, *AKR1B10*, and *RGS4*. Female livers showed suppression of core metabolic pathways including PPAR, AMPK, insulin signaling, cholesterol metabolism, and fatty acid degradation, while males exhibited modest downregulation centered on steroid and terpenoid backbone biosynthesis. All mitochondrially encoded subunits of oxidative phosphorylation, spanning Complex I to Complex V, were downregulated only in females, indicating impaired oxidative phosphorylation. Key metabolic transcription factors (TFs) were repressed in a sex-specific manner, with *SREBF1* silenced in males and *HNF4A* in females, accompanied by promoter hypermethylation and concordant repression of their target genes. Whole genome methylome profiling revealed a substantially larger burden of liver differentially methylated regions (DMRs) in females with only limited overlap between sexes. DMRs in both sexes were preferentially enriched in CpG islands, exons, and 3' UTRs, whereas core promoter DMRs showed little pathway signal, indicating that promoter proximal DMRs likely played a minor role in gene regulation compared with exon and 3' UTR. Liver CpG island methylation changes localized mainly to gene bodies, with a greater burden in females, pointing to regula-

tion through gene body architecture rather than promoter sites. Female DMRs were selectively enriched for liver centric metabolic and pro fibrotic programs such as TCA cycle, carbon metabolism, TGF beta, and Notch signaling, consistent with advanced liver disease, whereas males showed fewer endocrine or signaling oriented pathways, including cortisol synthesis and oxytocin signaling, that are less directly tied to hepatic metabolic injury. **Conclusions:** Ancestral BPA exposure produced sex-divergent liver disease in unexposed medaka offspring: females displayed advanced NAFLD-like transcriptional programs with suppression of core metabolic pathways and coordinated downregulation of mitochondrial OXPHOS complexes I-V, whereas males showed a muted response. Epigenomic profiling revealed substantially more DMRs in females, enriched in CpG islands within gene bodies, exons, and 3' UTRs, implicating DNA methylation in sex-specific metabolic dysregulation with minimal changes at promoters.

PS 4644 Inorganic Arsenic Reprograms a Placental miRNA-mRNA Axis Governing Structural and Stress Adaptation

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Background and Purpose: Chronic exposure to inorganic arsenic (iAs) in drinking water remains a primary global public-health concern and is linked to pregnancy complications and long-term disease risk in offspring. The placenta, a key mediator of nutrient exchange and endocrine and immune signaling, is highly sensitive to environmental insults during early development. Proper placental function depends on coordinated molecular programs governing structural maintenance, vascular remodeling, and stress adaptation. However, the mechanisms by which low iAs exposure perturbs these processes remain poorly understood. We hypothesize that the mechanisms for iAs-promoted dysfunctional reprogramming of placental development involve disruption of placental miRNA-mRNA regulatory networks that orchestrate post-transcriptional developmental regulation. **Methods:** Female C57BL/6J mice were given 10 ppb sodium arsenite in drinking water two weeks before conception through embryonic day 16. Placental miRNA and mRNA expression profiles were integrated using sparse partial least squares (sPLS) for pairwise cross-omics correlation and the DIABLO framework for supervised multi-omics integration. Correlated molecular pairs ($|r| > 0.8$) were visualized by Circos plots, and enrichment of mRNA partners was used to infer pathways represented within each miRNA module. **Results:** Multivariate integration revealed a distinct molecular signature separating iAs-exposed from control placentas, driven by coordinated downregulation of five miRNAs, miR-450a, miR-467d, miR-92a, miR-15b, and miR-295. These miRNAs formed a tightly connected repressive axis, with correlated mRNA targets enriched for adhesion, mitochondrial, and oxidative-stress pathways, suggesting coordinated alterations in placental structural integrity and stress-adaptation programs. Within this network, miR-92a and miR-15b emerged as nodal regulators negatively correlated with Itga5 and other adhesion-related genes, implicating altered placental-endothelial interactions. In parallel, miR-450a, miR-467d, and miR-295 were inversely associated with stress-responsive transcripts, including Hspa5, Hyou1, Gpx3, Ldha, and Cox5a, indicating activation of endoplasmic reticulum and oxidative stress responses. Collectively, these interactions delineate an iAs-responsive regulatory network that coordinates placental structural integrity and cellular stress adaptation, which are fundamental to maintaining placental homeostasis. **Conclusions:** iAs exposure reconfigures a placental miRNA-mRNA network that bridges structural and stress-regulatory pathways. Given that the placenta acts as a critical interface between maternal and fetal environments, perturbation of this regulatory axis provides a mechanistic basis for how early-life exposure to iAs may translate into developmental vulnerability and adult disease risk. *Supported by NIEHS grants R01ES034760 (WT) and R01ES033519-05 (AB, WT).*

PS 4645 Unraveling Benzo[a]pyrene-induced epigenetic modifications in Japanese medaka

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Background and Purpose: The transmission of toxicant-induced phenotypes across generations has emerged as a critical topic in toxicology and epigenetics. Mounting evidence suggests that benzo[a]pyrene (BaP) and its metabolite BaP diol epoxide (BPDE) play a role in epigenetic regulation, potentially altering chromatin accessibility and gene expression patterns in germ cells through modification of epigenetic enzyme expression persistently affecting the offspring phenotype for three generations. Delineating epigenetic modifications in oocytes, sperm, ovary tissue and testis tissue will shed light on maternal and paternal contributions to epigenetic inheritance. It is hypothesized that epigenetic modifications in both, sperm and oocytes, contribute to the BaP-induced transgenerational phenotype. **Methods:** Ten pairs of adult medaka fish (*Oryzias latipes*; n=5) were exposed to solvent control or 1 µg/L BaP or for 21 days. Eggs were collected after the exposures ended and raised to 4 months old. This was repeated for 3 generations. Sperm, testis tissue, small oocytes, large oocytes and ovary tissue (n=5) were dissected from F3 adults and DNA extracted for 3piRAD, a refined EpiRAD/HELP single serial digestion assay with

MfeI, HpaII and MspI digestion. Libraries were sequenced with Illumina NovaSeq and data were filtered, cleaned, trimmed and mapped to the *O. latipes* reference genome. Methylation rates are calculated based on the proportion of reads mapping to methylated versus unmethylated fragments. Downstream analysis is conducted using R and Bioconductor packages, such as DSS, to identify differentially methylated regions (DMRs) between tissues or treatments. gProfiler was used for GO/KEGG pathway analyses. **Results:** Overall, ancestral BaP exposure significantly changed DNA methylation in a bi-directional manner in all tested tissues of the unexposed F₃ generation. A shift from hypermethylation to hypomethylation was particularly noted for sperm and testis tissue, while small oocytes, large oocytes and ovary tissue tended towards increased hypermethylation. These methylation profile changes were enriched for genes which were significantly associated with the human pathology pathways for abnormal skeletal morphology and abnormality of the skeletal system as well as abnormal brain morphology and abnormality of the nervous system. These pathologies have been as well identified on the phenotypic level. Distinct analyses of tissue type contrasts will shed further light on maternal and paternal contribution to BaP exposure modified epigenetic profile inheritance. **Conclusions:** The pronounced methylation differences observed in the F₃ generation indicate that ancestral BaP exposure in the F₀ lineage induced stable, heritable epigenetic alterations that persist across multiple generations in *Oryzias latipes*. Because F₃ individuals were never directly exposed to BaP, these methylation signatures represent true transgenerational epimutations, transmitted through the germline. The strong bias toward hypermethylation in sperm, contrasted with hypomethylation in oogenic and somatic gonadal tissues, suggests sex-specific epigenetic inheritance mechanisms. Similar patterns have been reported in mammalian and fish models following ancestral hydrocarbon, endocrine disruptor, or heavy-metal exposure, where F₃ progeny exhibited altered sperm methylation, differential expression of chromatin and developmental genes, and reduced fertility. The persistence of these methylation marks in the absence of direct exposure implies incomplete erasure or active maintenance during germline reprogramming, potentially via dysregulated DNA methyltransferase activity or chromatin remodeling factors. Collectively, these findings demonstrate that BaP exposure in ancestral lineages can reprogram the epigenome in a manner that is both tissue-specific and heritable, providing mechanistic evidence that environmental contaminants can drive long-term, transgenerational changes in reproductive epigenetic regulation.

PS 4646 Perinatal lead (Pb) exposure impacts on DNA methylation and hydroxymethylation in mouse hippocampus

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Background and Purpose: Environmental exposure to metals are important modifiable risk factors for neurological diseases. Epidemiological studies have found that exposure to lead (Pb) is associated with cognitive decline or Alzheimer's Disease and related dementias (ADRD). Toxicology studies have found that Pb exposure is linked to ADRD-associated molecular changes, pathology, and behavior. However, more understanding of the epigenetic consequences of Pb exposure in the brain, the specific tissue of interest for neurological outcomes, is necessary. In particular, hydroxymethylation is a less studied epigenetic modification enriched in brain tissue. **Methods:** C57BL/6J adult female mice 6-8 weeks of age were exposed to either control water or Pb acetate water (32 ppm) two weeks preconception and through weaning. At weaning (post-natal day 21), offspring were sacrificed to collect tissue, including hippocampus, and DNA was extracted from 6 control and 6 Pb exposed mice (3 male and 3 female in each group). We sequenced DNA on the Oxford Nanopore Technology PromethION platform, multiplexing two samples per flow cell. Basecalling was done in dorado software using the dna_r10.4.1_e8.2_400bps_sup@v5.2.0 basecalling model to measure methylation (5-mC) and hydroxymethylation (5-hmC) base modifications at CpG sites across the genome, and mapped to the mm10 mouse genome build. We used the dmrseq package to test for differentially methylated regions and differentially hydroxymethylated regions by Pb exposure. **Results:** We measured modifications at 14.4 million CpG sites with at least 10x coverage in all samples. Across these sites, we observed a global average of 69.3% methylation and 12.2% hydroxymethylation. Global methylation (0.35% higher in Pb exposed, p-value=0.4) and hydroxymethylation (-0.31% lower in Pb exposed, p=0.5) did not differ by treatment. Two regions were nominally associated (p-value<0.01) with Pb exposure-related DNA methylation changes in the hippocampus, in the genes *Grb10* and *Celf2*. At the *Grb10* region, Pb exposed mice had 12.9% higher DNA methylation than controls. At the *Celf2* region, Pb exposed mice had 15.7% higher DNA methylation than controls. For hydroxymethylation, 75 regions were nominally associated (p-value<0.01) with Pb exposure, including genes *Cdk12*, *Enox1*, *Crim1*, *Dlg1*, *Mir708*, *Tenm4*, *Nrg1*, *Cracd*, *Pcp4*, *Igfsf5*, *Ppip5k2*, *Pik3cb*, *Kcnma1*, *Aak1*, *Dab1*, *Rbfox1*, *Nell2*, *Syne2*, *Tbc1d9*, *Stxbp6*, *Klc1*, *D030068K23Rik*, *Plcl2*, *Eva1c*, *Chrm3*, *Rabl3*, *Rpl9*, *Lias*, *Klb*, *Sash1*, *Psen2*, *Ank2*, *Gm16958*, *Prpf18*, *Gad1l1*, *Herc2*, *Wdr7*, *Cckbr*, *Dym*, *Srrm4*, *Robo1*, *Bank1*, *Runx1t1*, *Rab3c*, *Gm5108*, *Mybbp1a*, *Ntrk2*, *Snappc3*, *Psp1*, *Gm3693*, *Slc8a3*, *Rnft2*, *Pam*, *Dock4*, *Magi1*, *Zp3*, *Slc8a1*, *2210408F21Rik*. Genes related to hydroxymethylation were enriched (FDR<0.1) in gene ontology pathways, including intracellular signal transduction, nervous system development, and ensheathment of neurons. **Conclusions:** This study identified several genes that may be epigenetically impacted in the brain by perinatal Pb exposure. These genes are involved in cell signaling and ensheathment of neurons, processes that may be disrupted by Pb exposure. Commonly used bisulfite conversion methods to measure

methylation do not allow for differentiation of 5-mC and 5-hmC, which have different biological functions and may be impacted differently by exposures. In our study, hydroxymethylation was found to be more strongly impacted, underlying the importance of its study in brain tissue and use of techniques to separately measure 5-mC and 5-hmC.

PS 4647 DNA Methylation Signatures for Fetal Alcohol Spectrum Disorder Prediction

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Background and Purpose: Prenatal alcohol exposure is a major preventable cause of neurodevelopmental disability and can lead to the spectrum of outcomes known as fetal alcohol spectrum disorder (FASD). Affecting 2-5% of North American children, the diagnosis of FASD remains challenging due to the lack of standardized diagnostic criteria and the wide variability in neurobehavioral manifestations. Meanwhile, prenatal alcohol exposure could induce widespread and persistent epigenetic disruption including DNA methylation (DNAm) alterations that may serve as molecular biomarkers of developmental toxicity. In this study, we developed and validated a DNAm-based prediction model to identify childhood FASD using non-invasive biospecimens, and further explored different DNAm patterns in healthy and FASD groups. **Methods:** We harmonized three public Illumina 450K datasets related to FASD through a unified pipeline. After batch correction, we constructed a buccal dataset consisting of 260 samples with 135 FASD cases. Another dataset of 134 peripheral blood samples was used as tissue-independent external validation. We first conducted feature selection using random forest model under multiple thresholds. The identified CpG features were then used to build machine learning models for FASD prediction. Model performances were evaluated through 5-fold cross validation. Metrics such as accuracy, area under the receiver operating characteristic curve (AUC-ROC), sensitivity, specificity, precision, and F1 score were used for model evaluation. Finally, we explored the co-methylation network patterns between FASD and control groups using graph learning. **Results:** Our random forest model achieved 81.1% accuracy, 89.0% AUC-ROC, 77.2% sensitivity, 85.6% specificity, 85.4% precision, and 80.7% F1 score in FASD prediction with the buccal dataset. External validation in peripheral blood yielded 77.9% accuracy, 86.3% AUC-ROC, 96.4% specificity, and 88.4% precision, while sensitivity dropped to 42.8% (F1=55.9%), potentially reflecting tissue-specific methylation differences. Preliminary network analysis demonstrated alcohol-induced alterations in coordinated methylation patterns. **Conclusions:** Our findings suggest that DNAm signatures may predict FASD using pediatric buccal samples, with preliminary evidence of cross-tissue applicability in peripheral blood. The developed model leverages DNAm data as biomarkers to complement existing behavioral and morphological criteria for FASD diagnosis. This study also contributes to molecular toxicology by exploring coordinated methylation patterns between FASD and control groups. Prospective validation in larger, clinically diverse cohorts is needed to further assess whether such methylation signatures could support early diagnosis.

PS 4648 Epigenetic alterations of histone post-translational modifications in response to alcohol

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Background and Purpose: Alcohol consumption prenatally is associated with various adverse reproductive health outcomes, such as fetal alcohol spectrum disorders which can span behavioral issues and developmental defects. In *Caenorhabditis elegans* it has furthermore been demonstrated with snRNA-seq that parental consumption of alcohol can alter the transcriptome in F1 and F3 generations without additional alcohol exposure, as well as promote reproductive health effects such as germline dysfunction and embryonic lethality (Truong et. al, 2023). Evidence also suggests that alcohol can directly alter histone acetylation levels (Mews et. al, 2019), potentially providing a mechanism for transmission of negative reproductive outcomes driven by ancestral alcohol exposure. This project aims to discern the mechanism through which multigenerational reproductive outcomes propagate employing the model nematode *C. elegans*. **Methods:** In this study, we cultured *C. elegans* in M9 media supplemented with OP50-1 bacteria at the L4 stage for 48 hours. Animals were exposed to USP grade ethanol at a dose of 0.5%, in accordance with previous literature. *C. elegans* are self-fertilizing, produce large broods and have a rapid generation time (~ 3 days) making them ideal for multigenerational studies. Additionally, their spatiotemporally regulated germline allows for analysis of multiple meiotic stages at one timepoint. To this end, adult P0 and F1 animals were dissected to analyze alterations in the germline to Pan H3ac, H3K9ac, H3K27ac and H3K56ac via immunofluorescent in response to ancestral alcohol. For western blotting analysis histones were enriched using an EpiQuik Total Histone extraction kit, and Pan H3ac, H3K9ac, H3K27ac and H3K56ac were analyzed quantitatively in Fiji. **Results:** Preliminary data shows that P0 animals show elevated levels of pan H3ac in response to 0.5% alcohol exposure via immunofluorescence, with differential levels also observed in the F1 generation. Western blot analysis also appears to demonstrate elevated H3K27ac in P0 animals, with differential expression also observed in

H3K9ac. **Conclusions:** Alcohol exposure appears to alter the histone PTM landscape in P0 animals, of which some of these effects can be observed over multiple generations. This potentially provides a mechanism for multigenerational phenotypes stemming from ancestral alcohol exposure.

PS 4649 Mitochondrial Dysfunction in Hemin-Induced Acute Kidney Injury is Mediated by Guanine Quadruplex Dynamics

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Background and Purpose: Proximal tubule cells are dependent on energy from mitochondria for their transporter activity and transcription of mtDNA is essential for oxidative phosphorylation. Previously, we identified that transcription by the mitochondrial RNA polymerase (POLRMT) is regulated by formation of guanine-quadruplexes (G4) in nascent RNA. Hemin is a known endogenous G4-stabilizing ligand, and it accumulates in the kidney under conditions associated with acute kidney injury (AKI) such as sepsis, rhabdomyolysis, and ischemia-reperfusion injury (IRI). The purpose of this study is to determine whether free heme during AKI stabilizes G4 structures in the mitochondria of renal proximal tubules, impairing transcription of mtDNA and contributing to mitochondrial dysfunction following injury. **Methods:** We used circular dichroism analysis and an *in vitro* primer extension assay to measure the stability of G4s and transcriptional changes induced by the presence of hemin. In a mouse model of kidney IRI, we measured the intracellular distribution and colocalization of G4s with heme oxygenase (HMOX1) expression in proximal tubules 6 hours to 4 weeks post-IRI. Using a machine learning algorithm to delineate proximal tubules within whole slide scans of cortical kidney biopsies from 6 human donors, we measured the abundance of cytoplasmic G4s in tubular versus glomerular tissue and correlated the intensity of cytoplasmic G4s with HMOX1 expression for each donor. We also treated human proximal tubule epithelial cells (PTCs) in serum-free culture with non-cytotoxic doses of hemin for 1-24 hours. Heme-bound G4s were biotinylated in live PTCs using a peroxidase reaction and probed with streptavidin to determine the intracellular distribution of G4s stabilized by hemin with confocal microscopy. Gene expression in PTCs was analyzed by qPCR and mitochondrial function was measured using Seahorse extracellular flux assay. **Results:** Hemin stabilized RNA G4s, evidenced by increased melting temperature of RNA G4 structures and impaired transcription by POLRMT in acellular assays. Proximal tubules from mouse IRI kidneys had higher HMOX1 expression, indicating the presence of free heme, and more G4 structures at 6-48 hours post-IRI, returning to contralateral control levels within 2 weeks. Biopsies from human donor kidneys had significantly higher cytoplasmic G4 abundance in proximal tubule tissue compared to glomeruli and a positive correlation ($r > 0.7$) was found between the expression of HMOX1 in each tubule and the appearance of cytoplasmic G4 structures. Human PTCs exposed to free hemin revealed a dose-dependent increase in G4 abundance which peaked at 1-6 hours. Intracellular distribution of heme-bound G4s was primarily cytoplasmic and colocalized with mitochondrial mass (Spearman coefficient > 0.5). Mitochondrial-encoded OXPHOS genes were downregulated by hemin exposure while nuclear-encoded transcription factors TFAM and TEFM were unaffected. Uncoupled oxygen consumption rates in PTCs were significantly decreased after 24 hours of hemin exposure. **Conclusions:** These results show that hemin stabilizes G4s in proximal tubule cells and impairs mitochondrial gene transcription and function. We conclude that stabilization of G4 structures and consequent dysregulation of POLRMT transcription is an additional mechanism mediating mitochondrial dysfunction in proximal tubules during hemin-induced AKI.

PS 4650 Genome-Wide Redistribution of Newly Synthesized Histones in Human Cells Following Formaldehyde Exposure

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Background and Purpose: Formaldehyde (FA), a reactive aldehyde widely present in the environment, is a known carcinogen associated with nasopharyngeal cancer and has also been implicated in myeloid leukemia and neurodegenerative disease. Recent studies indicate that exogenous FA induces only a modest increase in DNA adduct formation compared with the levels generated endogenously, suggesting that epigenetic mechanisms may play a key role in FA-mediated toxicity and disease. We previously reported that FA exposure inhibits the nuclear translocation of newly synthesized variant histone H3.3 from the cytoplasm. However, how this impairment influences the genome-wide incorporation of newly synthesized H3.3 into chromatin has remained unclear. **Methods:** To investigate the effects of FA exposure on global chromatin assembly of newly synthesized histones, we utilized a SNAP-tagged histone expression system to selectively label nascent histones. BEAS2B-SNAP-H3.3 stable cell lines were first treated with SNAP-Cell Block to quench preexisting SNAP-H3.3. After removing the blocking agent, cells underwent a "chase" period during which newly synthesized H3.3 accumulated in the presence or absence of 100 μ M FA for 24 hours. Following exposure, mono- and dinucleosomes were isolated, and nucleosomes containing newly synthesized SNAP-H3.3

were biotinylated with SNAP-Biotin ("pulse"). Biotinylated nucleosomes were then immunoprecipitated using streptavidin-conjugated beads. ChIP-seq libraries were prepared and sequenced on an Illumina NovaSeq X Plus platform to generate 2x150 bp paired-end reads. Sequencing reads were aligned to the human reference genome (hg38), and ChIP-enriched regions (peaks) were identified from deduplicated BAM files using MACS2. **Results:** The ChIP-seq analysis revealed extensive genome-wide changes in chromatin distribution of newly synthesized H3.3 following FA exposure. Occupancy analysis identified 46,548 H3.3 sites unique to controls, 139,608 unique to FA-exposed samples, and 78,992 shared loci, indicating both loss and gain of H3.3 incorporation across the genome. Differential peak analysis (DESeq2) further identified 1,603 regions with increased and 1,577 with decreased H3.3 occupancy ($p < 0.01$, $|\log_2\text{FC}| \geq 0.5$). Genomic annotation showed that sites with reduced occupancy were primarily located in distal intergenic and intronic regions, while those with increased occupancy were enriched near promoters, suggesting that FA exposure redistributes newly synthesized H3.3 from distal intergenic to promoter-proximal regions. Pathway and GO analyses linked these occupancy changes to mRNA splicing and RHO GTPase signaling, while ENCODE histone modification analysis associated the affected loci with H3K27me3, H3K27ac, and H3K79me2 marks. Notably, Ingenuity Pathway Analysis identified "cancer" as the top disease category, with head and neck carcinoma—including nasopharyngeal cancer, a known FA-related malignancy—among the most enriched terms. **Conclusions:** These results suggest that FA exposure induces a genome-wide redistribution of newly synthesized H3.3 that perturbs chromatin organization and gene regulatory pathways, potentially contributing to nasopharyngeal carcinogenesis.

PS 4651 Exploring Tissue-Specific DNA Co-Methylation Network dynamics During Human Aging

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Background and Purpose: Aging is a complex process with widespread molecular alterations across tissues. Recent multi-omics profiling has revealed nonlinear dynamics of individual biomarkers during aging. However, how the interactions and correlations among biomarkers change over time remains understudied. Meanwhile, DNA methylation (DNAm) exhibits systematic age-associated shifts that have enabled the development of epigenetic clocks capable of estimating biological aging. DNA co-methylation networks could further capture coordinated regulatory patterns. Yet, it remains unclear whether aging involves tissue-specific dynamic changes of DNAm co-methylation networks and how these dynamics unfold during human aging. We thus investigated age-dependent changes in DNA co-methylation network structures across multiple human tissues to characterize regulatory coordination patterns complementing current studies on nonlinear aging dynamics. **Methods:** We analyzed DNA methylation of 163 samples (ages 19-70 years) profiled on Illumina MethylationEPIC v2.0 across buccal epithelium, peripheral blood mononuclear cells, saliva, and dried blood spots. Tissue-specific age-predictive CpGs were identified through Random Forest feature selection with 5-fold cross-validation and importance-threshold optimization. Weighted co-methylation networks were constructed using absolute Pearson correlations for three age groups (19-30, 31-50, 51-70 years). Network properties were quantified via mean edge weight, average weighted degree, weighted clustering coefficient, and modularity. External validation employed whole blood samples (GSE40279, n=656, ages 19-101) and combined buccal datasets (GSE50759, GSE94876, GSE113018, n=180, ages 0-60). CpGs from widely used aging clocks (e.g., Horvath pan-tissue; Hannum blood; Johnson buccal) were used for comparison. Gene ontology and KEGG pathway enrichment analysis were conducted for tissue-specific CpGs. **Results:** We identified tissue-specific CpGs that could achieve robust age prediction ($R^2=0.69-0.87$, $\text{MAE}=4.52-7.23$ years) with random forest models. Network analysis on co-methylation networks under different age groups showed diverse patterns across tissues. For instance, mean edge weight, average weighted degree, and weighted clustering coefficient of buccal co-methylation networks declined from early to mid-adulthood and then increased in late adulthood. In contrast, PBMC, saliva, and DBS showed progressive declines in mean edge weight and average weighted degree across adulthood, while weighted clustering coefficient exhibited a shallow midlife rise followed by a late-life decrease. Modularity tended to decline across tissues, indicating erosion of coordinated community structure despite divergent connectivity patterns. Validation in whole blood dataset confirmed early-to-midlife mean edge weight and average weighted degree decline with late-life stabilization across multiple CpG selection strategies. Validation in buccal dataset showed higher mean edge weight, average weighted degree, and weighted clustering coefficient in childhood, decreasing through adolescence into adulthood. Functional enrichment identified several biological pathways including mitochondrial/apoptotic regulation. **Conclusions:** Our findings suggest tissue-specific nonlinear dynamics in DNA co-methylation networks. Mean edge weight and average weighted degree, which reflecting network connectivity, showed mid-life decline and late-life increase in buccal and progressive decline in blood-derived tissues, consistent with distinct tissue-specific aging patterns. Weighted clustering coefficient, capturing local network density, exhibited similar tissue-dependent nonlinear patterns. The modularity decline across tissues indicates common loss of coordinated epigenetic structure.

PS 4652 Placental miRNA responses to prenatal cannabis, tobacco and alcohol exposure in Rhode Island, US

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Background and Purpose: Tobacco and alcohol use are declining while cannabis use is on the rise in the US. These substances are associated with adverse maternal-child health outcomes, although the impact of cannabis is less clear. Dysregulated placental epigenetics is a potential mechanism underlying these effects. Past epidemiological studies have mostly examined effects related to any or no use of each substance in isolation which underestimates the effects of co-use, dose and trimester-specific contributions. While many previous studies have examined the effects on placental DNA methylation or gene expression, little is known about the short-lived epigenetic regulators, miRNAs, which can block protein translation, impacting multiple biological processes. This study examines the dose- and trimester-specific placental miRNA responses to prenatal cannabis, tobacco and alcohol co-exposures. **Methods:** An ethnographically diverse sample of 133 pregnancies, 29% reporting low income, were oversampled for cannabis use from New England (the Behavior and Mood in Babies and Mothers cohort). Small RNA sequencing was performed on placental samples with 621 miRNAs passing QC and processing. Daily cannabis, tobacco, and alcohol use during and 3 months prior to pregnancy were collected via timeline follow-back interviews and biochemically verified. Latent profile analysis (LPA) was used to identify dose-based classes of prenatal cannabis exposures and secondarily to identify co-use classes of cannabis, tobacco and alcohol. Untargeted miRNA-wide associations with dose-specific profiles of cannabis only use and co-use were examined using separate negative binomial regressions. Models adjusted for infant sex, socioeconomic status, race/ethnicity, maternal age, and two surrogate variables capturing unmeasured confounding. P-values were corrected for false-discovery rate (q-value < 0.10). Sensitivity of effects to confounding by gestational age was examined. Functional effects were searched in KEGG, Reactome, and WikiPathways. **Results:** Participants reported no substance use (44%), cannabis only (29%), tobacco only (4.5%), and co-use (23%) prenatally. LPA identified three exposure profiles, that broadly reflected high, moderate, and low exposures. By itself, prenatal cannabis use had dose-specific effects on placental miRNAs while adjusting for tobacco and alcohol use. High cannabis exposures were associated with altered miR-4521 ($\log_2$ fold change (LFC) = 1.16; $q = 0.08$; 1436 mRNA targets) and miR-3925-5p (LFC = -0.85; $q = 0.08$; 119 mRNA targets) while medium cannabis exposures had no associations compared to low exposures. Cannabis, tobacco and alcohol co-exposures altered placental miRNAs in a dose-dependent manner with more changes related to moderate exposures. Compared to low exposures, high exposures perturbed miR-421 (LFC = -0.23; $q = 0.09$) which potentially targets 1436 mRNAs. Medium exposures associated with downregulation of miR-6501-3p (LFC = -0.53; $q = 0.06$) and upregulation of miR-483-5p (LFC = 0.44; $q = 0.06$); miR-1180-3p (LFC = 0.59; $q = 0.06$); and miR-365b-5p (LFC = 1.50; $q = 0.06$). These miRNAs' mRNA targets had functional effects on RNA metabolism, cell response to stimuli and stress (Reactome); VEGF signaling (WikiPathways); and spliceosome, neurodegeneration and Alzheimer disease pathways (KEGG). After gestational age adjustments, only miRNAs associated with high exposures remained significant. **Conclusions:** Using exposure profiling and untargeted small RNA-seq, this is the first study to describe the link between prenatal cannabis, tobacco and alcohol co-exposures and placental miRNAs and functioning. In a birth cohort oversampled for cannabis use, this analysis identified miRNAs associated with high cannabis exposures as well as high and moderate co-use, although these may be driven by the effects of substance use on gestational age at birth. Processes (e.g., RNA metabolism, stress, VEGF, neurodegeneration) that influence placental growth, vascular signaling and neurodevelopment, were perturbed suggesting their altered regulation could affect pregnancy outcomes.

PS 4653 Metaboloepigenetic mechanisms of transgenerational alcohol-seeking behavior

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Background and Purpose: The fetal brain is particularly vulnerable to ethanol's neurotoxic effects. Exposure during fetal development can cause irreversible damage, leading to long-term cognitive and behavioral deficits that fall under the umbrella of fetal alcohol spectrum disorders (FASD). Human studies on the multi- and transgenerational effects of alcohol have suggested that neurobehavioral FASD features, including alcohol preference, can be passed across generations. However, the underlying mechanisms of transgenerational FASD inheritance remain largely unknown. **Methods:** We used the model organism *Caenorhabditis elegans* to test the hypothesis that ethanol metabolism directly leads to increased, transgenerationally stable histone acetylation that affects ethanol-related behaviors. Wild-type animals and animals carrying loss-of-function mutations in conserved ethanol metabolism genes (alcohol dehydrogenase, *adh-1*, and acetyl coenzyme A synthetase 2, *acs-19*) were exposed to physiological ethanol levels (0.05%, 0.1%, and 0.5%) and tested using behavioral and molecular assays including ethanol preference, crawling speed, food race, Western blotting, fluorescence microscopy, and mass spectrometry. **Results:** While ethanol acted as a mild aversive cue for naïve animals, 48-hour exposure to all tested doses induced attraction to ethanol. This switch from aversion

to attraction persisted to the F3 generation following a single parental exposure at the two higher doses. The ethanol preference switch was not merely a result of decreased locomotion, as crawling speed showed no decrease or increased 1 hour after exposure in P0, F1, and F3 generations. Animals tested on a more complex locomotory task (moving towards a food source) showed ethanol-dependent performance decreases at P0 and F1, though not F3, generations. Animals lacking functional *adh-1* or *acs-19* did not display an ethanol preference switch at P0, F1, or F3 generations, indicating that metabolism of ethanol into acetyl-CoA—the key molecule used to acetylate histones—is required for transgenerational ethanol-seeking behavior. Unbiased mass spectrometry of over 80 histone post-translational modifications revealed that H3K5ac, but not H3K9ac, increased in P0 and F3 generations after 0.5% ethanol exposure at P0. H3K27 modifications could not be examined due to protein sequence differences; however, preliminary Western blot and germline immunofluorescence results suggest that H3K27ac also increases upon 0.5% exposure in P0. **Conclusions:** Ongoing studies are validating the mass spectrometry results and identifying histone post-translational modifications to target for epigenomic analysis using CUT&TAG methods. We will also elucidate the epigenetic requirements, including timing and cellular distribution, that direct alcohol's effects on behavior across generations using pharmacological and RNAi-based approaches. This research will identify the metaboloeigenetic mechanisms underlying alcohol's transgenerational effects.

PS 4654 Effect of Theophylline on Respiratory and CNS Parameters in Sprague Dawley Rats

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Background and Purpose: Theophylline is a bronchodilator that acts through phosphodiesterase inhibition, adenosine receptor antagonism, and anti-inflammatory mechanisms. Its respiratory and behavioral effects were assessed following overstimulation of the immune system and cascading an inflammatory response in male and female Sprague Dawley rats by administering lipopolysaccharide (LPS). **Methods:** Respiratory parameters - respiratory rate, minute volume, and tidal volume - were assessed using plethysmography chambers alongside behavioral outcomes using Functional Observational Battery (FOB) and locomotor activity tests. Animals (4/sex/group) received vehicle, LPS, theophylline, or LPS/theophylline at 0, 3, 20, or 3/20 mg/kg. When co-administered, theophylline was given 2.5 hours after LPS to allow an inflammatory response to develop. LPS was delivered intranasally (0.1 mL/animal), while vehicle and theophylline were administered intraperitoneally (10 mL/kg). **Results:** Consistent with its known stimulant effects, a significant increase in mean respiratory rate was observed in animals administered theophylline (246%) and LPS/theophylline (211%), compared to the vehicle controls. Mean minute volume rose 168% and 134% in animals administered theophylline or LPS/theophylline compared to the vehicle control group. Tidal volume was unchanged in theophylline or LPS/theophylline groups, though LPS alone caused a transient decrease. These results support the expected pharmacodynamic profile of theophylline and suggest that LPS-induced inflammation may attenuate its respiratory stimulant effects. In behavioral testing, rats treated with theophylline showed consistent increases in distance travelled, ambulation, rearing, and time active, with reduced resting time across all 5-minute intervals over a 30-minute post-dose period. These behavioral effects were observed intermittently in the LPS/theophylline group. **Conclusions:** The results showed a more agile and ambulatory CNS behavior and bronchodilation in animals administered theophylline or co-administration of LPS and theophylline. Moderately elevated effects were observed in animals administered theophylline only.

PS 4655 N-Acetylcysteine (NAC) does not prevent symptoms of acute lung injury (ALI) in acrolein-exposed mice

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Background and Purpose: Since Yves Alarie characterized toxicant-associated respiratory depression (RD) in a mouse model in 1966, RD has been recognized as an immediate response to lung toxicant exposures that can be associated with subsequent acute lung injury. Non-invasive whole-body plethysmography (WBP) was originally used to measure RD, but the WBP technique has been criticized in recent years, for misinterpretation of the enhanced pause (Penh) variable. NAC, typically used clinically as a mucolytic within the airways, has been proposed as a therapeutic for oxidative stress and lipid peroxidation associated with lung exposure to acrolein, specifically to counteract depletion of the antioxidant glutathione (GSH) and to reduce the subsequent oxidative stress and lipid peroxidation. In this study, we used the machine learning technique of Principal Component Analysis (PCA) to unbiasedly assess the breathing parameters measured with WBP, in testing the efficacy of NAC as a medical countermeasure against acrolein. **Methods:** Five groups of mice given intratracheal (IT) acrolein [Naïve (null), 0mg/kg(vehicle), 2mg/kg, 4mg/kg, and 4mg/kg+100mg/kg NAC, IT] were assessed for toxicity responses every 24h with WBP, body weight, and Kaplan-Meier survival analyses over 96h, followed by postmortem bronchial alveolar lavage (BAL) cell counts, and lung histology, to determine whether

NAC could alter toxic responses to acrolein. PCA of WBP data was used to determine the rank and interaction of measured variables, including breathing rate (f), minute volume (MVb), and Penh. **Results:** All acrolein exposed subjects showed RD at 3 hours post-exposure, with the 4 mg/kg group exhibiting severe RD to a level of 50% (RD₅₀) of initial breathing frequency. Furthermore, the 4 mg/kg acrolein group had 50% mortality at 96h, thereby defining the LD₅₀, in these experiments. The eigenvalue of f was 0.97 in Principal Component 1 (PC1, which explained 98% of the variance in the model). MVb had an eigenvalue of 0.94 in PC2 (which explained 1.43% of the variance in model) and was depressed from 50 ml/min to 29 ml/min in all acrolein exposure groups. Penh had a 0.96 eigenvalue in PC3 (which explained only 0.14% of the variance in the model), with median values being elevated from 0.7 to 10 (unitless) after 24h, in both 4 mg/kg acrolein exposure groups. Both 4 mg/kg acrolein exposure groups demonstrated a statistically significant increase in total airway cells (from 1.43E⁵ naïve, to 2.57E⁵ 4 mg/kg acrolein and 3.99E⁵ 4 mg/kg acrolein + NAC), with a high proportion of neutrophils observed at 96h, as compared to controls. With NAC treatment of 4 mg/kg acrolein-exposed mice, the RD₅₀ did not improve, as compared to 4 mg/kg acrolein alone. Weight loss with NAC administration was comparable to 4 mg/kg acrolein alone, both reaching a loss of 20% at 72h. The mortality rate in the NAC treatment group, as determined by the survival slope, unexpectedly accelerated, reaching LD₅₀ within 72h, as compared to the 4 mg/kg acrolein alone group, at 96h. In the NAC treated group, Penh elevations were not statistically different from 4 mg/kg acrolein, alone. BALF total cell counts of the surviving NAC treatment group mice at 96h exceeded those of the control group but were not significantly different from those observed with 4 mg/kg acrolein treatment, alone. **Conclusions:** From the PCA analysis, f, MVb, and Penh were ranked respectively as the greatest contributors to model variance. While Penh displayed a unique trendline with scalar values of 10+ within the 4 mg/kg acrolein group, as a principal component (i.e., PC3), it was not the most informative factor describing acrolein lung toxicity effects. Furthermore, 4 mg/kg acrolein + NAC, as compared with 4 mg/kg acrolein alone, did not differentiate in any statistically significant manner. Thus, NAC treatments did not mitigate any symptom of acrolein toxicity at the clinically-based dosage of 100mg/kg, suggesting that: 1) NAC was ineffective against acrolein toxicity, 2) further experiments are needed to determine possible NAC effectiveness at higher doses, and 3) the administration of NAC in the lung against acrolein toxicity may be contraindicated.

PS 4656 Noninvasive repeated sampling reveals specific respiratory cytokine signatures for individuals who use electronic-cigarettes and tobacco dual-products

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Background and Purpose: The recommendation that electronic-cigarettes (e-cigs) can be used as a safer alternative and smoking cessation tool in most countries has led to an increase in use, despite emerging evidence suggesting use, especially in combination with other products (dual use), is associated with increased risk of long-term respiratory disease. New and attractive flavors, different device types and brands, and targeted marketing make e-cigs more appealing to young adult and youth populations, sparking increased curiosity for use. Dual use, or the use of e-cigs in combination with conventional cigarettes or other inhaled products (tobacco, CBD, THC etc.) has increased in prevalence, particularly in the youth and young adult populations, reaching concerning levels. There is limited understanding of the health effects associated with dual use, as it has not been as extensively studied as e-cigs or conventional smoking have independently. However, the combination of inhaling combustible product smoke and e-cigs aerosols could lead to increased pathological outcomes compared to either alone. Minimal longitudinal studies have been conducted to gather respiratory and immune outcomes of e-cig use. Likewise, with the emergence of dual tobacco use, limited data exists on the consequences or outcomes of such exposures, increasing the need for repeated-measure studies. **Methods:** Nasal epithelial lining fluid (NELF) was collected from a remote cohort (N = 64) of never tobacco users (n = 34), exclusive electronic cigarette users (n = 14), dual tobacco product users (n = 10), and cannabis users (n = 6), once weekly via video conferencing over four weeks, and analyzed via multiplex enzyme linked immunosorbent assays for 29 different inflammatory immune mediators. Salivary cotinine was collected to assess nicotine content between groups. Sex was added as a cofactor to determine whether main effect sex differences were present within and between groups. **Results:** Cotinine analyses showed e-cig and dual tobacco product users did not display significant differences in average cotinine but had significantly more nicotine exposure compared to never users. Moreover, dual tobacco product users displayed increased correlations between cotinine and protein concentrations. Inflammatory cytokines differed among e-cig, dual tobacco products and never users. However, inflammatory markers did not change over time. E-cig and dual product users exhibited increased proinflammatory markers compared to never users. Chemokine profiles of Eotaxin, TARC, MCP-1, and MCP-4 were specifically elevated in dual product users only compared to never users. Main effect sex was identified in 24 of 29 tested analytes. Sex differences were eliminated, introduced, and enhanced when product user status was considered compared to never users. No significant variability was observed in cytokine levels within groups when assessing differences by collection week. **Conclusions:** E-cig and dual tobacco product use

elevates inflammatory cytokines, with dual tobacco product use displaying distinct changes in chemokine profiles compared to exclusive e-cig users. Sex as a variable should be considered when analyzing data, as user status influenced the elimination and introduction of sex differences.

PS 4657 Quenching the flame: VCAM neutralizes age enhanced neuroinflammation

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Background and Purpose: Ambient air pollution is recognized as the second leading risk factor for global mortality, accounting for more than 8.1 million deaths per year and contributing significantly to the global noncommunicable disease burden. Recent unprecedented heatwaves, exemplified by record-breaking temperatures and extended wildfire seasons, have further amplified ambient particulate pollution and associated health hazards, particularly in vulnerable populations such as older adults. Over 90% of the world's population lives in regions exceeding World Health Organization air quality standards, with India and China disproportionately affected, but high exposures and associated risks also persist in westernized nations where particulate matter (PM) is a prevalent contributor to disease. As our climate warms, wildfire-derived PM has become increasingly frequent and severe, affecting respiratory and extrapulmonary organ systems, leading to cardiovascular and neurological symptoms. Among the most concerning consequences is neuroinflammation, particularly in aging populations, where cumulative exposures and vascular remodeling exacerbate susceptibility. Air pollution is implicated in a spectrum of health outcomes, notably including neuroinflammatory disorders such as Alzheimer's disease, Parkinson's disease, and stroke, for which elderly cohorts appear at heightened risk due to age-associated changes in vascular and immune regulation. Mechanistically, inhaled pollutants can access and damage the central nervous system via direct and indirect pathways, provoking neuroinflammation through upregulation of pro-inflammatory mediators including tumor necrosis factor alpha (TNF) and vascular cell adhesion molecule 1 (VCAM1), a mediator of leukocyte adhesion and vascular inflammation. VCAM1, expressed primarily on endothelial cells, is of particular interest to investigate as it plays an important role at the blood-brain barrier (BBB) and the lining of the gut and is especially elevated in aged mammals. **Methods:** The present study investigated whether VCAM1 mediated immune cell trafficking contributes to neuroinflammatory responses following wildfire smoke exposure in aged animals. Eighteen-month-old female mice (human equivalent $\approx$ 60 years) were exposed to either filtered air or woodsmoke (500 μ g/m³ PM, 4 h per day, every other day for 2 weeks) while receiving intraperitoneal injections of either a VCAM1 neutralizing antibody or IgG isotype control. Tissues were harvested post-exposure for flow cytometric and molecular analyses of immune activation and inflammatory biomarkers across multiple organ systems, including the brain. Data were analyzed using two-way ANOVA to assess the interactive effects of smoke exposure and VCAM1 blockade. **Results:** Flow cytometric and immunosay analyses demonstrated that wildfire smoke exposure induced significant activation of leukocyte adhesion markers, as well as augmented neuroinflammatory profiles, specifically in animals receiving control injections. Wildfire smoke exposure upregulated VCAM1 ($p=0.0451$) and LFA ($p=0.0029$) expression on circulating leukocytes and increased the abundance of CD11b⁺ myeloid immune cells ($p=0.0007$), indicating systemic macrophage activation. VCAM1 blockade effectively attenuated these pathological immune responses and normalized organ level outcomes such as cecal hypertrophy ($p=0.0032$). **Conclusions:** These data demonstrated that VCAM1 mediated endothelial signaling contributes to wildfire smoke induced immune activation in aged animals. The unexpected observation that smoke exposure enlarged the cecum parallels clinical observations of microbiome disruption in chronically exposed human cohorts and may serve as an indicator of microbial imbalance which was normalized by VCAM1 blockade, suggesting possible gut-vascular-brain interactions in the inflammatory response. This work supports a model in which air pollution driven neuroinflammation in aging is contingent upon VCAM1 mediated leukocyte-endothelial interactions, positioning VCAM1 as a promising therapeutic target to mitigate vascular and neurological consequences of ambient particulate exposure under climate change-enhanced pollution scenarios.

PS 4658 Modelling the lung and the COPD pathophysiology in a Mechanically stimulated small airways Lung-on-Chip

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Background and Purpose: Chronic Obstructive Pulmonary Disease (COPD) remains a global health challenge in both industrialized and developing countries, with no curative therapies available. Despite cigarette smoke is recognized as one of the major risk factors for COPD, different air pollutants may contribute to the pathology onset and progression. To develop innovative treatments, it is thus fundamental to advance the understanding of disease's mechanisms, also through advanced *in vitro* models that accurately mimic COPD. In this context, organs-on-chip (OoC) have emerged as promising tools, especially to mimic alveolar structures subjected to

mechanical stimulation. However, these models lack the integration of small airways physiology with a 3D environment that can be mechanically activated to better reflect natural respiratory motion. Here we present a COPD-OoC model, generated by co-culturing human bronchial epithelial cells (HBECs) and bronchial smooth muscle cells (BSMCs) in a 3D controlled microenvironment, where a physiological-like mechanical stimulation was applied. **Methods:** BSMCs were organized as 3D microtissues, while HBECs are lined along the muscles to generate epithelial monolayers. The stromal and epithelial compartments were firstly optimized by culturing the cells for 14 days both in static and dynamic (i.e. cyclic sinusoidal 10%uniaxial strain, 16bpm) conditions. The optimized compartments were finally combined in a 5-channels device encompassing two BSMC microtissues separated by HBECs monolayer, which was subjected to air-liquid-interface. The obtained mechanically active system has been exposed to pro-inflammatory cytokines (e.g. IL-13) and/or to cigarette smoke extracts (CSE) to assess their effect in inducing COPD traits and/or impair cell viability, assessing lung toxicity. **Results:** For the stromal compartment, the optimal cell seeding density resulted a 50x10⁶cells/mL of BSMC monoculture, which led to a higher cell viability and interconnectivity. Cell elongation and expression of BSMCs markers at protein levels, such as α -SMA, β -tubulin and Collagen I were enhanced in mechanically stimulated conditions. Concerning the epithelial compartment, a seeding density of 10x10⁶cells/mL yielded optimal cell viability and barrier-like structure formation, as demonstrated by ZO-1 expression. The optimized compartments were then combined in the 5-channels device, where the model correctly developed in 10 days. BSMCs expressed α -SMA and Collagen I and exhibited increased elongation when subjected to mechanical stimulation. Epithelial cells formed structured monolayer and expressed specific markers of epithelial cells sub-populations, such as MUC5B (i.e. acinar cells), FOXJ1 (e.g. ciliated cells), and SCGB1A1 (i.e. acinar cells). The obtained mechanically active system was proven able to respond to pro-inflammatory cytokines and to cigarette smoke extracts: NHBE showed an enhanced elongation and higher α -SMA expression, especially when subjected to cigarette smoke extracts. **Conclusions:** The lung-on-chip incorporating stromal and epithelial compartments, enabled the creation of a dynamic 3D human bronchial model that mimics the active mechanical environment of the lung. BSMC and NHBE were successfully cultured within the system and exposure of the model to CSE elicited an inflammatory response characteristic of COPD. This model offers a powerful tool for assessing lung toxicity and studying disease mechanisms, to finally accelerate drug discovery.

PS 4659 Fiber Diameter Distributions of Modern Synthetic Vitreous Fibers (SVFs): Implications for Exposure and Risk Assessment

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Background and Purpose: Synthetic vitreous fibers (SVFs) are a class of materials that have major uses in commercial and residential insulation for thermal, fire, noise, and vibration protection, and are critical materials for energy conservation. SVFs are composed of an amorphous silicate arrangement with no discernable crystalline structure. Fiber dimension is one of the key characteristics of the 3Ds (dose, dimension, durability) of fiber toxicology, and fiber diameter is an important determinant of fiber respirability. The purpose of this analysis was to characterize the fiber diameter distribution and other physical attributes of a range of modern synthetic vitreous fibers (SVFs). **Methods:** Twenty-two samples of glass, stone/rock, and slag SVFs were selected from samples obtained from 17 different plants from eight different manufacturers. Physical characterization of SVFs included fiber diameter and size distribution by scanning electron microscopy (SEM, Tescan MIRA3), surface area by Brunauer, Emmett, and Teller (BET) analysis, and density by gas pycnometry. SVF samples were prepared by sieving fibers through a 63 μ m size mesh to reduce fiber length and remove shot. BET analysis involved degassing 0.5-1 g SVF (in duplicate) under vacuum at 45°C for 16 hours prior to analysis. The BET specific surface area (m²/g) was determined using Krypton gas (77 °K) and five relative pressures (i.e. 5-point BET) within the linear region of the adsorption isotherm. Fiber density measurements included milling and then drying $\sim$ 20 g SVF at 110°C for at least 2 hours and allowing the material to cool to room temperature in a desiccator. After insertion of a determined mass of material in the pycnometer, 50 He purges were conducted before analysis, and the average of seven measurements at 17 psi were reported. Samples for SEM analysis were prepared from subsamples of material mixed with a Turbula mixer, suspended in filtered IPA, vacuum deposited onto 0.2 μ m polycarbonate filter, and mounted on a SEM stub with colloidal graphite. Samples were then coated with a thin layer of carbon by evaporative deposition under vacuum. The lengths and widths of 700-800 individual fibers for each SVF sample were measured from an automated montage of high-resolution SEM images. **Results:** Shot ($>63 \mu$ m) was present in seven samples with masses ranging from approximately between 25-50% of the original SVF sample. Density measurements varied by SVF type and generally ranged from 2.4 to 2.9 g/cc. Surface area also differed across SVF types with an approximate range from 0.1 m²/g to slightly greater than 0.5 m²/g. Greater than 15,000 fibers were counted for fiber diameter distributions across the 22 SVF fiber samples. Fiber diameter size distribution analyses indicated that modern SVF samples were comprised of fiber diameters both less than and greater than the fiber diameter threshold of 3 μ m for respirability. **Conclusions:** A fiber diameter distribution analysis was conducted on 22 modern glass, stone/rock, and slag SVFs to evaluate fiber physical characteristics important for potential respirability. Fiber diameter distributions indicated that SVFs were comprised of both non-respirable

and respirable fiber diameter sizes. The fiber physical analyses (dimension, density, and surface area) are intended to serve as informative inputs to 1) fiber deposition/dosimetry modeling (e.g., multiple-path particle dosimetry model) and exposure/risk analysis of occupational settings involving the manufacture of different SVFs, and 2) new approach methods (NAMs) for measuring *in vitro* fiber dissolution and predicting *in vivo* fiber biopersistence.

PS 4660 Human macrophage secretory response to woodsmoke condensate exposure is dependent on polarization state, sex, and estrogen receptor activation

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Background and Purpose: Wildfires have increased in frequency and severity over the past few decades, and wildfire risk is predicted to increase in the coming decades due to rising ambient temperatures and human encroachment into wildland areas. Exposure to ambient wildfire smoke (WFS) and wood smoke (WS)/WS condensate (WSC) (often used as an experimental surrogate for wildfire smoke) are associated with respiratory morbidity and mortality and can modulate inflammatory and oxidative processes in a variety of cell types and model systems. Importantly, controlled exposure studies in humans, rodents, and epithelial cells have recently demonstrated that sex can influence cellular responses to inhaled toxicant exposure. However, no studies to-date have investigated sex-specific or estrogen-dependent effects of WS on macrophages, a critical phagocytic cell type responsible for responding to infection and injury in the lungs. This study evaluated macrophage polarization state (M0, M1, M2), estrogen receptor signaling, and sex as potential sources of interindividual variability in response to WS exposure. **Methods:** Human monocyte-derived macrophages differentiated into M0, M1, and M2 polarization states were used as a model for respiratory macrophages. Monocytes were isolated from venous blood ($n = 5$ females, $n = 4$ males) and cultured for six days with M-CSF, resulting in M0 macrophages. Cells were then treated for 24 hours with interferon gamma and lipopolysaccharide to generate M1 macrophages, IL-4 to generate M2 macrophages, or no additional stimuli to maintain an M0 phenotype. A subset of macrophages were also treated with either 1 nM 17 β estradiol (E2) (an estrogen receptor alpha and beta agonist) or 1 μ M ICI 162,780 (fulvestrant, an estrogen receptor antagonist) during the differentiation process. Polarized macrophages were treated with 25 μ g/cm² smoldering red oak wood smoke condensate (WSC) for four hours followed by fresh media for two hours. Following the two-hour period with fresh media, the media was collected for cytokine/chemokine analysis, and cells were assayed for cytotoxicity and phagocytic capacity. Linear mixed effects models and principal component analysis were used to investigate the effects of sex, polarization state, and estrogen receptor treatment on macrophage responses to WSC. **Results:** WSC did not elicit significant cytotoxicity in comparison with the vehicle control, but did significantly decrease phagocytosis across macrophage polarization states, donor sexes, and estrogen receptor treatments. At baseline without WSC or estrogen treatment, M2 macrophages from male participants secreted significantly more IL-17A and TNF- β than M2 macrophages from female participants, while M0 macrophages from female participants secreted significantly more IL-7 than M0 macrophages from male participants. With estrogen treatments and no WSC, ICI treatment significantly increased expression of many secreted proteins, such as IL-6, IFN- α , and IL-1 β in all polarization states and both sexes, while estradiol had minimal effects on secreted protein concentrations. With WSC, pro-inflammatory protein secretion was significantly increased in M0 and M2 macrophages not exposed to E2 or ICI. Notably, these WSC effects were muted in E2 and ICI treated cells. **Conclusions:** Our findings demonstrate baseline differences in macrophage secretory phenotypes between males and females and that the estrogen receptor plays a role in macrophage secretory phenotype and cellular responses to WSC exposure. WSC was also found to decrease phagocytic capacity in all experimental groups, suggesting a general functional suppressive effect with exposure. Ongoing studies are focused on characterizing the transcriptional response of these cells using the same cell culture and exposure paradigm. Future studies are needed to further investigate these mechanisms and determine their impact on respiratory morbidity and mortality following inhaled toxicant exposures and ultimately inform public health and therapeutic approaches to managing health risk associated with WFS exposure.

PS 4661 *In vitro* immune off-target toxicity assessment using morph_ONE for antibody drug conjugates (ADCs).

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Background and Purpose: Antibody drug conjugates (ADCs) have revolutionized targeted cancer therapy by delivering potent cytotoxic agents directly to cancer cells. However, off-target toxicities remain a major limitation, with interstitial lung disease (ILD) emerging as one of the most serious adverse events. ILD associated with ADCs is characterized by inflammation and fibrosis of the pulmonary tissue, which can lead to progressive respiratory impairment and, in severe cases, fatal outcomes. The underlying mechanisms are not fully understood but may involve immune-mediated responses, non-specific uptake, and systemic exposure to the cytotoxic payload.

The study aimed to (i) optimise and validate human *in vitro* system (alveolar macrophages) for predicting the pulmonary toxicity observed in *in vivo* toxicity studies of ADCs, and (ii) understand the impact of the ADC vs individual components on respiratory inflammation and other potential off-target effects characterized by changes in macrophage morphology. **Methods:** ImmuPHAGE™ (alveolar macrophage-like cells, ImmuONE, UK) were exposed to ADCs of known off-target lung toxicity: trastuzumab deruxtecan, brentuximab vedotin and sacituzumab govitecan in a range of concentrations 0.001 - 100 μ g/mL for 24 h, 48 h, 72 h and 144 h. Following incubation, cells were stained with a cocktail of fluorescent dyes to identify cellular features (nucleus, cytoplasm, mitochondrial activity, membrane permeability). Images were captured using the InCell Analyser 6000 and analysed using InCell Toolbox v1.9.2. Supernatant from cell cultures was assessed for proinflammatory cytokine panel (IFN- γ , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF- α) using MSD cytokine assay system. **Results:** Cell health deterioration, manifested by increase in membrane permeability, decrease in cell count and mitochondrial activity, was affected in a dose and time dependent manner for drugs alone but not for the ADC, indicating that drugs as a standalone treatment display cytotoxic effect on macrophages. While the ADCs did not affect cell health, individual cell data analysis showed changes in macrophage morphology, namely foamy macrophage appearance, especially following 72 h incubation. Increased area of vacuoles and increased number of vacuoles were reported in dose response manner. Cytokine analysis revealed increased secretion of proinflammatory cytokines, especially IL-6 and TNF- α . **Conclusions:** This study showed that the high content imaging assay, morph_ONE, is a suitable tool for identifying changes in macrophage health and morphology resulting from ADCs treatment. Implementing this tool in the early stages of ADC development may help to detect potential off-target toxicity in the airways before progressing to animal studies, aiding candidate selection and increasing the chances of success in the drug development process.

PS 4662 Synergistic Toxicity of E-Cigarette Flavoring Aldehydes and Nicotine in Lung Epithelial Cells

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Background and Purpose: Flavored ENDS aerosols contain aldehydes, ketones, and esters, as well as nicotine. The mixture toxicity of these ENDS-derived chemicals on the lungs is not well understood. We hypothesized that mixtures of flavoring aldehydes, or aldehydes with nicotine, would exert synergistic toxicity in lung epithelial cells compared to individual components. **Methods:** Bronchial epithelial cells (BEAS-2B) were treated with individual aldehydes-benzaldehyde (B), furfural (F), decanal (D), salicylaldehyde (S), citral (C), p-tolualdehyde (T), 2,5-dimethylbenzaldehyde (DB), benzaldehyde(B), cinnamaldehyde (C), and veratraldehyde(V), at 10 μ M - 2.5 mM, and in combination of two aldehydes at varying ratios (1:1- 1:49), to assess their individual and combined toxicological effects on lung epithelial cells. Effects of nicotine were determined by combining aldehyde with S-nicotine (N). Twenty-four hours post-treatment cytotoxicity was measured with MTT assays. Elicited inflammatory response was determined by a Luminex 17-plex cytokine panel. Individual chemical treatment-induced dose-response and synergism of combined chemicals were determined using CompuSyn analysis. **Results:** B:F combination showed no cytotoxicity; however, IL-6 and IL-8 levels were significantly decreased. B:D caused significant cytotoxicity, decreased IFN- γ , MCP-1, IL-5, IL-6, and IL-8, but increased GM-CSF, IFN- γ , and MCP-1. B:S showed significant cytotoxicity and a significant decrease in MCP-1, IL-6, and IL-8. B:T caused significant cytotoxicity and decreased MCP-1, IL-6, IL-7, and IL-8. B:DB caused significant cytotoxicity, increased IL-6, while decreasing TNF- α and IL-7. Combining nicotine with aldehydes, such as N:C caused significant cytotoxicity proportional to the increased cinnamaldehyde ratio in the mixture. N:C caused a decrease in MCP-1 but elevated levels of MIP-1 β , IL-6, and IL-8 with increased nicotine concentration. **Conclusions:** Binary mixtures of ENDS flavoring aldehydes, as well as aldehyde-nicotine combinations, caused synergistic and ratio-dependent cytotoxicity with differential inflammatory responses. These findings highlight the importance of mixture toxicity analysis for ENDS toxicity assessment and suggest that even low-level chemical combinations can exacerbate pulmonary toxicity. This study was funded by the National Institutes of Health R00ES033835.

PS 4663 A Comparative Analysis of Approaches for Identifying Biologically Safe Doses for Asbestos

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Background and Purpose: The fields of pharmacology and toxicology have long embraced that “the dose makes the poison”. Despite OSHA and EPA’s claim that there is “no safe dose” and reliance on the linear no-threshold (LNT) model for carcinogens, there is always a dose at which no adverse effects are reasonably expected. For years, statisticians have opined that “direct statistical confirmation of a threshold from human data is virtually impossible” due to differences in susceptibility between people (Hodgson and Darnton 2000). An understanding of the mechanism of action for asbestos carcinogenesis, in combination with the epidemiology data, supports the existence of exposure thresholds below which increased mesothelioma

and lung cancer risk are de minimis. Asbestos-induced carcinogenesis is primarily driven by chronic inflammation, with the weight of evidence indicating that asbestos is not directly DNA-reactive, immunosuppressive, or estrogenic. A biologically safe dose (BSD), below which adverse health effects are unlikely to deviate from the background incidence of disease, is generally accepted because inflammation is a reversible effect. In recent years, several approaches have been developed to identify BSDs for each asbestos fiber type. **Methods:** A targeted, non-systematic literature review was conducted to identify recent approaches for deriving asbestos BSDs. Four methodologies were reviewed: No Observable Adverse Effect Level (NOAEL) Approach, Benchmark Dose (BMD) Approach, Central Tendency Approach, and Fiber Burden Approach. The linear no-threshold (LNT) model, as applied by regulatory agencies such as the EPA and OSHA, was reviewed for comparison. **Results:** The NOAEL approach identified best-estimate NOAELs of 250-379 f/cc-years for chrysotile, 2-5 f/cc-years for amosite, and 0.6-1 f/cc-years for crocidolite (Beckett et al. 2023). Applying a safety factor of three, appropriate given the robust epidemiological and toxicological data for asbestos, yielded threshold doses of 105, 1.2, and 0.25 f/cc-years for the respective fiber types. The benchmark dose (BMD) extrapolation approach utilized updated cohort information to derive estimated doses corresponding to an observable response (Stevens et al. 2025). A theoretical 1-in-1,000 mesothelioma risk was then extrapolated below the experimental range, resulting in threshold doses of approximately 100 f/cc-years for chrysotile, 2 f/cc-years for amosite, and 0.3 f/cc-years for crocidolite. The central tendency modeling approach fit several models to available epidemiological data to estimate a central tendency threshold for chrysotile (Goodman et al. 2025). Applying potency ratios across fiber types produced cumulative thresholds of 90 f/cc-years for chrysotile, 1 f/cc-year for amosite, and 0.25 f/cc-years for crocidolite. In contrast, the fiber burden approach, as currently implemented by some experts, yielded considerably lower dose estimates: 0.015 f/cc-years for crocidolite, 0.075 f/cc-years for amosite, and 7-10 f/cc-years for chrysotile. This threshold dose is based on a risk level that “doubles” the background risk of developing mesothelioma. These values were derived based on a background rate of mesothelioma of 1 per million-years, although recent epidemiology studies suggest that a more appropriate background rate is closer to 5 per million-years. It is also noteworthy that the crocidolite-exposed cohort relied upon to derive these values (Reid et al. 2007) has limitations. Updating this approach with the latest mesothelioma background risk estimates and epidemiological data would yield BSDs that begin to approach those from the other methods. **Conclusions:** The figures derived using NOAEL/3, BMD, and Central Tendency are remarkably consistent, considering they relied on different studies and used different approaches to reach these conclusions. These three methods indicate reasonable BSDs of 100 f/cc-year, 1-2 f/cc-year, and 0.3 f/cc-year for chrysotile, amosite, and crocidolite, respectively. An updated assessment using the fiber burden approach is more consistent with these values. Given that asbestos induces mesothelioma through repeated inflammation, these approaches likely overestimate risk, suggesting a potential threshold above the derived BSDs. Significant evidence of potency differences between asbestos fiber types indicates the need for fiber-type-specific occupational exposure limits.

PS 4664 Inhaled Vitamin D as a Prophylactic Against Environmental Lung Disease and Respiratory Infection

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Background and Purpose: People of color are disproportionately affected by environmental lung diseases and respiratory infections, likely due in part to increased prevalence of vitamin D deficiency (VDD). In addition to health and nutritional disparities caused by socioeconomic and structural inequities, higher levels of melanin in people of color leads to decreased subcutaneous vitamin D synthesis, the primary route of vitamin D generation. VDD has been linked to increased severity and occurrence of environmental lung diseases, such as asthma and COPD, and increased risk of influenza and COVID infection, indicating that the disproportionate impact of environmental lung diseases and respiratory infections on people of color may be due in part to increased prevalence of VDD. While vitamin D has been proposed as a prophylactic treatment for environmental lung disease and respiratory infections, studies of oral vitamin D supplementation have produced mixed results. Inhalation offers the advantage of delivering vitamin D directly to the respiratory epithelium, potentially providing superior protection compared to oral supplementation. We therefore investigated the role of vitamin D inhalation as a prophylactic strategy against ozone exposure and H1N1 infection in human airway cells and evaluated potential antiviral and immunomodulatory mechanisms. **Methods:** We exposed well-differentiated, primary human bronchial epithelial cells (HBECS) at air-liquid interface to ozone or H1N1 influenza virus (A/California/07/2009), following administration of 10 µL of 10 µM vitamin D to the apical compartment. Apical treatment was delivered via aerosol in a novel multi-well *in vitro* exposure system, On-Plate Aerosol Delivery Array (OPADA). Prophylactic effects of vitamin D were evaluated through bulk RNA-sequencing, RT-qPCR, cytokine secretion, and oxidative stress endpoints. Differential expression analysis was performed along with unbiased analyses such as multi-dimensional scaling and hierarchical clustering. Gene set enrichment analysis (GSEA) and Ingenuity Pathway Analysis (IPA) were used to reveal potential mechanisms behind vitamin D's protective effects. **Results:** We found that pre-treatment with aerosolized vitamin D significantly reduced H1N1 viral load in HBECS, as measured by decreased expression of influenza genes. Vitamin D pretreatment also reduced virus-induced expression of antiviral interferon-stimulated genes (ISGs) while modulating cytokine

responses indicative of reduced inflammation. Similarly, GSEA revealed that vitamin D treatment attenuated viral inflammatory responses, immune dysfunction, and cellular damage pathways, while boosting pathways related to ciliary function. We also found that vitamin D pre-treatment attenuated ozone-induced upregulation of inflammation- and oxidative stress-related genes such as IL-8, FFAR2, COX-2, and NFKB2. Mechanistic investigation revealed that vitamin D reduced lipid peroxidation and glutathione oxidation induced by ozone, indicating that vitamin D may act as a membrane antioxidant. **Conclusions:** Here, we provide studies demonstrating that inhaled vitamin D: 1) reduces H1N1 viral load and decreases aberrant inflammatory responses, and 2) attenuates oxidative stress and inflammatory gene expression induced by oxidant pollutants such as ozone. Our data suggest inhaled vitamin D may be a feasible prophylactic strategy for protecting against influenza infection and environmental lung disease and may have utility in reducing health disparities associated with VDD in people of color. Ongoing studies aim to understand the pharmacokinetics of inhaled vitamin D, further elucidate mechanisms by which inhaled vitamin D provides protection, and explore the utility of vitamin D inhalation in humans.

PS 4665 Mast Cells Modulate Early Pulmonary Inflammatory Responses to Amorphous Silica Nanoparticles

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Background and Purpose: Amorphous silica nanoparticles (SiNP) are considered an occupational concern in both industrial and agricultural settings. While crystalline silica is well known to drive strong pulmonary inflammation, the inflammatory potential of amorphous silica is less understood. Mast cells are early responders in the lung and can influence acute inflammatory and tissue-damage responses to particle exposures. This study examined the role of mast cells in the acute pulmonary response to SiNPs using wild-type (WT) and mast cell-deficient (Kit^{W-sh}) mice. **Methods:** WT and Kit^{W-sh} mice received a single exposure to either vehicle (PBS) or SiNPs (~200 nm) at 5 mg/kg via oropharyngeal aspiration. Lung tissue and bronchoalveolar lavage fluid (BALF) were collected after 24 hours. BALF cytosins and differential cell counts were performed to assess inflammatory cell recruitment. Lung tissue was examined by H&E staining to evaluate structural injury, and flow cytometry was used to characterize immune cell populations within the lung. Lipidomic profiling was also performed on BALF samples and human mast cells (HMC-1.2) were exposed to SiNPs *in vitro* for degranulation assessment and lipidomic analysis. **Results:** SiNP exposure produced an acute inflammatory response in WT mice, characterized by shifts in BALF immune cell composition, including increased neutrophils and eosinophils accompanied by a reduction in macrophages. Similarly, innate and adaptive immune cell populations in WT lung tissue indicated a broader inflammatory recruitment compared to Kit^{W-sh} mice. In contrast, Kit^{W-sh} mice displayed a muted inflammatory profile across multiple cell types, suggesting the absence of mast cells attenuates early-stage pulmonary responses. Although SiNPs induced measurable degranulation in HMC-1.2 cells, lipidomic profiling in both BALF and HMCs revealed limited treatment-related differences, with only a few lipids showing nominal changes. **Conclusions:** Overall, the reduced responses in Kit^{W-sh} mice, together with SiNP-induced degranulation in HMCs, indicate mast cell involvement in early signaling events that may influence downstream pulmonary inflammation.

PS 4666 Tobacco-Flavored E-Cigarette Aerosol Suppresses Immune-Resolution Pathways and Initiates Early Lung Remodeling Following Sub-Chronic Inhalation Exposure

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Background and Purpose: Electronic nicotine delivery systems (ENDS) are widely marketed as safer alternatives to combustible cigarettes; however, toxicological data defining the chemical composition, aerosol characteristics, and biological consequences of sub-chronic flavored ENDS exposure remain limited. This study evaluated the combined pulmonary toxicity of tobacco-flavored ENDS aerosols with and without nicotine to identify early adverse outcome pathway events linked to chronic lung disease. **Hypothesis:** Sub-chronic inhalation of tobacco-flavored ENDS aerosols disrupts immune-resolution signaling and initiates ECM remodeling, with nicotine acting as a mechanistic amplifier through cholinergic suppression of inflammatory and reparative pathway. **Methods:** Male and female C57BL/6J mice were exposed to tobacco-flavored ENDS aerosols ±6 mg/mL nicotine (51 mL/puff; 2 puffs/min; 2 hr/day for 30 days). Aerosol size distributions, including number and mass concentrations, were monitored using a scanning mobility particle sizer and an optical particle sizer, while ENDS liquids were analyzed via thermal desorption DART-HRMS to characterize semi-volatile and non-volatile constituents. Post-exposure biological endpoints included lung mechanics, lung inflammatory response assessed by flow cytometry and cytokines, as well as proteomic and genomic profiling. **Results:** Tobacco and tobacco+nicotine aerosols generated significantly elevated ultrafine particle concentrations (10⁸-10⁹ particles/cm³), with dominant size modes between 30-150 nm and mass peaks at 0.1-0.3 µm, indicating strong deep-lung deposition potential. Chemical analysis revealed complex mixtures containing aldehydes, ketones, esters, alcohols, N-heterocycles, monoterpenes, sweeteners, nicotine, nico-

tine derivatives, and unidentified constituents, with tobacco-containing formulations showing the greatest chemical complexity. Both nicotine-free and nicotine-containing ENDS exposures suppressed key immune-recruitment and resolution mediators (IL-1 β , CCL3, CCR1/CCR2, PIR-A/B, LYN) and reduced fibroblast-activation markers (OSM, TNFRSF1 β). BALF exhibited increased epithelial-stress and ECM-remodeling proteins (complement D, CRP, CD26, acidic FGF, Gas6, pro-MMP9, PAI-1). Lung-tissue protease-antiprotease imbalance and impaired clearance were evident through reductions in MMP-9, MPO, CCL6, and eotaxin. Nicotine enhanced pro-MMP9 and PAI-1 elevations and increased static compliance while reducing elastance, indicating greater susceptibility to remodeling. **Conclusions:** These findings demonstrate that sub-chronic inhalation of tobacco-flavored ENDS aerosols produces early toxicological signatures of immune-resolution failure, protease imbalance, and ECM remodeling. Nicotine further amplifies these trajectories, suggesting ENDS use may predispose the lung to chronic lung disease-related outcomes even in the absence of overt inflammation. The study was supported by the National Institutes of Health, NIH/NIEHS R00ES033835.

PS 4667 Silica content-dependent epithelial injury and fibroblast activation following engineered stone particle exposure in an *in vitro* co-culture alveolar model

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Background and Purpose: Engineered stone (ES) is widely used for kitchen and bathroom countertops. This composite material, made of ground quartz with pigments and synthetic resin, has gained popularity due to its aesthetic and lower maintenance compared to natural stones. Respirable crystalline silica from engineered stone fabrication poses a significant occupational hazard, leading to progressive and irreversible silicosis. Recent cases underscore the urgent need to elucidate its pathogenic mechanisms. When silica particles reach the alveoli, macrophages activate and initiate inflammatory signals that promote fibroblast proliferation and collagen accumulation, leading to fibrosis. ES with high silica content exacerbates these responses by increasing lung particle burden and sustaining inflammation. However, few studies have examined how the varying silica content in ES influences lung injury pathways related to silicosis. Using an *in vitro* co-culture system to model the distal lung microenvironment, we investigated how silica content affects epithelial barrier integrity, intercellular signaling, and fibroblast activation, to elucidate early events connecting ES particle exposure to silicosis progression. **Methods:** Based on established *in vitro* models, rat alveolar type II (AT II) RLE-6TN epithelial cells and NR8383 macrophages were seeded in the apical compartment, and lung fibroblast RFL-6 in the basolateral compartment, of a co-culture system to mimic alveolar microenvironment. Commercially available ES samples with high (> 40%) or low ($\leq$ 40%) silica content were tested, and a positive control pure crystalline silica (Min-U-Sil 5, diameter $\leq$ 5 μ m). With an air-liquid interface system, AT II cells and macrophages were exposed to 0, 1.0, 2.5, and 5.0 μ g/cm² ES particles, followed by incubation with fibroblasts. Cell viability and membrane integrity, and fibroblast activation were assessed at 24, 48, and 72 h post-exposure by measuring lactate dehydrogenase release, transepithelial electrical resistance (TEER), and α -smooth muscle actin (α -SMA) immunofluorescence staining, respectively. Particle size distribution of ES suspensions was characterized by dynamic light scattering (DLS). **Results:** DLS showed ES particle median diameters of 618 - 678 nm, a size range capable of reaching alveolar regions. AT II cells exposed to ES exhibited a 110%, 240%, and 290% increase in TEER at 24, 48, and 72 hours post-exposure, respectively, at the highest concentration. In comparison, control and Min-U-Sil 5 exposures produced approximately 200%, 400%, and 500% increases in TEER at the same time points. Immunofluorescence analysis of α -SMA in fibroblasts showed a dose-dependent response, with increased fluorescence intensity observed after exposure to high-silica ES. Cell viability remained within normal ranges, indicating that observed effects arose from structural and molecular alterations rather than cytotoxicity. **Conclusions:** Exposure to ES particles impaired epithelial barrier integrity in AT II cells, independent of silica content. Fibroblast activation increased with higher silica content, suggesting that particle composition influences fibrotic remodeling. While intercellular crosstalk between epithelial cells, macrophages, and fibroblasts is inferred from these responses, further studies are underway to confirm the underlying signaling mechanisms. Overall, these findings provide mechanistic insight into ES-induced disruption of alveolar homeostasis and advance understanding of the early events contributing to ES-associated silicosis, identify potential therapeutic targets, and inform exposure thresholds relevant to occupational risk.

PS 4668 Toxicity of Algal Species Associated with Florida Freshwater Cyanobacterial Blooms To Human Small Airway Epithelial Cells

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Background and Purpose: Shifting climates and other environmental factors have been associated with increasing harmful cyanobacterial blooms in freshwater bodies globally, including the southeast United States. Many of these blooms are responsible for the release of an array of various cyanotoxins, including microcystins (MC), a class of cyclic peptides, that have been widely studied for their detrimental effects on aquatic organisms and humans. Microcystin-LR (MC-LR) is one of the most common congeners from this class of cyanotoxins with well-known hepatotoxicity, typically associated with oral exposure. However, the respiratory effects of MC-LR as a result of inhalation exposure are unknown, despite abundant evidence indicating aerosolization of MC-LR from bodies of water. This research gap led us to investigate the toxicity of algae with and without MC-LR on small airway epithelial cells, hypothesizing that the presence of MC-LR would produce the greatest toxicity. **Methods:** *Microcystis aeruginosa* (MA) with high levels of MC-LR and *Florianema aeruginum* (FA) with no-detectable MC-LR were isolated from freshwater bodies in Florida and are maintained in culture. Total MC production from each strain was quantified by ELISA and levels of individual MC congeners (i.e. MC-LR) were quantified by Liquid Chromatography Mass Spectrometry (LC/MS). Lyophilized cyanobacterial aliquots were lysed with three freeze-thaw cycles, particulate matter was sedimented by centrifugation and then the soluble cyanobacterial suspension supernatant was quantified and added to the growth media of cultured SAEC at increasing concentrations (120, 240 and 480 ng MC-LR/mL media) (2-3 replicates/experiment, >3 repeat experiments). As a control, purified MC-LR was also included at a concentration that mirrored what was present in the highest *Microcystis* dose (200 μ g/mL). After a 48-hour exposure, the viability of SAEC was characterized by flow cytometry using Calcein-AM (violet) to label live cells with intact membranes, CellEvent caspase 3/7 reagent (green) to label cells undergoing apoptosis and Sytox AADvanced dead cell stain to detect cells with broken membranes. Extracellular vesicles (EV) were also collected and purified from SAEC media to explore this as a unique mechanism. Extracellular vesicles were characterized by nanoCFM for number and diameter. **Results:** Quantification of MC-LR content in cyanobacterial lysates indicated 119 μ g microcystin/mg MA biomass and no detectable MC-LR in FA. SAEC post exposure to MA or to MC-LR show significant decrease of live cell count (-15.3 $\pm$ 2.3%, P=0.0085 and -21.5 $\pm$ 7.6%, P=0.0013) and increasing number of apoptotic (13.7 $\pm$ 1.8%, P=0.0002 and 19.1 $\pm$ 5.8%, P<0.0001) and dead cells (5.1 $\pm$ 1.3%, P=0.047 and 6.0 $\pm$ 2.4%, P=0.044) at 200 μ g/mL MA and 500 ng/mL MC-LR. There were no significant changes to SAECs in the presence of FA. EV size profiles showed that the spread of the diameter is seen for both MA and FA at 200 μ g/mL extract. This is interesting because MA produces MC-LR and FA does not. In a follow up experiment, the same doses were used again, but this experiment included purified MC-LR at a high dose. Again, the low dose of MA did not change the diameter, but the higher doses starting at 100 μ g/mL did increase the spread in diameters of the exosomes. Purified MC-LR also increased the diameter spread. But in this particular experiment, FA at 200 μ g/mL did not. This data suggests that the exposures to the toxins are causing the cells to put out exosomes with altered structure. **Conclusions:** This work presents some of the first characterizations of toxicity of cyanobacterial extracts and MC-LR to human small airway epithelial cells. Importantly, these data support that the toxin, MC-LR seems associated with the death of SAEC and secretion of EVs. These important observations support that the lung may be a site of toxicity of inhaled harmful algal blooms and associated toxins, and that secretion of exosomes could present as a novel mechanism of action. The results of this study add to the limited reports on how MC-LR may directly affect the respiratory system. Future work is aimed at directly understanding the mechanism of action behind MC-LR toxicity of SAECs.

PS 4669 Pulmonary Pathophysiologic Response to Acute Inhalation of Chloropicrin

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Background and Purpose: Chloropicrin (trichloronitromethane, CP) is a potent pulmonary irritant and soil fumigant historically used as a chemical warfare agent. Despite its continued agricultural use and potential for accidental or intentional release, the dose-response, temporal progression of lung injury, and sex dependent acute susceptibility to inhaled CP remain poorly defined. We used an acute 4 hr exposure in an inhaled CP dose response in mice to characterize the cellular targets of toxicity in the lung by sex, the LD50 and innate immune response in lung tissue. **Methods:** Male and female C57BL/6 mice were exposed via nose-only inhalation to 0.75, 1.5, 3, 6, or 12 parts per million (ppm) CP vapor for 4 hours (n=6/sex/group/timepoint) and were monitored for up to 3 days. Survival and weight loss were recorded. Regional focal intrapulmonary toxicity was assessed using histopathology in high resolution resin sections. Bronchoalveolar lavage fluid (BALF) was collected for analysis of total protein, immune cell differentials and cell death. **Results:** Weight loss was evident within 24 hours and correlated with exposure concentration and mortality.

Both sexes exhibited concentration and time dependent lethality, with females showing greater sensitivity. At 3.0ppm, mortality reached 25% in females compared with 10% in males, and at 12ppm, mortality was higher, with nearly complete lethality in females (92%) versus 56% in males. BALF protein content and total cell counts increased in a concentration dependent manner. In BALF, both sexes showed an up to a tenfold increase in total cells by 24-48 hours post exposure. Neutrophils increased in a concentration and time dependent manner. At 48 hours, neutrophils rose to $12.0 \pm 15.8\%$ and $31.2 \pm 14.4\%$ in females, and $28.6 \pm 26.7\%$ and $33.9 \pm 25.4\%$ in males at 6 and 12 ppm, respectively. By 72 hours, neutrophils increased to $12.9 \pm 7.7\%$ and $62.0 \pm 20.5\%$ in females and $18.4 \pm 13.2\%$ and $59.8 \pm 28.6\%$ in males at 6 and 12 ppm. Neutrophilic infiltration was comparable between sexes at 24 hours but trended higher in females at later timepoints and at higher doses (6 and 12 ppm). Dead epithelial cells in BALF peaked at 2 days post exposure. Histologic evaluation confirmed bronchiolar club cells as the primary targets of CP induced lung injury with pronounced vacuolization and cell loss over time post exposure. **Conclusions:** Nose only CP exposure produced reproducible, concentration dependent acute airway epithelial club cell injury in C57BL/6 mice and revealed sex differences in mortality that correspond with increased neutrophil and lavage protein levels. The selective vulnerability of bronchiolar club cells identifies a site-specific pathologic target relevant to inhalation threat scenarios that expands the current knowledge of acute cellular targets in the lung. This model establishes essential baseline data to guide both mechanistic long-term injury and repair studies being conducted in our lab, and the development of medical countermeasures for acute high dose CP exposure. Funded by: NIH grants: R01 ES034419, T32 ES007059 (to SA) T32HL007013 (to MD and JM), and with support from P30 ES023513

PS 4670 Loss of Cytochrome P450 Reductase (CPR) in Alveolar Type II Cells and Hepatocytes Exacerbates Hyperoxic Lung Injury in Mice

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Background and Purpose: Supplemental oxygen is primarily used in the management of acute respiratory distress syndrome (ARDS), a chronic lung disease affecting more than 200,000 adults and leading to 80,000 deaths in the US each year. Although supplemental oxygen is intended to treat hypoxemia (low blood oxygen) in ARDS patients, prolonged oxygen exposure (hyperoxia) alters alveolar morphology, thus causing acute lung injury (ALI). Hyperoxia has been shown to cause lung injury in humans and animal models by increasing reactive oxygen species (ROS) leading to oxidative stress, inflammation, and structural alterations to lung morphology. Our preliminary data in hyperoxia treated mice has shown significant reduction in the mRNA expression of cytochrome P450 reductase (CPR). CPR is the obligate electron donor to microsomal cytochrome P450 enzymes and is highly expressed in both lung and liver. Prior work suggests that hepatic P450 pathways mitigate hyperoxic lung injury, implicating extra-pulmonary detoxification in lung protection. We hypothesized that CPR in lungs (alveolar type II (ATII)) cells and liver is required to alleviate hyperoxia-induced lung injury and inflammation. **Methods:** Adult conditional CPR knockout mice with ATII-specific deletion (SPC promoter; lung-CPR-KO) or hepatocyte-specific deletion (albumin promoter; liver-CPR-KO), and their wild-type (WT) littermates were exposed to room air (RA) or $>95\% O_2$ for up to 72 h. Lung injury was assessed by lung weight/body weight (LW/BW) ratios and hematoxylin-eosin histopathology. Lung and liver mRNA levels of CPR, pro-inflammatory cytokines (TNF- α , IL-6) and oxidative stress response genes (NQO1, HMOX1) were quantified by qPCR, and CPR/CYP1A activity was measured enzymatically. **Results:** In WT mice, hyperoxia significantly increased LW/BW ratios (unpaired t-test; $p < 0.05$); lung TNF- α and IL-6 expression (unpaired t-test; $p < 0.05$), while significantly reducing pulmonary CPR mRNA by $\sim 35\%$ (unpaired t-test; $p < 0.05$), indicating that lung CPR is downregulated during oxygen toxicity. ATII-specific CPR deletion further exacerbated hyperoxic lung injury, with lung-CPR-KO mice displaying significantly higher LW/BW ratios (unpaired t-test; $p < 0.05$), augmented TNF- α and IL-6 expression (unpaired t-test; $p < 0.05$), and marked induction of NQO1 and HMOX1 (unpaired t-test; $p < 0.05$) compared with WT under hyperoxia, consistent with amplified inflammation and oxidative stress. Hepatocyte-specific CPR deletion similarly increased susceptibility to hyperoxic lung injury. Liver-CPR-KO mice exhibited markedly reduced hepatic CYP1A enzyme activity (unpaired t-test; $p < 0.05$), higher LW/BW ratios (unpaired t-test; $p < 0.05$), and more severe alveolar and perivascular edema and hemorrhage, along with increased pulmonary pro-inflammatory mediator expression (TNF- α and IL-6) compared with WT controls (unpaired t-test; $p < 0.05$). **Conclusions:** These data identify CPR as a critical determinant of host defense against hyperoxic lung injury in both pulmonary (ATII epithelial) and hepatic compartments. Loss of CPR in either site amplifies lung inflammation, oxidative stress, and tissue damage, supporting a dual-organ model in which local epithelial CPR and extra-pulmonary (hepatic) detoxification cooperate to constrain hyperoxic ALI. Targeting CPR-dependent pathways, or preserving CPR function in at-risk patients, may offer novel strategies to prevent or attenuate hyperoxic lung injury.

PS 4671 Transport of Airborne Asbestos Fibers in the Ambient Air: Effects of Fiber Type and Debris Binding on Fiber Dispersion

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Background and Purpose: Mechanical cutting of asbestos cement (AC) pipe releases airborne fibers whose transport behavior is poorly characterized. This study quantified the dispersion of chrysotile and crocidolite fibers generated from high-velocity pipe cutting, using spatially resolved air sampling and scanning electron microscopy. While different methods have been proposed for estimating AED, few (if any) are able to adequately account for all the physical properties of fibers which are likely to affect transport. This study is novel in that it may be the first one to evaluate fiber transport at short distances from the source in an open environment, differentiating between chrysotile and crocidolite. **Methods:** Particulate plumes assessed in this study were generated through the cutting of a historical AC pipe containing approximately 30% chrysotile and 10% crocidolite asbestos. Six cuts were made in the pipe over a 30-minute sampling period, releasing approximately 115 g of material into the air. High velocity air sampling pumps were placed in concentric circles around the operator cutting the pipe. Samplers were placed at 28 different locations in concentric circles around the pipe cutter. Air samples were collected at distances of 8 to 95 ft from the source at a height of 4 ft above ground level. Two cassette samples were collected at each of the 28 sample locations around the point source for a total of 56 samples collected over the course of the study. **Results:** Fiber concentrations were highest (peak of 4.92 f/cc; 3.82 f/cc for chrysotile and 1.10 f/cc for crocidolite) along the visible dust plume direction (east) within 8 feet of the cutting interface and total asbestos exposure declined exponentially with distance (2.19 f/cc at 25 ft; 0.73 f/cc at 50 ft; 0.39 f/cc at 75 ft; 0.13 f/cc at 95 ft). At 90° from the plume axis, fiber concentrations at the 8-foot sampler were reduced by approximately 35%. Fiber concentrations were less than 0.7 f/cc beyond 90° from the plume axis. Aerodynamic equivalent diameter (AED) was strongly associated with deposition distance (crocidolite: $\beta = -0.046 \text{ ft}^{-1}$, $p = 3.48 \times 10^{-5}$; chrysotile: $\beta = -0.039 \text{ ft}^{-1}$, $p = 5.97 \times 10^{-4}$) while fiber length was not (crocidolite: $\beta = 0.00043 \text{ ft}^{-1}$, $p = 0.83$; chrysotile: $\beta = 0.00081 \text{ ft}^{-1}$, $p = 0.41$). Crocidolite fibers, which exhibited higher AEDs (crocidolite GM: 3.7; chrysotile GM: 2.0), settled more rapidly and were detected over shorter distances than chrysotile (crocidolite decay constant: 0.049 ft⁻¹; chrysotile decay constant: 0.044 ft⁻¹). A high proportion of fibers were bound to cement debris (chrysotile: 86%; crocidolite: 68%), and these bound fibers exhibited slightly steeper decay profiles (chrysotile: $\beta = -0.032$ vs -0.026 ; crocidolite: $\beta = -0.053$ vs -0.045). **Conclusions:** Empirical transport patterns aligned with AED-based theoretical models but diverged significantly from Gaussian plume assumptions, underscoring the limitations of traditional dispersion models for short-range, ground-level fiber emissions. These findings support the use of AED as a mechanistic predictor of fiber transport and highlight key considerations for exposure assessment in still-air environments. From an exposure perspective, the results highlight three key considerations. First, the combination of steep decay and directionality implies that short-range, directional sampling may be sufficient to characterize worst-case exposure under similar conditions. The "rule of thumb" relationship, presented in Donovan et al. (2011), between distance from a point source and fiber concentration can underestimate fiber concentrations in the plume window if the point source is directionally releasing fibers with significant velocity and significantly overestimate concentrations for locations not in that axis. Second, the predominance of debris-associated fibers—many of which settle rapidly—suggests that airborne residence time for a substantial fraction of released fibers may be limited, particularly for those with higher AED or bound to heavier debris. Finally, while crocidolite is often viewed as more hazardous due to its biopersistence and its high carcinogenic potency, its faster decay rate and shorter transport range may reduce its relative contribution to downwind exposures in similar scenarios.

PS 4672 Fluorotelomer and Perfluoro Aldehyde Lung Cell Toxicity: Implications for Indoor Exposure to Volatile PFAS

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Background and Purpose: Fluorotelomer alcohols (FTOHs) are a volatile subclass of per and poly-fluoroalkyl substances (PFAS) that represent the largest fraction of gas-phase PFAS in indoor air. Various industrial and consumer products, like certain food contact materials, and treated textiles and upholstery, are common sources of emissions. Atmospheric oxidation of FTOHs by hydroxyl radicals produces intermediate aldehydes, mainly fluorotelomer aldehyde (FTAL) and perfluoro aldehyde (PFAL), before terminating into perfluoro carboxylic acids (PFCAs). Yet, the inhalation exposure to and toxicity of the intermediate FTOH atmospheric degradation products is not well-understood, and previous *in-vitro* studies show FTAL compounds are more toxic than their terminal PFCA products. This study aims to examine cytotoxicity in lung cells by establishing LC_{50} values for 4:1 PFAL, 4:2 FTAL, 6:1 PFAL, 6:2 FTAL, 10:1 PFAL, and 10:2 FTAL through *in-vitro* assays, and characterize their chemical reactivity with the important antioxidant biological nucleophile glutathione (GSH) to investigate its contribution to the overall mechanism of toxicity. The central hypothesis is that FTALs will exhibit the highest cytotoxicity as reflected by the lowest LC_{50} values compared to FTOH and PFAL; the cytotoxicity of the aldehydes will decrease with decreasing chain length, and will correlate with GSH adduct

formation. **Methods:** Cytotoxicity was assessed by lung cell viability after 24 hour exposures of 4:1, 6:1 and 10:1 PFAL, and 4:2, 6:2, and 10:2 FTAL on 96-well plates of BEAS-2B cells, immortalized human epithelial lung cells, using a XTT assay measuring light absorbance of metabolized salts. Cells were exposed to FTOHs, FTALs, and PFALs in cell media with less than 1% v/v DMSO or ACN depending on solubility and includes a vehicle control. To predict reactivity with biological nucleophiles, electrophilicity indexes (ω) were calculated using ionization energy and electron affinity derived from orbital energies computed at the DFT/B3LYP/6-311+G(d, p) level of theory. Calculated electrophilicity will be compared to GSH depletion via detection of adducts in chemical assays of aldehyde standards incubated with GSH. Standard aldehyde-GSH adducts will be characterized using LCMS, and exposed cells will be screened for aldehyde-GSH adducts. **Results:** 4:2 FTAL, 6:2 FTAL, and 4:1 PFAL significantly reduced lung cell viability in the micromolar range in a dose dependent manner with LC_{50} 2 μ M, 40 μ M, and 2500 μ M, respectively. The chain length of the tested aldehyde compounds influenced the cell viability, demonstrating increasing cytotoxicity with decreasing chain length, in line with previous studies on aldehydes derived from PFAS. The remaining aldehydes to be tested are expected to follow this trend. Electrophilicity indexes rank as PFAL>FTAL>FTOH and are expected to correlate positively with GSH adduct formation. **Conclusions:** 4:1 PFAL had a considerably higher LC_{50} than 4:2 FTAL, implying the alpha carbon unique to FTAL may drive cytotoxicity. Despite higher electrophilicity index ranking, 4:2 FTAL is more cytotoxic than 4:2 PFAL, showing increasing electrophilicity index does not correlate with higher toxicity. 4:2 FTAL had a lower LC_{50} than 6:2 FTAL; shorter chain lengths may allow for greater uptake and ready reaction with thiol containing biological nucleophiles such as GSH, with FTAL being attacked at the alpha carbon by thiol containing molecules, while the longer chain compounds may be sterically hindered and thus decrease their reactivity. The results from this study imply that intermediate oxidation products from atmospherically oxidized FTOHs have their own inhalation exposure risks in addition to terminal PFCAs and parent compounds, and warrants further investigation to fully elucidate inhalation risks posed by FTOHs.

PS 4673 Sex chromosome dosage as a mechanism of neonatal pulmonary oxygen toxicity

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Background and Purpose: Preterm birth (<37 weeks gestational age) affects about 10% of pregnancies globally, and premature infants are predisposed to early life mortality and a variety of long-term adverse sequelae. Premature infants often rely on therapeutic interventions such as surfactant administration, mechanical ventilation, and supplemental oxygen (hyperoxia) for survival. Although recognized as a lifesaving treatment, hyperoxia contributes to alveolar simplification and pulmonary vascular dysfunction that is pathognomonic of bronchopulmonary dysplasia (BPD). BPD is the most common lung disease of prematurity, and surviving infants are at higher risk for diseases such as reactive airway disease and emphysema. BPD has a clinically recognized male bias for disease incidence and severity, underscoring an unmet need to identify molecular mechanisms of sex-specific pathobiology. Our lab previously discovered that mice with XX chromosomes, regardless of gonadal sex, had preserved alveolarization in a BPD mouse model. It is unknown if mice with XX chromosomes were protected because they lacked a Y chromosome, or because they had twice as many X chromosomes relative to XY mice. The objective of this study is to determine the role of sex chromosome dosage on lung development and response to neonatal hyperoxic lung injury. **Methods:** To interrogate sex chromosome dosage, we used the XY* mouse line that has XX, XO, XY, and XXY genotypes. Neonatal XY* mice were exposed to hyperoxia (95% FiO₂, PND 1-5) or room air (21% FiO₂) and euthanized on PND 7 or PND 21. This exposure paradigm was chosen to parallel the sacular stage of lung development when a human preterm infant would receive supplemental oxygen in the NICU. Bulk RNA sequencing was used to assess changes in the pulmonary transcriptome, including differentially expressed genes (DEGs) and biological pathways ($\log_2FC = 0.6$; FDR = 0.05) as a function of sex chromosome dosage (n = 4 pups/genotype/treatment). **Results:** We previously characterized alveolar development at PND 21 in XY* mice and identified an interaction between the Y chromosome and treatment for radial alveolar count (P = 0.0171), and an interaction between X chromosome dosage and treatment (P = 0.0392). To interrogate the pulmonary transcriptome at PND 21, we used bulk RNA sequencing and found a subtle transcriptional response to treatment. Therefore, we sought to investigate the acute transcriptional response (PND 7) that may instead be driving our observed phenotype. We identified 216 common DEGs (15%) at PND 7 to hyperoxia. We then compared mice with a Y chromosome (XY and XXY), without a Y chromosome (XO and XX), with one X chromosome (XO and XY), and with two X chromosomes (XX and XXY). We found that mice with two X chromosomes had the most DEGs (308) under hyperoxia, followed by mice lacking a Y chromosome (148), mice with only one X chromosome (144), and mice with a Y chromosome (57). Using GSEA, we identified 78 common pathways in response to hyperoxia, including humoral immune response and positive regulation of vascular endothelial growth factor production. Mice with two X chromosomes showed unique pathways in hyperoxia response such as modulation of inflammatory response, nitric oxide pathways, and lipid metabolism. **Conclusions:** Mice with a Y chromosome had worse outcomes after neonatal hyperoxic lung injury. Ongoing works are interrogating transcriptional response at single cell resolution at PND 7 and assessing pulmonary function at

PND 21 using FlexiVent. A comprehensive investigation of sex-specific responses to respiratory exposures informs our understanding of lung development, cumulative exposures, and long-term outcomes.

PS 4674 Aerosolized Microcystin from Harmful Algal Blooms drives congener-dependent inflammation and cytotoxicity in human airway cells

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Background and Purpose: Harmful algal blooms (HABs), primarily resulting from cyanobacterial overgrowth, are becoming more frequent and now pose serious environmental threats by releasing microcystin toxins into both water and air. Of the more than 300 identified microcystin variants, microcystin-leucine arginine (MC-LR) and leucine alanine (MC-LA) are frequently detected in both water and air during HAB events, highlighting inhalation as a significant but underappreciated route of exposure. This study investigated the physiological and inflammatory impacts of aerosolized microcystins on human airway cells. **Methods:** Primary human nasal and bronchial airway epithelial cells (hAEC) from healthy and asthmatic donors were grown at the air-liquid interface (ALI) and fully differentiated to mimic *in vivo* airway characteristics. The differentiated cells were exposed apically to aerosolized microcystins (1 μ M of MC-LR or MC-LA) or a saline control, using the expoCube targeted aerosol exposure system, which delivered a precise dose of 13 μ g/m² of each microcystin to the cell surface. Inflammatory responses were quantified at both the gene and protein levels: gene expression was measured by qRT-PCR, and protein concentrations were determined using ELISA. NF- κ B activity was assessed using A549-Dual reporter lung cells treated with 1 μ M of microcystin toxins with or without IL-1 β (0.5 ng/ml). Cytotoxicity was quantified by measuring LDH released into the conditioned media from both primary hAEC and A549 lung cells after 24-hour exposures to 1 μ M and 10 μ M of each toxin. **Results:** A 3D *in vitro* model of primary human nasal and bronchial airway epithelial cells showed full differentiation with functional cilia and mucociliary clearance. Exposure to 1 μ M MC-LR or MC-LA did not significantly alter cell morphology, while 10 μ M MC-LR caused minimal changes and 10 μ M MC-LA caused significant cell death. In healthy primary human airway cells, 1 μ M MC-LR increased *TNF- α* and *IL-6* levels (1.8-fold and 1.9-fold, respectively; p<0.001), with even greater increases observed in asthmatic cells (*TNF- α* : 3.5-fold; *IL-6*: 2.6-fold; p<0.001). In A549 cells, NF- κ B activity rose modestly (~10%, p<0.01) with MC-LR and substantially (~50%, p<0.01) with MC-LA, the latter being significantly higher than MC-LR (~40%, p<0.001). When stimulated with IL-1 β , NF- κ B activity further increased (~40% with MC-LR, ~70% with MC-LA; p<0.0001), highlighting a stronger pro-inflammatory response to MC-LA. **Conclusions:** Aerosolized microcystin exposure in human airway cells from nasal and bronchial origin induced pro-inflammatory genes, with asthmatic cells showing increased sensitivity. MC-LA triggered stronger pro-inflammatory and cytotoxic responses than MC-LR, posing higher respiratory risks for vulnerable groups. These findings highlight the potential exacerbation of airway inflammation by aerosolized MCs, particularly in vulnerable populations.

PS 4675 Hazard characterization of 2-ethyltoluene in rats and mice following whole-body inhalation exposure

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Background and Purpose: Onshore crude oil production in the United States has been increasing steadily over the last decade. Production begins by removing petroleum from underground reservoirs, followed by fractional distillation to separate crude oil into useful components, such as gasoline and diesel. The naphtha component is separated at a boiling point of 60-180°C and contains aromatic alkylbenzenes, such as trimethylbenzene, ethyltoluene isomers, and xylene mixtures. These alkylbenzenes are used in a variety of products, including gasoline mixing and blending, paints, sealants, and other industrial coatings, and combustion processes. Between 5 - 10 billion pounds of naphtha was produced in the U.S. in 2019, making it a high-production volume chemical. Thus, due to its widespread use, understanding occupational and general population exposures to the chemical constituents of this crude-oil fraction is essential for protecting human health and safety. Previously, a 2-week inhalation study in Sprague-Dawley (SD) rats and B6C3F1 mice, identified 2-ethyltoluene (2-ET) as the most potent isomer, causing high mortality and prominent liver, lung, and nasal lesions. Thus, we designed a guideline sub-chronic inhalation study of 2-ET at several concentrations in mice and rats to further characterize these health effects. **Methods:** Male and female SD rats and B6C3F1/N mice (10 animals/sex/species) were exposed to 0, 3.13, 6.25, 12.5, 25, 50, and 100 ppm of 2-ET via whole-body inhalation for 6hrs/day over 13 weeks. Following exposure, survival, body and organ weights, and histopathology were assessed. **Results:** The results from this study indicated that the nose and brain might be target organs for 2-ET toxicity. In B6C3F1/N mice, there was a trend for a significant treatment-related body-weight decrease in male mice only. We observed significant decreases in terminal organ weights compared to controls that occurred in a dose-response manner across males and females, specifically at 100ppm expo-

sure levels for the liver (~12%), kidneys (~20%), and in the epididymis (~16%) and ovaries (~31%), although these changes did not manifest with histologic correlates. In SD rats, terminal body weights were significantly decreased in males and females at ≥ 12.5 ppm. Histopathology revealed test-article related lesions in the nasal cavity and the olfactory lobes of the brain, in both SD rats and B6C3F1/N mice of both sexes. Nasal lesions included olfactory epithelium atrophy, olfactory nerve atrophy, chronic active inflammation, concretions, and metaplasia of the olfactory epithelium. Respiratory epithelium hyperplasia was observed in rats but not in mice. Olfactory bulb atrophy was evident in the brain for both rats and mice. Nasal lesions were present at all doses starting from 3.13 ppm, whereas brain lesions appeared at ≥ 6.25 ppm. We were unable to identify a no observed effect level (NOEL) since changes were observed at every tested concentration of 2-ET. These findings indicate that 2-ET is a potent isomer that elicits adverse effects in the nasal cavity and olfactory lobes of the brain following inhalation exposures at concentrations of 3.13ppm or higher. **Conclusions:** This study provides valuable insights into the health effects of 2-ET, advancing potential risk assessment and read-across methodologies, as other structurally related compounds do not elicit the same potency and toxicity as 2-ET. Further studies are underway to identify a NOEL and to characterize potential bioactive metabolites of 2-ET that may contribute to nasal and brain toxicity. These insights help bridge critical data gaps needed to better assess human health risks and strengthen public health preventions against this ubiquitous environmental compound.

PS 4676 6PPD drives acute airway inflammation and macrophage shifts toward remodeling

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Background and Purpose: N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine (6PPD) is a widely used rubber antioxidant, yet its respiratory toxicity and immunological mechanisms following airway exposure remain largely unclear. Macrophages play a central role in the initiation and resolution of lung inflammation, and their M1 and M2 polarizations strongly influences respiratory disease outcomes. Here, we investigated the temporal dynamics of pulmonary inflammation and macrophage polarization induced by 6PPD in both *in vivo* and *in vitro* studies. **Methods:** Mice received a single intratracheal instillation of 6PPD and were sacrificed on days 1, 4, 7, and 14. Lung weight, inflammatory cell profiles, and cytokine/chemokine levels were assessed along with histopathological evaluation. In parallel, RAW264.7 macrophages were exposed to 6PPD for 24 hours to determine polarization patterns via flow cytometry. **Results:** *In vivo*, 6PPD exposure at day 1 increased lung weight and eosinophil counts, accompanied by elevated interferon- γ (IFN- γ), interleukin-5 (IL-5), monocyte chemoattractant protein-1 (MCP-1), and vascular endothelial growth factor (VEGF). By day 4, prominent macrophage aggregation appeared in lung tissue. *In vitro*, 6PPD induced a dose-dependent shift in macrophage polarity, promoting M1 polarization at lower concentrations and M2 polarization at higher concentrations. **Conclusions:** Collectively, these findings demonstrate that 6PPD triggers acute eosinophilic inflammation with progressive macrophage recruitment, alongside a concentration-dependent polarization response. Given that M1-to-M2 transitions are associated with airway remodeling and fibrosis, repeated or high-level exposure to 6PPD may contribute to chronic respiratory pathology. These results reveal a previously unrecognized immunological mechanism of 6PPD and support its classification as a potential respiratory toxicant.

PS 4677 N-acetyltransferase 2 acetylator phenotype modifies N-acetylation, DNA damage and oxidative stress responses of 2-amino-9H-pyrido[2,3-b]indole

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Background and Purpose: 2-Amino-9H-pyrido[2,3-b]indole (AaC) is a heterocyclic amine carcinogen which induces liver and blood vessel tumors and aberrant crypt foci, an early biomarker of colon cancer, in mice. (AaC) is present at high levels in tobacco smoke. Human exposure also occurs following dietary ingestion of well-done meats and exposures to diesel fumes. AaC is genotoxic in human lymphoblastoid and peripheral blood lymphocytes cells when assessed by comet and micronucleus assays and it forms high and persistent levels of DNA adducts in primary human hepatocytes. The high levels of AaC in tobacco smoke and its hepatic bioactivation may contribute to development of liver and gastrointestinal tract cancers in smokers. AaC bioactivation is catalyzed by cytochrome P450-mediated (CYP1A2) N-hydroxylation of the exocyclic amine group followed by O-acetylation catalyzed by N-acetyltransferase 2 (NAT2) to form the acetoxy derivative which binds covalently to DNA leading to mutagenesis and potentially carcinogenesis. A genetic polymorphism in NAT2 yields rapid, intermediate and slow acetylator phenotypes. Epidemiological studies suggest a role for human NAT2 genetic polymorphism in cancer risk following heterocyclic amine exposures in tobacco smoke and consumption of well-done meats. **Methods:** The role of NAT2 acetylator phenotype in AaC N-acetylation, DNA damage response and oxidative stress were investigated in rapid, intermediate, and slow acetylator cryopreserved human hepatocytes and in

nucleotide excision repair-deficient (UV5) Chinese hamster ovary (CHO) cells stably transfected with human CYP1A2 and either NAT2*4 (rapid acetylator) or NAT2*5 or NAT2*7 (slow acetylator) alleles. Cryopreserved human hepatocytes possessing two NAT2 alleles associated with rapid acetylation activity were classified as rapid acetylators, samples possessing two NAT2 alleles associated with slow acetylation activity were classified as slow acetylators. Samples possessing one allele associated with rapid acetylators and one allele associated with slow acetylators were classified as intermediate acetylators. AaC N-acetylation was quantified by separation and quantification of AaC and its N-acetyl-AaC by reverse phase high performance liquid chromatography. CHO cells treated with AaC were assessed for DNA damage response using phospho-histone H2A.X (Ser139) antibody and RedDot™2 Far-Red Nuclear Stain for normalization to cell number. CHO cells and cryopreserved human hepatocytes treated with AaC were assessed for oxidative stress response using the 2',7'-dichlorodihydrofluorescein diacetate fluorescence assay. **Results:** Levels of AaC N-acetylation and oxidative stress response were NAT2 phenotype-dependent in human cryopreserved human hepatocytes. Rates were significantly highest in rapid acetylators, lower in intermediate acetylators and lowest in slow acetylator cryopreserved human hepatocytes ($p < 0.0001$). Treatment of CHO cells with AaC resulted in dose-dependent DNA damage and oxidative stress responses that were significantly highest in CHO cells transfected with CYP1A2 and rapid acetylator NAT2*4 compared to un-transfected UV5 CHO cells, or UV5 CHO cells transfected with CYP1A2, CYP1A2/NAT2*5 or CYP1A2/NAT2*7 ($p < 0.0001$). **Conclusions:** N-acetyltransferase 2 acetylator phenotype modifies AaC N-acetylation, and DNA damage and oxidative stress responses following AaC treatment. Although N-acetylation of AaC may compete with N-hydroxylation, the O-acetylation of the N-hydroxy-AaC appears to be more impactful towards determining levels of DNA damage and oxidative stress responses. These laboratory results support epidemiological studies reporting a modifying effect of human NAT2 genetic polymorphism in cancer risk from tobacco smoke and consumption of well-done meats.

PS 4678 Effect of lead on endothelial cell viability and junctional integrity and the potential protective role of dapagliflozin

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Background and Purpose: Cancer is the second leading cause of death globally, primarily due to metastasis. Transendothelial migration of tumor cells across the vascular barrier is a key step, regulated by the integrity of endothelial cell junctions. Adherens junctions, with VE-cadherin as a crucial component, maintain vascular integrity, and disruption facilitates tumor cell migration. Environmental lead (Pb) exposure impairs endothelial function by increasing oxidative stress and reducing nitric oxide (NO) bioavailability. Dapagliflozin (DAPA), a sodium-glucose co transporter 2 (SGLT2) inhibitor, may protect endothelium from toxic insults by reducing oxidative stress, preventing cell death, and promoting survival. This study examines the impact of lead and DAPA on endothelial viability and junction integrity, addressing whether DAPA can mitigate lead's adverse effects. **Methods:** Primary human umbilical vein endothelial cells (HUVECs) were cultured in endothelial growth medium supplemented with bovine brain extract and plated in 384-well plates to ensure reproducible monolayer formation. Cells were dosed with lead acetate trihydrate at ten concentrations ranging from 0.08 to 40 μ M for time points at 48 hours and 96 hours and subsequently stained with the nuclear stain Hoechst 33342 for quantitative assessment of cell number. In parallel, a dose-response experiment measuring differences in cell number was conducted with DAPA at concentrations from 0.08 to 10 μ M, after which 10 μ M was selected as the concentration for all subsequent co-dosing assays. Three lead doses were selected—0.16 μ M, 1.25 μ M, and 10 μ M and each was tested both independently and in combination with 10 μ M DAPA. Cell number was quantified through high-content imaging using a CX5 microscope and CellProfiler 4.2.1, with cell counts normalized as percent of average control values for comparative analysis. Junctional integrity was evaluated by immunofluorescence staining for VE-cadherin, with automated measurement of edge intensity to assess changes in junction localization. Statistical analyses for the initial dose-response experiments were performed using boxplots and t-tests in R across replicates and conditions, while Wilcoxon rank-sum tests were employed for co-dosing experiments to compare cell counts and VE-cadherin edge intensity across groups. **Results:** Lead exposure reduced HUVEC cell counts in a concentration-dependent manner, with cytotoxic and anti-proliferative effects observed at 40 μ M ($p < 0.05$). DAPA alone promoted an increase in cell counts relative to untreated controls. In co-dosing experiments, DAPA exerted a marked rescue effect across all the tested doses, elevating cell counts to near-control levels at 96 hours (10 μ M $p < 2.3e-05$; 1.25 μ M $p < 2.5e-12$; 0.16 μ M $p < 0.02$). VE-cadherin immunofluorescence analysis revealed that Pb alone did not cause substantial changes in junction integrity, as edge intensity remained largely stable across different Pb doses and time points. In contrast, DAPA co-treatment led to notable increases in VE-cadherin edge intensity at both moderate and high lead exposures (1.25 μ M and 10 μ M), most significantly at 96 hours ($p < 2e-16$). **Conclusions:** DAPA confers dose-dependent protection on HUVEC viability over time, and restores VE-cadherin-mediated junction organization, especially following 96 hours of exposure. Importantly, DAPA's restorative effects on VE-cadherin organization and barrier integrity were present even in settings where total cell counts did not fully recover, underscoring the potential for pharmacological intervention to preserve junctional function under toxic stress conditions. These findings support DAPA's therapeutic potential for preserving endothelial barrier func-

tion following environmental lead exposure. Ongoing experiments are evaluating the effect of lead on endothelial cell junctions using functional transwell migration assays with breast cancer cells to directly assess whether DAPA mitigates lead-induced endothelial toxicity and limits metastatic migration, further establishing its relevance in cancer-related vascular toxicity.

PS 4679 Personalized ω -3 Fish Oil Clinical Trial Supplementation Decreases Pro-Inflammatory Adipokine Secretion and *In Vitro* Mammary Stem Cell Self-Renewal

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Background and Purpose: Adipose inflammation associated with obesity is a driver of cancer risk and mortality. Adipose tissue secretes cytokines, termed adipokines, which regulate inflammation, metabolism, and other biological processes and are dysregulated during obesity. Mammary stem cells are a target of adipokines and the expansion of this stem cell population is a hypothesized driver of breast cancer risk. Dysregulated secretion of cytokines in obesity may promote the expansion of breast stem cells. Dietary ω -3 polyunsaturated fatty acids are used to alleviate symptoms of inflammatory diseases, such as rheumatoid arthritis. Thus, an ω -3 rich fish oil intervention may be an effective strategy to reduce breast cancer risk by alleviating adipose tissue inflammation and decreasing secretion of mediators that regulate breast stem cell proliferation. To test this, we conducted a clinical trial examining the impacts of a personalized fish oil intervention on adipose inflammation and breast stem cell outcomes. **Methods:** Fifteen clinically obese (BMI ≥ 30 kg/m²) women were enrolled in a clinical trial, of which 13 were able to successfully complete. Prior to fish oil dosing, blood was collected as well as subcutaneous adipose tissue via needle aspiration. Participants then received a personalized fish oil dosage designed to deliver a serum EPA: ω -3 ratio of 0.3. After 12 weeks, participants returned and blood and subcutaneous adipose were collected. Total lipids were extracted from serum and fatty acids measured by GC-MS. Adipose tissue was cultured for 48 hours and the resulting adipose conditioned media (ACM) collected. One sample was excluded due to culture contamination. Twenty-two serum cytokines and adipose-secreted adipokines were measured using Luminex or ELISA. Effects of ACM from pre- and post-treatment samples on mammary stem cells were tested using the mammosphere assay using a pool of normal human breast epithelial cells from reduction mammoplasty donors. Differences between pre- and post-treatment were determined using paired t-tests and a statistical significance cutoff of 0.05. **Results:** Twenty-eight fatty acids were detectable in most serum samples and concentrations of ω -3 fatty acids were increased after 12 weeks of fish oil supplementation. Eicosapentaenoic acid (20:5 ω -3), docosahexaenoic acid (22:6 ω -3), and docosapentaenoic acid (22:5 ω -3) were increased from 0.32 to 2.25 mol% ($p < 0.001$), 0.50 to 1.31 mol% ($p = 0.0012$), and 0.34 to 0.76 mol% ($p < 0.001$) respectively. The EPA:AA ratio also increased from 0.06 to 0.46 ($p < 0.001$). Of 17 circulating cytokines detected, 6 were significantly altered. Serum C-reactive protein decreased by 29.3% (4.65 μ g/ml to 3.27 μ g/ml, $p = 0.0016$). Circulating adiponectin, complement factor 2, and osteopontin decreased by 8.5% ($p = 0.013$), 9.1% ($p = 0.029$), and 7.68% ($p = 0.033$). Serum RBP-4 increased by 179% ($p = 0.0007$) and CCL-2 by 17.0% ($p = 0.021$). Of 16 adipokines measured in the adipose conditioned media (ACM), 2 decreased significantly, including C-reactive protein by 57.6% ($p = 0.0043$), CXCL-5 by 81.7% ($p = 0.025$). Primary mammosphere formation was not different when cultured with pre- and post-fish oil ACM (1.95% vs. 1.81%, $p = 0.38$). However, secondary mammosphere formation efficiency decreased when treated with post-fish oil ACM compared to pre-fish oil ACM (1.75% vs. 1.40%, $p = 0.03$). **Conclusions:** Personalized daily fish oil supplementation incorporated ω -3 fatty acids into blood lipids of trial participants after 12 weeks of treatment. Multiple inflammatory factors and adipokines were decreased after fish oil treatment. Secondary mammosphere formation was decreased when treated with post-fish oil ACM, suggest that altering secreted adipokines reduces breast stem cell self-renewal. Our results show that fish oil may reduce inflammatory adipokine secretion which, within the breast microenvironment, may decrease mammary stem cell expansion and thus breast cancer risk.

PS 4680 JNK Inhibition by HLN Disrupts Mitochondrial Proton Motive Force to Induce Ferroptotic Cell Death in Colorectal Cancer Therapy

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Background and Purpose: Mutations within MAPK signaling, including dysregulated JNK activity, promotes colorectal cancer (CRC) progression and therapy resistance. The JNK pathway is a central regulator of cellular stress and mitochondrial integrity. While its activation is associated with survival signaling and redox balance, its inhibition can dismantle metabolic homeostasis. Ferroptosis, an iron-dependent oxidative death pathway, represents an emerging strategy to overcome apoptosis resistance in CRC. In this study, we hypothesized that HLN* functions as a potent JNK inhibitor that drives ferroptotic cell death in CRC cells through disruption of mitochondrial bioenergetics and collapse of the proton motive force (PMF). **Methods:** CRC

cell lines (CT26, COLO-205, and HCT116) were treated with HLN to determine IC₅₀ values by SRB. CFA were conducted to investigate HLN's impact on the long-term proliferative potential of CRC cells. ROS generation was measured using DCFDA assay. Ferroptosis induction was assessed by lipid peroxidation MDA assay, GSH depletion and GST activity. Labile iron levels were assessed by ferro-orange assay. Mitochondrial function was evaluated via JC-1 potential assay to quantify PMF integrity as well as DiSC3(5) assay. RNA-seq and GO enrichment analyses were performed to identify pathways downstream of JNK inhibition, focusing on ferroptosis and oxidative stress. Western blotting analyzed phospho-JNK levels & other JNK pathway related proteins. To further delineate the mechanism of ferroptosis induction, key proteins were assessed using western blot such as GPX4, xCT and DMT1 expression. Mechanistic rescue assays used NaHCO₃ and NAC. For preliminary *in vivo* validation, an orthotopic CRC model was established by injecting luc-labeled CT26 cells into the cecal wall of BALB/c mice ($n = 5$ per group). After tumor establishment (day 7), mice received HLN (10 mg/kg daily oral administration) or vehicle control. Tumor growth was monitored by bioluminescence imaging. **Results:** HLN treatment exhibited potent cytotoxicity across CRC cell lines, with IC₅₀ values ranging from 1 to 4 μ M as determined by SRB assay. CFA showed a dose-dependent reduction in long-term proliferative capacity. HLN markedly increased intracellular ROS, confirming oxidative stress. This was accompanied by a significant increase in lipid peroxidation, depletion of reduced GSH, and suppression of total GST activity. Ferro-orange staining revealed a rise in labile iron pools, reinforcing the iron-dependent oxidative phenotype characteristic of ferroptosis. HLN profoundly impaired mitochondrial function. JC-1 and DiSC3(5) assays both demonstrated mitochondrial depolarization and collapse of the cytoplasmic proton-motive force (PMF), indicating bioenergetic failure. Western blot confirmed JNK pathway inhibition. Transcriptomic and GO enrichment analyses further identified enrichment in oxidative-stress and toxic-response pathways, alongside downregulation of antioxidant regulators. These transcriptional findings were corroborated at the protein level, where HLN decreased expression of GPX4, xCT, and increasing the iron transporter DMT1, implicating suppression of cystine uptake, glutathione synthesis, and iron handling. Pretreatment with N-acetylcysteine (NAC) or NaHCO₃ effectively rescued cells from ROS accumulation, restored GSH content, and mitigated HLN-induced cytotoxicity, confirming that oxidative stress downstream of JNK inhibition is central to its ferroptotic action. In the orthotopic CRC model, HLN treatment resulted in a reduction in tumor bioluminescence relative to control. No significant body-weight loss or gross toxicity was observed, underscoring tolerability. **Conclusions:** Our findings identify JNK inhibition as a novel upstream trigger for ferroptosis via mitochondrial dysfunction and PMF dissipation. By suppressing JNK-dependent antioxidant and metabolic repair pathways, HLN destabilizes mitochondrial potential, amplifies ROS accumulation, and silences the GPX4-SLC7A11 axis, driving lipid peroxidation, mediated ferroptotic death. This study defines a JNK-PMF-ferroptosis axis as a critical vulnerability in CRC and introduces HLN as a first-in-class small molecule targeting this energy-redox interface for therapeutic exploitation. *Due to Intellectual property issue, the name of the compound is not provided

PS 4682 Mechanisms of Hexavalent Chromium-Induced Carcinogenesis: DNA Double Strand Breaks and DNA Repair Inhibition in a Guinea Pig Model

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Background and Purpose: Hexavalent chromium [Cr(VI)] is an environmental and occupational health hazard, and a known lung carcinogen. Despite the exposure risks, it is unclear how Cr(VI) causes lung cancer. Prolonged Cr(VI) exposure induces DNA double strand breaks and inhibits their repair, leading to chromosome instability, a hallmark of lung cancer. Specifically, Cr(VI) inhibits RAD51, a key effector protein in the homologous recombination repair pathway. This mechanism has been well characterized in cell culture assays and was recently translated to a rat model. However, rats have different bronchial bifurcation patterns from humans, which is critical because Cr(VI) particles impact and form tumors at these sites in humans. In contrast, guinea pigs share these bronchial bifurcation patterns with humans, making them a more translatable model to study how Cr(VI) causes lung cancer. Our goal was to translate the mechanism of Cr(VI)-induced DNA double strand breaks and DNA repair inhibition to guinea pig lungs. We hypothesized acute Cr(VI) exposure would increase the levels of DNA double strand breaks and increase DNA repair, while subchronic exposure would increase DNA double strand breaks and inhibit their repair. **Methods:** 10-11-week-old Hartley guinea pigs were exposed to zinc chromate via oropharyngeal aspiration for an acute (24 h) or subchronic (90-day) exposure. Zinc chromate was used as a representative particulate Cr(VI) compound, as epidemiological and toxicological data show it causes lung cancer in humans. At the end of each exposure, animals were euthanized and lungs were dissected. A piece of left upper lobe lung tissue was analyzed for metal contents by

inductively coupled plasma mass spectrometry to confirm Cr reached the lungs. The right middle lobe was used for immunofluorescence staining. DNA double strand breaks were measured using the gamma-H2AX, and homologous recombination repair was assessed using RAD51. Z-stack images were obtained using a confocal microscope, and nuclear gamma-H2AX and RAD51 foci were quantified. **Results:** Cr levels increased in Cr(VI)-exposed groups, indicating our exposures reached the lungs of guinea pigs. In addition, zinc levels were also measured because we used zinc chromate as our representative Cr(VI) compound, but as expected, we did not observe significant changes in zinc levels. When comparing animals acutely and subchronically exposed to Cr(VI), acute Cr(VI) exposure induced DNA double strand breaks and DNA repair, whereas subchronic exposure also caused DNA double strand breaks but inhibited repair. This effect of increased Cr(VI)-induced increased DNA double strand breaks and inhibited repair after subchronic Cr(VI) exposure was largely observed in bronchial regions but not in alveolar regions. **Conclusions:** This study establishes a new human-relevant guinea pig model to study Cr(VI)-induced carcinogenesis and translates the mechanism of Cr(VI)-induced DNA double strand break formation and repair inhibition to guinea pig lungs. In addition, results in this study showed bronchial regions are more sensitive to this mechanism than alveoli, which is consistent with previous studies showing Cr accumulates in the bronchial regions where it forms tumors. Future studies will assess how Cr(VI) inhibits DNA repair pathways to drive lung carcinogenesis. This work was supported by the National Institute of Environmental Health Sciences grant R35ES032876 (JPWSr.) and a fellowship from "la Caixa" Foundation LCF/BQ/EU22/11930060 (IM).

PS 4683 KRAS Variant-Dependent Effects of PFOS and PFHxS Exposure in Pancreatic Ductal Adenocarcinoma Cells

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are known as "forever chemicals" based on their persistence in biota and the environment. Perfluorooctanesulfonate (PFOS) is one of the most prevalent PFAS, previously used to produce non-stick materials and concentrated in aqueous film-forming foams used in firefighting. PFOS was removed from many products and processes due to hazards, and it was replaced by a shorter chain (6-carbon vs. 8-carbon) analog of PFOS known as perfluorohexanesulfonate (PFHxS). PFAS have been correlated with increased risk for several cancers, but their role in pancreatic carcinogenesis remains poorly understood. Pancreatic ductal adenocarcinoma (PDAC) is among the deadliest cancers with activating KRAS mutations present in approximately 90-95% of cases. To assess the impact of PFOS and PFHxS on oncogenic and clinically relevant KRAS variants, isogenic PANC1 cells expressing KRAS G12D, KRAS G12R and KRAS Q61R mutations were exposed to a concentration range of each compound. KRAS variant-specific signaling in PDAC differentially activates effector pathways such as MAPK, PI3K, and RAL-GDS, influencing tumor cell behavior. Therefore, variant-specific differences in proliferation, gene expression, and morphology in response to PFAS were measured. **Methods:** Isogenic KRAS mutant PANC1 cells were generated using CRISPR-mediated knock-in approaches allowing for controlled interrogation of KRAS variant-specific responses to PFAS. Cells were exposed to 6.25-200 μ M of PFOS and PFHxS for 48 hours, and proliferation was measured using the PrestoBlue assay. Epithelial-to-mesenchymal (EMT) marker gene expression was quantified by quantitative PCR (qPCR). Variant-specific transcriptomic and morphological feature analyses were conducted using RNA-sequencing coupled with Cell Painting, respectively. **Results:** Both PFOS and PFHxS increased cell proliferation in a concentration-dependent manner, with cytotoxicity only observed at 200 μ M PFOS. KRAS G12R and KRAS Q61R variants displayed greater proliferative responses to PFHxS than KRAS G12D. qPCR for EMT-related genes revealed variant-specific effects, with PFOS more than doubling TWIST1 expression in KRAS G12D cells and PFHxS increasing TWIST1 in the same manner in KRAS G12R cells. **Conclusions:** These results suggest that PFOS and PFHxS promote pro-tumorigenic signaling in a KRAS variant-specific manner. Ongoing RNA sequencing and Cell Painting analyses will integrate transcriptomic and morphological responses to PFAS exposure. Collectively, these data will identify candidate biomarkers of PFAS exposure in genetically predisposed individuals, aiding early detection and risk stratification. They will also reveal molecular signatures that inform patient selection and risk modeling in environmental epidemiology. Finally, they may uncover pathways or vulnerabilities that can be targeted to mitigate the synergistic effects of PFAS exposure in KRAS-driven cancers.

PS 4684 A Mode of Action (MOA) and Human Relevance Framework (HRF) Analysis for Nasal Tumors Induced by Methyl Isothiocyanate (MITC) in Sprague Dawley Rats

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Background and Purpose: Methyl isothiocyanate (MITC; CAS # 556-61-6) is formed in soil following application of soil fumigants, including metam sodium and metam potassium. Exposure to MITC via inhalation following application of the fumigants has been assessed in human health risk assessments, most notably for workers and bystanders. MITC induced nasal tumors in Sprague Dawley rats exposed by inhalation to a high concentration (20 ppm), but not at 0.5 and 5 ppm. To investigate the MOA for the tumors and their relevance to humans, a study was conducted to understand the nature and severity of the acute and subacute responses to inhaled MITC in the rat nasal mucosa, at tumorigenic and non-tumorigenic concentrations. **Methods:** Seven-week-old male Crl:CD(SD) rats (8/group/time point) were exposed to filtered air (control) or MITC (0.5, 5 or 20 ppm) by whole-body inhalation for 6 hours per day for 1 day, 1 week (5 exposures per animal), or 4 weeks (5 days per week). Exposures were conducted using 1000-L stainless steel whole-body chambers. One day after the end of the last scheduled exposure, the animals were fasted overnight and sacrificed. Additional male rats (8/group) were similarly exposed for 5 days/week for 4 consecutive weeks, but sacrificed four weeks after the last exposure (recovery group). Two hours before the scheduled termination, the rats received an intraperitoneal injection of 50 mg/kg body weight bromodeoxyuridine (BrdU). Nasal tissues were taken at necropsy, fixed in 10% neutral-buffered formalin and processed for light microscopic examination of histopathology and quantitative morphometric analysis of BrdU-labelled nasal epithelial cells in regions that correlate with previously determined sites of nasal tumors. Tissue blocks were taken from four locations throughout the nasal airways (T1, T2, T3, and T4). The MITC-induced lesions identified for semiquantitative severity scoring were inflammation, epithelial hyperplasia in transitional and respiratory epithelium, squamous cell metaplasia in transitional and respiratory epithelium, epithelial degeneration and necrosis in transitional and respiratory epithelium, olfactory sensory neuron apoptosis in olfactory epithelium, olfactory sensory neuron loss resulting in atrophy of olfactory epithelium, and respiratory metaplasia of olfactory epithelium. Morphometric measurements, such as unit length BrdU labeling index in nasal epithelium (ULLI), were performed at a select anatomic site (maxilloturbinates in T1 section) that corresponded to an intranasal region with MITC-induced epithelial lesions. Data for the BrdU ULLI were statistically analyzed using a one-way ANOVA and Tukey's HSD post-hoc test. Semi-quantitative data from histopathological scoring of inflammation and epithelial hyperplasia were analyzed by the Mann-Whitney test and the Kruskal-Wallis test to compare the effects of single and multiple days of exposure. The available data on MITC toxicity were evaluated using the International Programme on Chemical Safety framework for determining MOA for the tumors and their relevance to humans. **Results:** Analysis of data from this study as well as other available toxicity data (acute and repeat dose inhalation studies in rats at various concentrations and durations) indicated that a biologically plausible MOA leading to nasal tumors at the 20 ppm exposure level involved the following key events: 1) direct cytotoxicity of MITC to the nasal mucosa (degeneration, necrosis/apoptosis); 2) consequent increased cell proliferation to compensate for MITC-induced cytotoxicity; 3) persistence of squamous cell metaplasia of the respiratory mucosa as an adaptive response to the cytotoxic insult; and 4) development of tumors, primarily squamous cell carcinomas. The analysis also examined and excluded several alternate MOAs such as genotoxicity, estrogenicity, and immunosuppression. **Conclusions:** The data informed that the tumorigenicity of MITC has exposure thresholds and is not likely to pose a carcinogenic risk to humans under normal use patterns and corresponding exposure levels.

PS 4685 Estrogenic Endocrine Disrupting Compounds Increase Cell Proliferation and Cancer Stemness in Estrogen Receptor Positive Breast Cancer Cells

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Background and Purpose: Estrogenic endocrine disrupting compounds (EDCs) found in plastics, drinking water, and food are able to bind to the estrogen receptor (ER). Excessive estrogen signaling drives proliferation, progression, and the formation of breast cancer stem cells (CSCs) in ER-positive breast cancer. Thus, we hypothesized that estrogenic EDCs mimic estrogen (E2) in breast cancer cell lines. **Methods:** Three estrogenic EDCs routinely found in human biosamples were selected for analysis: bisphenol-A (BPA), diethyl-hexyl phthalate (DEHP), and alpha-zeranol (α ZAL). We assessed proliferation, transcriptional reprogramming, and CSC formation in several breast cancer cell lines. **Results:** E2, BPA, and α ZAL significantly increased cell proliferation in ER-positive, but not in ER-negative cell lines. This was reversed with the administration of an ER-antagonist, ICI 162,780. BPA and α ZAL upregulated estrogen target genes (PGR, TFF1) and increased levels of cell cycle protein. Whole-transcriptome RNA sequencing analysis revealed that BPA and α ZAL altered the expression of genes in pathways related to cell division, DNA repair, and estrogen signaling, with a substantial transcriptional overlap between EDC and estrogen

treatments. Additionally, in the ER-positive cell line MCF-7, BPA and α ZAL increased the proportion of CSCs, defined as the CD24^{low}/CD44^{high} expressing subpopulation.

Conclusions: Overall, these data indicate that BPA and α ZAL act as functional estrogen mimics in breast cancer cells, activating canonical estrogen signaling pathways and promoting stem-like characteristics. Exposure to EDCs is nearly unavoidable; therefore, understanding their estrogenic activities is crucial for assessing environmental contributions to breast cancer risk. This work provides mechanistic insight into how environmental EDCs may influence ER-positive breast cancer biology.

PS 4686 Carcinogenicity of vinyl chloride monomer: A weight-of-evidence review by cancer sub-type

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Background and Purpose: The United States Environmental Protection Agency (EPA) designated vinyl chloride monomer (VC) as a High-Priority Substance for risk evaluation under the Toxic Substances Control Act (TSCA) with cancer as a key health outcome. However, the evidence for the relationship between VC and cancer varies by sub-type. The objective of this review was to independently assess the quality of the available epidemiological and toxicological literature to determine the weight of evidence for the relationship between VC exposure and site-specific cancer risk. **Methods:** A review of English-language literature was conducted in PubMed and Scopus databases through October 7, 2024, and compared with reference lists from Agency for Toxic Substances and Disease Registry, Texas Commission on Environmental Quality, and EPA Integrated Risk Information System assessments of VC. Inclusion criteria were met when the study included relevant population (e.g., human, animal), exposure (e.g., VC via oral, inhalation, or dermal route), comparator (e.g., unexposed epidemiological controls, sham or vehicle control), and outcome (e.g., cancer, genotoxicity, carcinogenicity). The quality of each study was evaluated using the Draft Systematic Review Protocol Supporting TSCA Risk Evaluations for Chemical Substances. Studies were numerically scored from 1 to 4 (1 indicating highest quality) on methodological and analytical characteristics, including study participation, exposure characterization, outcome assessment, potential confounding, and statistical analysis for epidemiological studies, as well as test substance, test design, exposure characterization, test organisms, outcome assessment, confounding control, and data analysis for toxicological studies. Individual metric scores were averaged to provide an overall ranking of *high* ($1 \leq X < 1.7$), *medium* ($1.7 \leq X < 2.3$), *low* ($2.3 \leq X \leq 3$), or *critically deficient* (score of 4 on any metric). **Results:** A total of 96 epidemiological and 31 toxicological studies that assessed the association between VC exposure and cancer outcomes/carcinogenicity were evaluated. The 54 human incidence and mortality studies received the following ratings: 21 high-quality, 25 medium-quality, 6 low-quality and 2 critically deficient. In 13 occupational cohort studies, there was a statistically significant positive association between VC exposure and cancer of the liver or intrahepatic bile ducts. Seven high-quality occupational cohort studies and one medium-quality ecological study assessed hepatocellular carcinoma (HCC) and/or angiosarcoma of the liver (ASL). A statistically significant association with VC exposure was reported in all occupational cohorts for HCC and 5 for ASL. Overall, results were negative for cancers of the brain and central nervous system, respiratory system, hematopoietic and lymphatic, skin (including malignant melanoma), breast, and connective and soft tissues. Among 42 human genotoxicity studies, 3 were high-quality, 28 were medium-quality, 7 were low-quality, and 5 were critically deficient. Generally, high-quality studies indicated genotoxicity among workers exposed to VC for at least one year, including increased micronuclei frequency, decreased relative telomere length, and decreased expression of shelterin component genes relative to controls. Animal genotoxicity studies assessed included 7 *in vivo* studies (2 medium-quality, 3 low-quality, and 2 critically deficient), and results reinforce the observation of clastogenicity observed in humans. The 24 animal carcinogenicity studies were largely high- ($n=8$) or medium-quality ($n=10$). Animal studies similarly report common findings of liver cancers, including HCC and ASL, with less consistent observations of lung tumors. **Conclusions:** Collectively, the weight of evidence supports a positive association between VC exposure and cancers of the liver, but not other cancer types. The existing evidence is limited by the absence of occupational cohort studies at contemporary exposure levels. Additional and/or updated occupational cohort studies would provide data to address the potential for human health risk from VC exposure under current conditions.

PS 4687 A Quantitative Weight-of-Evidence Evaluation of Rodent Data Demonstrates Sufficient Evidence for a Lack of Carcinogenic Potential of Acetaminophen

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Background and Purpose: Acetaminophen is a common antipyretic/analgesic used in the treatment of fever and minor pain, headache, myalgia, and dysmenorrhea. Although previous regulatory assessments on genotoxicity and carcinogenicity have

concluded that acetaminophen is not a carcinogenic hazard, toxicological and regulatory evaluations continue to be conducted due to new data and the widespread use of this medication. In 2024, International Agency for Research on Cancer (IARC), a specialized cancer agency of the World Health Organization, announced the intent to re-evaluate the carcinogenic hazard potential of acetaminophen. Numerous regulatory bodies have emphasized the need for a weight-of-evidence (WoE) approach toward assessing preclinical carcinogenicity data and potential relevance for human health hazard and risk evaluation. Therefore, the objective of this analysis was to inform the IARC review by performing a comprehensive quantitative weight-of-evidence (QWoE) analysis of the preclinical carcinogenicity data in rodents exposed to acetaminophen. **Methods:** We previously developed a QWoE framework using prespecified scoring criteria for preclinical acetaminophen data to characterize study outcomes with considerations for biological relevance of the response to adverse outcomes (*outcome score*) and the strength of methods and study design (*methods score*). The *outcome score* considers a potential adverse outcome based on (1) statistical significance, (2) dose-response, (3) reliability/relevance/magnitude, and (4) plausibility. Considerations for the *methods score* included (1) experimental design, (2) details/reliability of measurement(s), (3) data transparency, and (4) translational/methodological relevance. The current analysis uses this framework to characterize potential carcinogenic outcomes—specifically focused on apical endpoint measures (i.e., neoplastic lesions and tumors)—following exposure to acetaminophen in rodent studies. Following a systematic literature search, data from identified rodent studies were extracted and processed through the QWoE framework with a focus on two main carcinogenic endpoints—tumor-promoting potential and tumorigenicity (all tissues)—with additional endpoint analysis on tumorigenicity for specific tissues of interest, including liver, kidney, urinary bladder, hematopoietic tissues, and all other tissues. An entry, with an individual *outcome score* and *methods score*, was provided for each sex, species/strain, and exposure duration in an individual study for each applicable endpoint. **Results:** A total of 191 QWoE entries were documented from *in vivo* rodent studies identified in 28 publications across the carcinogenic endpoints of this QWoE analysis. For each endpoint, the mean *outcome score* and *methods score* were calculated for total entries and for entries segregated by sex to assist in determining relative data quality and potential adversity. For the tumorigenicity (all tissues) endpoint, the QWoE analysis demonstrated a mean ($\pm$ SD) *outcome score* of 0.4 ± 0.8 (out of 4)—indicating a lack of biological relevance and reliability of reported adverse effects—and a mean ($\pm$ SD) *methods score* of 2.2 ± 0.9 (out of 4)—indicating a moderate quality of evidence. Similar results were found for tumor-promoting potential and tissue-specific tumorigenicity endpoints as well as analyses segregated by sex. **Conclusions:** This QWoE analysis on the *in vivo* rodent data demonstrated no consistent evidence of carcinogenic responses in males or females following exposure to acetaminophen at a range of doses, including therapeutic/non-toxic to toxic doses. The assessment incorporated well-conducted studies, including GLP-compliant rodent carcinogenicity bioassays and more recent tumor-modulation studies employing chemically induced and tumor-implantation models, encompassing both sexes, multiple rat and mouse strains, and a range of doses. Collectively, these data provide no consistent or biologically relevant evidence that acetaminophen is carcinogenic. Accordingly, the evidence is sufficient to support the conclusion that acetaminophen is not a carcinogenic hazard in rodents.

PS 4688 Genotoxic and estrogenic exposures target distinct tissue structures in early breast carcinogenesis

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Background and Purpose: Breast cancer is the most common invasive cancer diagnosis in the US and worldwide, and rising rates among younger people in particular suggest that environmental exposures play a role. Genotoxic and estrogenic exposures increase risk in humans and rats, particularly during developmental windows of susceptibility such as puberty, and co-exposure may magnify effects. To describe early steps in breast carcinogenesis following exposures that are both estrogenic and genotoxic, we are assessing molecular biomarkers of effect in their spatial context in a rat model. **Methods:** In pilot studies, we exposed pubertal rats to mammary carcinogens that are genotoxic (N-nitroso-N-methylurea, NMU; acute), estrogenic (ethinylestradiol, EE2; chronic), or both (NMU during EE2; or 7,12-dimethylbenz(a)anthracene, DMBA; acute). Using immunofluorescence, H&E, and whole mounted mammary glands, we assessed DNA damage, proliferation, and tissue morphology across the mammary gland 1-day and approximately 1-week after genotoxicant exposure. **Results:** Both NMU and DMBA caused DNA damage, with the strongest γ H2AX signal in immature terminal end buds (TEBs) and little effect in mature tissue, which is consistent with the known sensitivity of the rapidly proliferating TEBs to genotoxic carcinogens. Proliferating Ki67+ cells were concentrated in TEBs in all groups, but EE2-exposed mammary glands showed increased proliferation relative to controls only in mature tissue while DMBA-exposed mammary glands exhibited a trend for increased proliferation mainly in developing regions. Consistent with estrogen-mediated proliferation, EE2-exposed mammary glands had larger TEBs and greater epithelial density in both mature and developing regions, with the most prominent differences from control in mature tissue. NMU+EE2 co-exposure resulted in patterns of DNA damage similar to those of NMU exposure alone and patterns of proliferation and tissue composition similar to those of EE2 exposure alone. Importantly, we found that since treatment-related effects occurred in specific locations (prolifera-

tive TEBs for genotoxicants, mature tissue for EE2) experimental approaches that did not separate these would have missed important biological nuances. **Conclusions:** This study lays the foundation for describing breast cancer dose-response relationships for exposures that are genotoxic and estrogenic. We found that distinct regions in the mammary gland are differentially susceptible to induction of DNA damage and proliferation by genotoxic and estrogenic agents, respectively. Our findings underscore the importance of assessing effects of chemicals with attention to their spatial context, because the mammary gland shows varying patterns of responses across the tissue. Further work will extend these experiments to include a longer 1-month timepoint to measure persistent effects of exposures, and we will use 6-plex immunofluorescence to measure DNA damage, proliferation, and cell death in various epithelial cell types. Measuring molecular effects in specific cell types *in situ* over time will allow us to identify which cells and tissue structures are most vulnerable to genotoxic and estrogenic exposures separately and together, and how those effects eventually lead to cancer.

PS 4689 Second Trimester Butanoate Metabolism in Pregnancy Linking Chemical Exposure to Incident Maternal Breast Cancer within 15 years

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Background and Purpose: A lower ratio of estriol to the sum of estrone and estradiol (estriol ratio) in the 3rd trimester predicted maternal breast cancer within 15 years of pregnancy (DOI: 10.1210/jc.2016-3476). To further elucidate the underlying biological mechanisms, we analyzed prospective serum samples collected during the 2nd trimester and evaluated associations between the maternal metabolome and the subsequent third-trimester estriol ratio. This allows us to investigate early pregnancy metabolic pathways that may contribute to hormonal dysregulation and elevated breast cancer risk in the years following pregnancy. **Methods:** Metabolites in 2nd trimester pregnancy serum were extracted and analyzed using established untargeted metabolic profiling in 566 pregnant women residing near Oakland, California 1959-1967 (Child Health and Development Studies) of whom 36 were diagnosed with breast cancer within 15 years. High resolution mass spectrometry was performed using C18 and negative electrospray ionization. Statistical analysis and pathway enrichment screened for 2nd trimester metabolic changes associated with the 3rd trimester estriol ratio. We used Rodin for untargeted analysis (<https://rodin-meta.com>) to fit a linear regression model predicting the 3rd trimester estriol ratio moderated by subsequent breast cancer within 15 years. Conditional logistic regression estimated associations of maternal breast cancer with 2nd trimester metabolites, 3rd trimester estriol ratio and age. **Results:** We found that exposure to exogenous chemicals annotated as amino-nitrophenols and 4-nitrophenol (PNP) were inversely associated with the 3rd trimester estriol ratio in future cases ($p=0.0004$) but not in controls. Independently, metabolites in butanoate pathway, fatty acid oxidation and branched chain amino acid also predicted the 3rd trimester estriol ratio only in future cases. Succinate semialdehyde, an intermediate that connects the breakdown of the gamma-aminobutyric acid (GABA) to the butanoate metabolism and the larger tricarboxylic acid (TCA) cycle, was significantly associated with PNP. Increased level of 2nd trimester SSA significantly strengthened the association of the 3rd trimester estriol ratio with subsequent maternal breast cancer (logistic regression, $p=0.0009$). Moreover, in jointly adjusted models the 2nd trimester SSA and PNP interactions with 3rd trimester estrogens remained independent, both being significant predictors of maternal breast cancer. **Conclusions:** Exposure to amino-nitrophenols and SSA levels in 2nd trimester contributed to the link between estriol ratio and subsequent cancer, suggesting that endocrine disruption contributes to the incidence of breast cancer within 15 years of pregnancy via multiple mechanisms.

PS 4690 Consideration of Implementation for Postmarketing Carcinogenicity Studies Based on a Retrospective Analysis of Approved New Drugs by U.S. FDA in the Past Ten Years

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Background and Purpose: Postmarketing requirements (PMRs) refer to all mandated postmarketing studies or clinical trials. According to FDA guidance "Postmarketing Studies and Clinical Trials—Implementation of Section 505(o)(3)" and the ICH S1 guideline "Guideline on the Need for Carcinogenicity Studies of Pharmaceuticals", carcinogenicity testing is not required prior to market approval for pharmaceuticals developed to treat certain serious diseases. However, these studies are expected to be conducted after approval. Notably, the guidelines do not provide specific examples of which serious diseases warrant postmarketing carcinogenicity studies. This research aims to systematically analyze postmarketing requirements for carcinogenicity studies in the U.S., clarify how such studies are proposed, and offer insights to support pharmaceutical companies in drug development as well as regulatory agencies in drug evaluation. **Methods:** This study delved into 465 drug approvals by U.S. FDA from 2015 to 2024, either as new molecular entities (NMEs) under New Drug Applications (NDAs), or as new therapeutic biologics under Biologics License Applications (BLAs), and examined all the 25 FDA approvals that contain postmarket-

ing requirements for carcinogenicity studies in the past ten years, leveraging review documents from FDA, comprehensive data on product characteristics, all post-marketing requirements and commitments for carcinogenicity studies were extracted. **Results:** This study found that the overriding consideration for all postmarketing carcinogenicity studies were the indication of these drugs, which were life-threatening or severely debilitating diseases, and there was unmet medical need for therapies to treat these diseases, which were cystic fibrosis (CF), Duchenne muscular dystrophy (DMD), spinal muscular atrophy (SMA), carcinoid syndrome diarrhea, early stage HER-2 positive breast cancer, amyotrophic lateral sclerosis (ALS), seizures associated with Lennox-Gastaut syndrome (LGS) and Dravet syndrome (DS), hereditary transthyretin-mediated amyloidosis (hATTR amyloidosis), HIV-1 infection, Lambert-Eaton myasthenic syndrome (LEMS), extensively drug-resistant (XDR) and treatment-intolerant/nonresponsive (TI/NR) multidrug-resistant (MDR) tuberculosis, acute hepatic porphyria (AHP), primary hyperoxaluria type 1 (PH1), chronic idiopathic constipation (CIC), Alagille syndrome (ALGS), hypertrophic cardiomyopathy (HCM), epidermolysis bullosa (EB), low-grade gliomas (LGGs), and warts, hypogammaglobulinemia, infections and myelokathexis (WHIM) syndrome. For example, Alagille syndrome (ALGS) is a rare (incidence of 1 in 30,000 to 1 in 70,000), autosomal dominant, multi-organ disease. Bile duct paucity in the liver occurs in approximately 90% of patients with ALGS. The disease can progress to cirrhosis leading to liver failure. Before the approval of the drug Livmarli, there was no U.S. Food and Drug Administration (FDA)-approved medical therapy for treatment of pruritus in patients with ALGS. Additionally, the available nonclinical and clinical data for these drugs supported such a strategy, for example, besides the indication, the justification of request for deferral of carcinogenicity studies of the drug Eteplirsen was: 1) Eteplirsen was not genotoxic in the ICH standard battery of assays: *in vitro* bacterial mutation and mammalian chromosome aberration, and *in vivo* mouse bone marrow micronucleus; 2) No pre-neoplastic or other proliferative lesions were observed in the 39-week toxicity study in monkey; 3) No treatment-related clinically significant adverse events have been observed in clinical studies of > 3 years of once-weekly IV administration of eteplirsen. **Conclusions:** Postmarketing carcinogenicity studies strategy speeds the availability of pharmaceuticals for life-threatening or severely debilitating diseases, especially where no satisfactory alternative therapy exists. Our analysis of drug approvals in the U.S. FDA reveals diverse drug patterns approved for post-marketing carcinogenicity studies, and provides clear cases. This study provides valuable insights for regulatory decision-making in a dynamic pharmaceutical landscape. Balancing the risks and rewards of conditional approvals is crucial in ensuring both patient safety and timely access to innovative treatments.

PS 4691 Readaddiction to deaddiction drug A case study on Buprenorphine Poisoning

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Background and Purpose: Opioid substitution therapy is the predominant measure in the National Drug De-addiction programme in India, and Buprenorphine is the key drug used in the therapy, which itself is an opioid and has less tendency to abuse and physical dependence. This drug is used in agonist substitution treatment—a method for addressing addiction by substituting a more potent full agonist opioid, such as heroin, with a less potent opioid, i.e., Buprenorphine. **Methods:** Patients who have been brought to the emergency department of AIIMS Bathinda in Altered Mental status and with a history of drug abuse were considered in the study. The Blood toxicological analysis was advised. Quantification was achieved by selected ion monitoring of m/z 468.4 (BUP), 414.3 (NBUP) and LC-MS/MS method was developed for the simultaneous quantification of BUP and NBUP in serum. For reference, the median (maximum) concentration in BUP dependent OST based patients is 4.2 µg/L (102 µg/L) for BUP, 74.7 µg/L (580 µg/L) for NBUP. **Results:** Personal history of the patients revealed addiction to a drug being distributed at the hospital. 24 per cent of the total subjects considered were found addicted to Buprenorphine-Naloxone fixed drug combination. The toxicology analysis result shows positive for norbuprenorphine with average levels of 1200 microgram/L. The patients were stabilised and the average duration of stay in the hospital was found to be one week. **Conclusions:** Buprenorphine misuse and diversion is a complex issue. Research indicates that though diversion of buprenorphine does take place at times, the possibility of harms is substantially reduced with the Buprenorphine-Naloxone formulation. It must also be noted that wider and easier availability of treatment would reduce the risk of diversion. Research also indicates that much of the diverted use of medications takes place for therapeutic purposes (easing withdrawal and controlling craving). No specified time limit for substitution is one of the major cause of drug diversion and misuse. However, treatment providers need to be vigilant regarding misuse and diversion, ascertain the reasons for the same and take steps to minimize it.

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Background and Purpose: The contamination level of cannabis in the illicit market - including unlicensed production (i.e., black market cannabis) within a legalized state and products that failed compliance testing in the gray market - is largely unknown. Our objectives were to i.) develop an analytical method to quantify a panel of common illicit drugs in cannabis and ii.) characterize the scale and type of illicit drug contaminants in the cannabis. To the authors' knowledge, this is the largest study of illicit drug contaminants in black- and gray-market cannabis in the Southwestern U.S. to date. **Methods:** We surveyed the production environment and contaminant sources of illicit cannabis. Maricopa County Sheriff's Office (MCSO)'s High Intensity Drug Trafficking Areas Task Force conducted 37 seizures and collected 79 cannabis dried flower samples between 2023 and 2024. While 90% of seized cannabis in Arizona was estimated to come from California, a few grow operations were discovered locally in Arizona. Additionally, U.S. Postal Inspection Services (USPIS) seized 39 cannabis dried flower samples between 2023 and 2024. The prepared samples were analyzed for the 17 analytes using an Agilent 6495 LC-MS-MS, including amphetamine, benzoylecgonine, cocaine, codeine, fentanyl, hydrocodone, hydromorphone, LSD, methamphetamine (MAMP), 3,4-methylenedioxymethamphetamine (MDMA/Ecstasy), morphine, oxycodone, oxycodone, phenylcyclohexyl piperidine, psilocin, psilocybin, 3,4-methylenedioxymethamphetamine. **Results:** Twenty-five (21.2%) out of 118 cannabis flower samples had detectable concentrations of illicit drugs. Only four of the 17 analytes tested were detected. The most commonly detected drug was cocaine (20.3%), followed by MAMP (3.4%) and MDMA (0.9%). Benzoylecgonine, the breakdown product of cocaine, was found in four samples containing cocaine. Three MAMP and one MDMA samples were also found to contain cocaine. The highest concentration of illicit drugs was cocaine at 15,200 ppm (or 1.5 % by weight). Only one positive seized sample (2.6%) was collected by the USPIS, while the remaining 24 positive samples were all collected by the MCSO at a detection rate of 27.8%. Notably, fentanyl was not detected in any of the 118 samples. This is consistent with a 2021 study in Connecticut that found fentanyl in illicit cannabis as a standalone case of unintentional contamination (Connecticut Overdose Response Strategy, 2021; Besthoff, 2022, NBC Connecticut. June 24). **Conclusions:** The presence of illicit drugs in black and grey market products highlights the contamination of the product being distributed. This research focused on illicit drugs, but other types of contamination are also likely to occur given the poor product preparation areas. Further research is needed to characterize the biological and chemical contaminants in illicit cannabis and their health risk to cannabis consumers.

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Background and Purpose: Consumer access to cannabinoids has increased significantly in recent years, thereby increasing the risk of developmental exposure to Δ^9 -tetrahydrocannabinol (Δ^9 -THC) and other cannabinoids. The goal of this study is to evaluate the impact of developmental cannabinoid exposure and determine the potential mechanism of action of behavioral and morphological deficits observed, using the zebrafish (*Danio rerio*) model. **Methods:** Wildtype (5D), *cnr1*^{-/-}, and *cnr2*^{-/-} embryos were exposed to increasing concentrations (0-2 μ M) of eight widely available cannabinoids from 6-96 hours post fertilization (hpf). Cannabinoids of interest included: Δ^9 -THC, Δ^{10} -THC, Δ^8 -THC, tetrahydrocannabinol (THCV), hexahydrocannabinol (HHC), cannabidiol (CBD), cannabigerol (CBG), and cannabinol (CBN). At 120 hpf, larval photolocomotor response and morphology, including body length, eye diameter, and the presence of edemas were evaluated. **Results:** Wild-type larvae exposed to Δ^9 -THC, Δ^8 -THC, THCV, HHC, CBD, and CBG showed dose-dependent reductions in photolocomotion. Body length and eye diameter were also reduced with higher exposure concentrations. The *cnr1*^{-/-} and *cnr2*^{-/-} fish showed lower baseline levels of activity than wild-type zebrafish. Nevertheless, several of the cannabinoids still caused a significant reduction in locomotor activity within the knock-outs, suggesting an alternative mechanism of action outside CB1 and CB2 for Δ^9 -THC, CBG, and HHC. Surprisingly, CBD and THCV did not reduce locomotion in *cnr1*^{-/-} fish, suggesting potential activity through CB1. THCV also failed to reduce locomotion in *cnr2*^{-/-} fish, suggesting potential activity through CB2. Minimal toxicity was observed with Δ^{10} -THC and CBN exposure, across all three genotypes. **Conclusions:** Together, these data highlight cannabinoid-specific neurodevelopmental toxicities. Most of the cannabinoids tested resulted in behavioral and morphological toxicities in the days following early embryonic exposure. Further work is needed to delineate the mechanism of toxicity and persistence of neurodevelopmental effects after early-life exposure to these consumer products.

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Background and Purpose: Ethanol is first metabolized to the toxic intermediate acetaldehyde and then converted to acetate by aldehyde dehydrogenase. One of the three enzymes involved in ethanol metabolism, cytochrome P450 2E1 (CYP2E1), exists in both endoplasmic reticulum (ER) and mitochondrial isoforms. ER localized CYP2E1 is well studied in alcohol metabolism, but far less is known about the function of mitochondrial CYP2E1. In rats, alcohol exposure increases CYP2E1 levels in brain mitochondria, yet the contribution of mitochondrial CYP2E1 to ethanol toxicity is unclear. This study aimed to determine how mitochondrial CYP2E1 influences organismal and mitochondrial responses to ethanol and its metabolites. **Methods:** Transgenic *Caenorhabditis elegans* were generated using CRISPR/Cas9 knock in to introduce human CYP2E1 and the human ER or mitochondrial cofactor required to direct CYP2E1 activity to that compartment. Wild-type N2 worms were used as a negative control, as they lack CYP2E1 expression. Worms were exposed for four hours to increasing concentrations of ethanol (0 to 500 mM), acetaldehyde (0 to 100 mM), or acetate (0 to 100 mM). Locomotion and lethality were quantified, and oxygen consumption rates (OCRs) were measured using the Resipher system. Additional assays included hydrogen peroxide lethality and initial glutathione measurements. **Results:** Ethanol caused dose dependent inhibition of motility in all strains, with mitochondrial CYP2E1 worms showing greater inhibition at 250 mM. Acetaldehyde exposure reduced motility more strongly in CYP2E1 expressing strains than in wild type worms, although LD50 values were similar across strains (24 to 49 mM). Acetaldehyde caused a dose dependent inhibition of respiration, with mitochondrial CYP2E1 worms showing the greatest reduction at 25 mM ($p < 0.0001$) and 50 mM ($p < 0.05$). Hydrogen peroxide lethality was similar across strains (LD50 values 2.7 to 4.5 mM), suggesting reactive oxygen species are not the sole drivers of acetaldehyde toxicity. Acetate impaired motility in both CYP2E1 strains at intermediate doses, with mitochondrial CYP2E1 worms showing significantly reduced movement at higher concentrations. Acetate increased OCR in most strains, but mitochondrial CYP2E1 suppressed this increase. **Conclusions:** These findings indicate that mitochondrial CYP2E1 increases sensitivity to ethanol and its metabolites in ways that extend beyond the initial oxidation of ethanol. Preliminary data suggest that CYP2E1 expressing worms have elevated baseline glutathione levels, and ongoing work is focused on defining oxidative stress responses and metabolomic changes to further clarify the role of mitochondrial CYP2E1 in alcohol related toxicity.

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Background and Purpose: Two species (a rodent and a non-rodent) are generally used to support clinical development and licensing of new medicines. Whilst there are opportunities to reduce to a single species in later-development studies for biologics that follow the ICH S6(R1) guideline (e.g. if toxicities in earlier studies are "similar") two species are currently required for small molecules and other drug modalities that are developed according to the ICH M3(R2) guideline. Following on from previous work to support wider uptake of opportunities to use a single species within ICH S6(R1), the UK National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) has recently initiated a project to determine whether there are cases where a single species could also be sufficient for later-development studies for molecules following ICH M3(R2), without compromising human safety. **Methods:** An international working group consisting of 44 experts from various pharmaceutical companies, contract research organisations and regulatory agencies was convened, and a survey designed to collect detailed information on studies conducted to support first-in-human (FIH) trials and later development for molecules developed under ICH M3(R2). Survey respondents were asked to provide details on the study designs and the toxicities observed (including severity, monitorability, reversibility, and their impact on decision-making and/or clinical programme). They were also asked for information on the similarity of toxicity findings across the two species and the two study phases, and to provide their opinion on whether - in hindsight - the same decisions could have been made using only one species for later development studies. **Results:** Data were collected from 17 companies for 75 molecules, covering various therapeutic areas including nervous system (23), anti-infectives (11) and oncology (10). Of these, 33 molecules were active in development, 16 marketed and 26 stopped during development (mainly for strategic and/or business reasons). For each molecule, data on at least two first-in-human enabling and two later-development studies was provided (one rodent and one non-rodent for each study phase). Species used were mainly rat (74) as rodent, and dog (50) as non-rodent. Toxicity findings occurred in ~90% of the studies. However, only ~30% were considered a concern for human safety and <20% had an impact on the clinical programme. Looking retrospectively at the study data, survey respondents indicated that the same decisions could have been made using only one species for later-development studies for 75% of the molecules, almost equally split between the choice of rodent only and non-rodent only. A series of blinded exercises were conducted

within the working group to assess if/when/how these decisions could be taken prospectively and revealed that the choice of using a single species for later-development studies could have been made prospectively for at least 60% of the molecules, without missing toxicities of concern for humans. **Conclusions:** The data analysis suggests there may be opportunities where using a single species for later development studies could be appropriate also for molecules developed following ICH M3(R2), without compromising human safety. Ongoing work aims now to identify the common factors driving the choice of a single species / two species approach, in order to develop recommendations and create an evidence base to inform regulatory guidance.

PS 4696 Pharmaceutical Safety Considerations for Cereblon-Recruiting Targeted Protein Degraders

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Background and Purpose: Cereblon (CRBN)-recruiting molecular glue degraders (MGDs) and proteolysis-targeting chimeras (PROTACs), collectively referred to as CRBN-recruiting targeted protein degraders (CRBN-TPDs), have shown great promise for exploring previously intractable drug targets across a range of indications. However, this class of drugs poses unique challenges related to off-target neosubstrate identification and risk-assessment. Hence, there is a need for a scientific consensus on rigorous, consistent, and effective methods for the assessment of the safety of CRBN-TPDs. Here, we provide an overview of the endogenous functions of CRBN and key learnings on potential organ toxicities and translational considerations for off-target neosubstrate degradation based on data from immunomodulatory imide drugs, next-generation MGDs and PROTACs. **Methods:** An extensive literature review was performed to assess CRBN-TPD safety testing strategies, and key considerations for CRBN-TPD development and recommendations for safety testing were identified. RNA-Seq expression data from nonclinical species were obtained (NCBI accession number: PRJNA516470). Human data were curated from the Genotype-Tissue Expression project. **Results:** Key challenges include species-specific sequence differences in CRBN and its neosubstrates, variability in neosubstrate expression patterns, and the off-target effects associated with diverse CRBN-TPD structures. Based on this assessment, we suggest best practices for monitoring on-target versus off-target degradation from CRBN-TPDs, such as profiling on- and off-target degradation using proteomics, assessing *in vivo* relevance across toxicology species, and applying predictive *in vitro* models for teratogenicity and other toxicities. Furthermore, we highlight gaps in the understanding of CRBN's full biological role and potential unintended consequences such as proteasome saturation, neosubstrate sequestration, and an altered immunopeptidome. **Conclusions:** Standardized safety assessment strategies and deeper mechanistic insights will support the development of CRBN-TPDs as well as novel E3 ligases and other degradation modalities.

PS 4697 In Vitro Hemolytic Potential of Cyclodextrins: A Species Comparison

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Background and Purpose: Cyclodextrins—including HP- β -CD, HP- γ -CD, RM- β -CD, and SBE- β -CD—are widely used as excipients in nonclinical formulations due to their ability to enhance solubility and stability of poorly soluble compounds. However, since their potential to induce hemolysis may vary by species and concentration, a rapid *in vitro* evaluation of hemolytic potential was conducted across several commonly used species in nonclinical safety testing. **Methods:** *In vitro* hemolysis assays were performed using fresh whole blood collected from fasted rats, rabbits, minipigs, and dogs. Pooled heparinized blood samples were exposed to increasing concentrations of several commercially available cyclodextrins including the following: random methylated β cyclodextrin (RM- β -CD), hydroxypropyl γ cyclodextrin (HP- γ -CD), sulfobutylether β cyclodextrin (SBE- β -CD), and hydroxypropyl β cyclodextrin (HP- β -CD). RM- β -CD was tested at 13.3, 23.3, and 33.3 mg/mL, while HP- β -CD, HP- γ -CD, and SBE- β -CD were evaluated at 23.3, 40.8, and 58.3 mg/mL. Triton X-100 (8.3 mg/mL) served as a positive control, and 0.9% saline was the negative control. For each assay, 100 μ L of the test formulation was added to 500 μ L of whole blood, followed by incubation for 30 minutes at room temperature. Hemolysis was quantified via spectrophotometric analysis of plasma hemoglobin using a Beckman Coulter AU480 clinical chemistry analyzer. **Results:** RM- β -CD induced significant hemolysis at 13.3 mg/mL in whole blood from rats, minipigs, dogs, and rabbits. HP- β -CD induced hemolysis at 23.3 mg/mL in rat and rabbit blood, and at 40.8 mg/mL in blood from minipigs and dogs. SBE- β -CD caused hemolysis at 40.8 mg/mL in rats and at 58.3 mg/mL in rabbits but did not induce hemolysis in minipigs or dogs at any concentration tested. HP- γ -CD did not induce hemolysis in any species at the concentrations evaluated. Across all species tested, rat-derived whole blood consistently was more sensitive to cyclodextrin-induced hemolysis. The findings with HP- β -CD are consistent with prior studies

showing hemolysis in rats following exposure to 35% HP- β -CD both *in vitro* (50 μ L added to 500 μ L whole blood) and *in vivo* (10 mL administered via short IV infusion). **Conclusions:** HP- β -CD and related cyclodextrins exhibit species- and concentration-dependent hemolytic potential *in vitro*, with rats demonstrating heightened sensitivity across common laboratory species tested. Species-related differences in hemolytic response highlight the need for tailored formulation strategies with cyclodextrins to ensure tolerability and study integrity.

PS 4698 Monitoring Muscle Fiber Toxicity with Transcriptional Biomarkers

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Background and Purpose: Drug-induced myopathies can be caused by different medications, such as statins and fibrates, and may originate from the energy metabolism perturbations. Muscle tissue that requires significant energy supply is particularly sensitive to these effects, and as fibers of type I and type II rely on differential metabolic pathways, they can have different susceptibility to the injury. **Methods:** In this study, we performed RNA sequencing on archived quadriceps samples from dogs treated for 9 months with a discontinued development compound with histopathological evidence of muscle degeneration. **Results:** Differential gene expression analysis revealed consistent changes in gene markers of type I and type II fibers in opposite directions, suggesting underlying changes in muscle fiber type composition. To confirm these findings, a single-nuclei RNA-Seq study was performed, with more than 17,000 nuclei isolated and sequenced. All major muscle cell types were identified in this study based on their established corresponding gene markers. Particularly, separate type I and type II myocyte nuclei were detected, and markers of the corresponding slow and fast fibers were confirmed. In addition, we observed upregulation of synaptic markers in regenerating myocytes in the injured tissue samples, which might be attributed to newly forming neuromuscular junctions. **Conclusions:** These RNA markers can be used for detection and monitoring of fiber type-specific myopathies in future drug development.

PS 4699 A 3D Heart-on-Chip For Cardiotoxicity Screening: Assessing Drug-Induced Alterations In Cardiac Electrophysiology and Contractility

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Background and Purpose: Cardiotoxicity detection remains a critical step in the drug development pipeline (DDP). To enable more predictive preclinical *in vitro* studies, the combined use of advanced technologies such as Organs-on-Chip (OoCs) and human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) holds great promise. This approach offers the opportunity to generate physiologically relevant, human-based data that better translate to clinical outcomes. By using animal models indeed, cardiovascular adverse effects that go undetected can result in cardiotoxic compounds reaching the market, while findings of cardiac issues in animals often lead to the premature discontinuation of promising drug candidates without confirmation of their true clinical risk. In this scenario, we present uHeart, a 3D cardiac *in vitro* model developed by integrating human iPSC-CMs within a beating OoC platform equipped with multiparametric readouts for cardiotoxicity assessment. uHeart has been applied in pharmacological studies to demonstrate its applicability in different contexts of use (CoUs) in cardiac safety, demonstrating its capability in detecting drug-induced changes in electrophysiology and contractility. **Methods:** uHeart microtissues are developed by using hiPSC-CMs and human fibroblasts (Fs) embedded in fibrin hydrogel at a density of 100x10⁶ cells/ml (75%-25%, CMs-Fs ratio) and cultured in uBeat® Stretch platforms for 7 days. The system provides to the obtained microtissues a physiological mechanical stimulation that mimics the heart-beat (i.e. 10% strain, 1Hz). uHeart was qualified for QT-prolongation and pro-arrhythmia detection by following the ICH S7B guidelines, using 11 drugs listed in the Comprehensive *in vitro* Proarrhythmia Assay (CiPA). Drug-induced alterations were evaluated at incremental doses, by analysing the field potential (FP) morphology (i.e., beating period-BP, spike amplitude-AMP, FP duration-FPD) and arrhythmic events. The model was further exploited for evaluating the effect of 19 reference compounds that affect contractility by acting on calcium homeostasis, on sodium channel and on the sarcomere. Drug-induced changes were evaluated at incremental doses by analysing contractility parameters from video analysis by means of MuscleMotion (e.g., beating period-BP, contraction time-CT, relaxation time-RT, contraction amplitude-CA, contraction velocity-CV). **Results:** The mechanical stimulation promoted the formation of microtissues exhibiting spontaneous and synchronized beating (coefficient of variation of the beating period-BP < 25%; ~85% yield). The analyses on field potential (FP) (i.e., BP, spike amplitude, FP duration) revealed that the microtissues responded to drugs acting on single (i.e. Nifedipine, Mexiletine and Dofetilide) and multiple (e.g. Quinidine, Terfenadine, Sotalol, Ranolazine) ion channel. Overall, uHeart demonstrates 83.3% sensitivity and 100% specificity in predicting QT-prolongation. Moreover, uHeart showed a superior outcome with respect to 2D models in detecting arrhythmic events and detect toxicities of molecules that resulted false-negative in animal models. The analyses on contractile parameters (e.g., beating period-BP,

contraction time-CT, relaxation time-RT, contraction amplitude-CA, contraction velocity-CV) highlighted that uHeart has 65% sensitivity and 100% specificity in predicting inotropic effects. Notably, uHeart outperformed traditional 2D models in detecting the effects of drugs targeting the sarcomere, underscoring the importance of using a 3D model to tackle effect of specific group of compounds. These results highlight uHeart enhanced relevance and potential for application in more specific CoUs. **Conclusions:** uHeart is a highly functional 3D human cardiac model, developed in accordance with the 3Rs principles, designed to advance preclinical cardiac safety assessment. Extensive pharmacological testing has demonstrated the model's robust suitability for cardiotoxicity evaluation across a range of CoU. This positions uHeart as a promising preclinical tool for predictive cardiac toxicity screening in drug development.

PS 4700 Development of a Guinea Pig Telemetry Model for Early QT Prolongation Screening in Compliance with ICH S7B Best Practices

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Background and Purpose: Nonclinical evaluation of drug-induced QT interval prolongation is essential for predicting proarrhythmic risk in humans. Regulatory guidelines, including ICH S7B, recommend *in vivo* QT studies using non-rodent species such as dogs or monkeys. However, these models present challenges in early drug development due to high costs, ethical considerations, and operational complexity. Guinea pigs offer a promising alternative because of their manageable size, ease of handling, and cardiac ion channel expression profiles that closely resemble those of humans. Although *ex vivo* studies using guinea pig hearts have demonstrated their utility in QT assessment, *in vivo* telemetry-based models remain insufficiently developed and lack standardization for early screening. In this study, we aimed to establish a telemetry-based QT assessment model in guinea pigs in accordance with the best practices outlined in the ICH S7B Q&A document. Furthermore, we evaluated strategies to improve screening sensitivity and efficiency in guinea pig QT prolongation assays by incorporating best-practice concepts. Developing a physiologically relevant and cost-effective model is expected to enhance both sensitivity and operational efficiency in early-phase cardiac safety screening. **Methods:** Four male SPF Hartley guinea pigs aged 7 to 9 weeks were administered moxifloxacin orally at doses of 10, 30, and 100 mg/kg, following a Williams Latin square design. Electrocardiographic parameters (RR, PR, QRS, QT, QTc) were continuously recorded under conscious conditions using telemetry. Comparative analysis was performed using ECG data from dogs treated with the same compound to explore interspecies differences in pharmacodynamic response. **Results:** QT interval prolongation was not observed at the low (10 mg/kg) or medium (30 mg/kg) doses of moxifloxacin. However, a trend toward QT prolongation was noted at the high dose (100 mg/kg). Compared to canine data, guinea pigs did not exhibit a clear dose-dependent QT response, suggesting potential species-specific differences in cardiac sensitivity to moxifloxacin. **Conclusions:** These findings indicate that guinea pig telemetry may serve as a practical and physiologically relevant model for early QT assessment, offering a viable alternative to conventional non-rodent species. From a toxicological standpoint, the ability to detect drug-induced electrophysiological changes such as QT prolongation at an early stage may facilitate the identification of proarrhythmic risk prior to progression into later-phase studies. Looking ahead, the application of individualized QT correction (QTc_{adj}) based on the slope (β) derived from QT-RR relationships, along with the estimation of the minimum detectable difference (MDD), may enhance the model's quantitative capabilities and contribute to improved sensitivity and screening precision in early drug development.

PS 4701 Differentiating Pure hERG from Multi-Ion Channel Blockers Using a New Proarrhythmic Risk Biomarker: a Study Evaluating the Effects of QT-positive drugs on the JTpeak interval in Telemetered Dogs

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Background and Purpose: The JTpeak (JT_p) and Tpeak-to-Tend intervals (TpTe), representing cardiac ventricular early and late repolarization, respectively, have been proposed as a complementary, sensitive, and more specific biomarkers than the QT interval for assessing proarrhythmic risk in drug development. It aims to improve the differentiation between drugs that selectively inhibit hERG channels (associated with high proarrhythmic risk) and those that affect multiple cardiac ion channels (associated with minimal to no risk). As previously reported by Darpo et al. (2019) in human, the present study aimed to evaluate the effects of five QT-prolonging reference drugs, namely moxifloxacin, dofetilide, dolasetron, ondansetron and quinine, on JT_p interval in telemetered conscious dogs. TpTe interval, together with PR and QRS intervals reflecting, the effects on other cardiac ion channels, were also measured. Levocetirizine, a QT-negative control drug, was included for comparison. **Methods:** The study was performed in conscious male dogs (n=7) previously instrumented

with a telemetry implant for the measurement of arterial blood pressure and ECG. Each compound was administered orally at two dose levels using a crossover study design, with ECG monitored over a 24-hour period post-dosing. Dedicated pharmacokinetic sessions were performed to determine corresponding drug plasma exposures, and sensitivity parameters were calculated. JT_p and TpTe intervals were individually corrected (c) for heart rate variations, as it is done with QT. **Results:** Compared to vehicle, dofetilide (0.03 and 0.15 mg/kg), moxifloxacin (30 and 100 mg/kg), and ondansetron (10 and 30 mg/kg) produced dose-dependent increases in JT_p interval, consistent with predominant hERG channel blockade. However, no effect was observed on TpTe-c interval with moxifloxacin and dofetilide, whereas a slight increase was noted with ondansetron. In contrast, dolasetron (6 and 20 mg/kg) and quinine (20 mg/kg) did not affect the JT_p interval. Interestingly, dolasetron slightly and transiently increased TpTe-c at the highest dose, and also increased both QRS and PR intervals, reflecting its inhibitory effects on sodium and calcium currents. With quinine, a slight increase in QRS interval was observed. As expected, levocetirizine (2 and 10 mg/kg) showed no effect on ECG intervals, at any dose or timepoint. Total plasma drug concentrations for each compound increased dose-proportionally. In this study, the residual mean standard error (RMSE) for JT_p-c ranged between 2.3 and 4.4 msec, thus suggesting a very well-powered study. **Conclusions:** The JT_p interval was confirmed as a sensitive biomarker to differentiate between purported selective hERG blockers (moxifloxacin, dofetilide, ondansetron) and compounds with multi-ion channel activity (dolasetron, quinine). In dogs, additional endpoints such as TpTe interval may further complement classification, although further assessment remains to be performed. These findings support the relevance of JT_p interval assessment as a valuable parameter to complement QT interval measurements in cardiovascular safety pharmacology studies, enhancing the specificity and sensitivity of proarrhythmic risk evaluation.

PS 4702 Non-Drug Induced Spontaneous Convulsions in Different Nonclinical Laboratory Animals Used for Non-Clinical Drug Safety Evaluation

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Background and Purpose: Convulsions are occasionally observed during long-term toxicity and carcinogenicity studies in laboratory animals, but distinguishing whether these events are related to the test article or are spontaneous/procedural is crucial for accurate data interpretation. This review aimed to summarize the incidence and characteristics of convulsions in various commonly used laboratory animal species at the Suzhou testing facility, and to provide a reference for toxicologists and pathologists in differentiating test article-related effects from procedural or spontaneous findings. **Methods:** A retrospective review was conducted of studies performed at the Suzhou testing facility between 2018 and 2025. The analyzed studies included 7-day to 2-year carcinogenicity and toxicity studies on Sprague-Dawley (SD) rats, CD-1 mice, Tg.rasH2 mice, Beagle dogs, non-human primates (NHPs), Bama minipigs, Hartley guinea pigs, and New Zealand white rabbits. The occurrence, timing, and circumstances of convulsions were recorded and analyzed, with attention to study duration, sex, procedures (e.g., blood collection, dosing), and histopathological findings. **Results:** The results indicated that convulsions were most frequently observed in rats, particularly in the 2-year studies, with a highest incidence of spontaneous convulsions at 5.9% (males) and 4.41% (females). The percentages of convulsions in the 26-week and 13-week studies were 2.99% and 2.63%, respectively, with no significant sex differences. In the 4-week studies, symptoms were noted in only one male rat. In CD-1 mice and Tg.rasH2 mice during the 26-week studies, convulsions occurred mainly during blood collection or formulation administration procedures, suggesting procedural-related. In NHPs, convulsions were noted once each in a 2-month and a 6-month Intravenous Infusion study, while no occurrences were observed in Beagle dogs, guinea pigs, rabbits, or minipigs. Convulsions first appeared at 20 weeks in males and 14 weeks in females, with an average onset age of approximately 51 weeks across all animals during 2-Year Carcinogenicity study. Some animals had convulsions only once, while others experienced multiple episodes. No deaths were related to convulsions, and no related histopathological abnormalities were found in the brain upon examination. **Conclusions:** SD rats exhibited a higher frequency of convulsions compared to other species, especially in longer-duration studies. The convulsions observed in mice were mostly procedural, and other species showed minimal or no occurrences. No convulsion-related deaths or brain pathology were detected. The increased frequency of convulsions in SD rats may be influenced by environmental factors, age, and handling frequency. The compiled database will support toxicologists and pathologists in distinguishing between test article-related effects and spontaneous or procedural findings in future studies.

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Background and Purpose: Bi-Tox, a new molecular entity is formed by combining the two botulinum domains (Spytag and Spycatcher), which is being developed for chronic peripheral neuropathy. As per the CDC guideline, a mouse bioassay was performed to determine whether Bi-Tox can be excluded from the select agent list. **Methods:** Acute lethality (LD₅₀) of a single intraperitoneal administration of Bi-Tox and wild type Botulinum Neurotoxin A (WT BoNT/A) following a seven-day observation period was determined in CD-1 Mice (4 wk old, 15-30 g body weight) using 0.5% Human Serum Albumin in sterile water as a vehicle. Dose formulations were analyzed by SDS-PAGE for Bi-Tox concentration. **Results:** WT BoNT/A at 40 or 24 ng/kg resulted in 100% mortality within 1 or 2 days of administration, respectively. Administration of WT BoNT/A at 4, 0.8, or 0.6 ng/kg resulted in 100%, 80%, or 100% mortality/morbidity, respectively. However, no clinical signs were observed in animals dosed with 0.4 ng/kg of WT BoNT/A. In the formulation analysis of Bi-Tox, the band in the SDS-PAGE predicted to be Bi-Tox was correlated to an average of 43.8 µg/mL for the expected concentration of 82.8 µg/mL. Bi-Tox at 2120 or 212 ng/kg resulted in 100% or 90% mortality within 1 or 2 days of dose administration. In addition, 52.9 ng/kg or 26.5 ng/kg Bi-Tox exhibited 70% or 40% mortality, respectively, with all deaths reported within 3 days of administration. Bi-Tox associated clinical signs of ataxia, labored breathing, and wasp waist were observed in these dose groups. Further, 30% or 10% mortalities were reported in animals administered 13.2 ng/kg or 10.6 ng/kg of Bi-Tox, respectively. Animals administered Bi-Tox at 7.94 ng/kg exhibited clinical signs of rapid breathing (all mice) and wasp waist (3 of 10 mice). Following administration of Bi-Tox at 5.29 ng/kg, mice exhibited rough coat (4 of 10 mice), slight rapid breathing (7 of 10 mice) and slight wasp waist (3 of 10 mice), which were mild and inconsistent with botulism progression. Therefore, no further groups were administered with Bi-Tox. **Conclusions:** Based on this bioassay, the Lethal Dose 50 (LD₅₀) of Bi-Tox was determined to be 35.15 ng/kg, and the LD₅₀ of WT BoNT/A was 0.692 ng/kg. This observed LD₅₀ of Bi-Tox was approximately 51-fold higher than that of WT BoNT/A under the conditions employed in this study, which was not adequate enough for CDC to exclude Bi-Tox from the select agent list. (Supported by Contract No HHSN2712017000161 under HEAL/NCATS program).

PS 4704 Preclinical Toxicology Assessment of VT-109, a Novel Agent for Treatment of Acute Respiratory Distress Syndrome

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Background and Purpose: Acute Respiratory Distress Syndrome (ARDS) is marked by non-cardiogenic pulmonary edema, hypoxemia, and respiratory failure, leading to high mortality. The COVID-19 pandemic has underscored the urgent need for effective ARDS therapies. To address this, we developed VT-109, a synthetic cyclic peptide to reduce pulmonary vascular leakage by modulating pathological calcium signaling in endothelial cells. Our studies evaluated the toxicity and pharmacokinetics of VT-109 following intravenous administration in Crl:CD (SD) rats and Beagle dogs. **Methods:** Rat Study: VT-109 was administered at dose levels of 0, 3.75, 7 (7.5 in the PK cohort), and 15 mg/kg. The test article and vehicle (5% DMA, 10% PEG400, 1% Polysorbate 80) were delivered via tail vein injection once daily for seven consecutive days. Crl:CD (SD) rats were obtained from Charles River Laboratories. Evaluations included mortality, clinical observations, physical examinations, body weight, clinical pathology, plasma and lung drug concentration analyses, pharmacokinetic assessments, and gross necropsy. Dog Study: VT-109 was administered at dose levels of 0.25 and 1.25 mg/kg. The test article and vehicle (0.42% DMA, 0.83% PEG400) were delivered via cephalic vein injection once daily for seven consecutive days. Beagle dogs were obtained from Marshall BioResources. Evaluations included mortality, clinical observations, physical examinations, body weight, food consumption, clinical pathology, and plasma drug concentration analysis. **Results:** Rat Study: Repeated administration of VT-109 at 3.75, 7, and 15 mg/kg for seven consecutive days produced clinical signs in the high-dose group, along with changes in hematology parameters and fibrinogen levels across all dose groups. No gross lesions were observed at necropsy. Plasma VT-109 concentrations increased proportionally with dose, with peak levels (C_{max}) observed within 2 minutes post-dosing. On Day 1, the terminal elimination half-life (t_{1/2-λz}) ranged from 5.87 to 6.92 hours across the 3.75, 7.5, and 15 mg/kg groups. Systemic exposure was higher in males than in females at all dose levels. On Day 7, t_{1/2-λz} ranged from 6.19 to 8.51 hours, with males showing higher exposure at 7.5 and 15 mg/kg, but lower exposure at 3.75 mg/kg compared to females. VT-109 concentrations in lung tissue also increased with dose, with C_{max} occurring approximately 2 hours after the final administration. The terminal half-life in the lungs ranged from 8.01 to 125.88 hours in males (3.75 and 15 mg/kg) and from 6.31 to 15.50 hours in females (7.5 and 15 mg/kg). Overall, lung exposure was lower in males compared with females at the 7.5 and 15 mg/kg dose levels. Dog Study: There were no mortalities or clinical signs in any dose group. No significant changes were observed in body weight, food consumption, or clinical pathology parameters in male or female dogs. VT-109 had no effect on food consumption at any dose level. Plasma VT-109 concentrations increased proportionally with dose.

On Days 1 and 7, C_{max} was reached at varying time points (15 minutes, 30 minutes, or 1 hour) depending on dose level and sex. Plasma concentrations were measurable up to 6 hours post-dose. **Conclusions:** The no-observed-adverse-effect level (NOAEL) in rats was not established. The lowest-observed-adverse-effect level (LOAEL) was established at the VT-109 dose level of 3.75 mg/kg. The test article at doses of 0.25 and 1.25 mg/kg was well tolerated by Beagle dogs. The results of these dose-range-finding studies were used in the ongoing pivotal VT-109 studies. The U.S. Army Medical Research Acquisition Activity, 808 Schreider Street, Fort Detrick MD 21702-5014 is the awarding and administering acquisition office. This work was supported by the Assistant Secretary of Defense for Health Affairs, endorsed by the Department of Defense, in the amount of \$5,860,641, through the Peer Reviewed Medical Research Program under Award No. W81XWH-21-1-0639. Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the Department of Defense. In conducting research using animals, the investigators adhere to the laws of the United States and regulations of the Department of Agriculture.

PS 4705 Risk Assessment of Lens Opacities Observed in a 39-Week Toxicity Study of SNG-30 in Dogs

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Background and Purpose: In nonclinical safety evaluation of drug candidates, repeated-dose toxicity studies with durations exceeding the anticipated clinical dosing period are essential. Unexpected toxicities that cannot be detected in subacute repeated-dose toxicity studies can sometimes be revealed for the first time in chronic repeated-dose toxicity studies. For the compound SNG-30 developed by SHIONOGI, lens opacities were observed at the end of a 39-week repeated oral dose toxicity study in dogs, which were not detected in a 4-week repeated oral dose toxicity study. Therefore, to assess the human risk and development feasibility of this toxicity, additional tests were conducted during the 39-week repeated dose toxicity study, as well as additional *in vitro* studies to investigate the mechanism. These results were compared with toxicity information from other drugs known to cause lens opacities. **Methods:** SNG-30 was orally administered to male and female beagle dogs at doses of 0, 3, 20, and 1000 mg/kg/day for 39 weeks. Ophthalmological examinations were conducted at multiple time points (pre-dose, week 19, week 38, and 4, 8, and 12 weeks after withdrawal), followed by necropsy and histopathological examinations (week 39, 13 weeks after withdrawal). As an additional investigation, plasma concentrations of cholesterol precursors were measured at various time points (pre-dose, weeks 13, 27, 39, and 4 and 13 weeks after withdrawal). In addition, multiple tissues were isolated from untreated beagle dogs, and mRNA expression levels of the pharmacological target gene M were analyzed by real-time PCR. Furthermore, the effects of SNG-30 on various cellular function markers were evaluated in human cultured lens epithelial-like cells (B-3 cells) after 24 hours of treatment. **Results:** In the high-dose group of the 39-week repeated-dose study in dogs, no abnormalities were observed at the 19-week ophthalmoscopic examination, but lens opacities were detected at week 38. Histopathological examination also revealed degeneration of lens fibers in this group. During the 13-week withdrawal period, no recovery was observed, but there was no further progression of lens fiber degeneration. The exposure margin between the dose at which no lens opacity was observed and the estimated human therapeutic dose was approximately 20-fold. Mechanistically, the pattern of lens opacity observed in this study was relatively similar to changes reported for compounds with cholesterol synthesis inhibitory activity, and increases in plasma cholesterol precursors were observed at weeks 27 and 39 in the high-dose SNG-30 group. Furthermore, there was no information on the relationship between the pharmacological target and lens opacities, and target gene expression was not detected in the canine lens. Additional data from human lens epithelial cells and other studies did not confirm any effects on oxidative damage, glucose metabolism abnormalities, or membrane dysfunction, which have been reported as causes of lens opacities for other compounds. **Conclusions:** Lens opacities caused by SNG-30, which first became apparent in the 39-week repeated-dose oral toxicity study in dogs, resembled those caused by other drugs known to induce lens opacities through inhibition of cholesterol synthesis and were accompanied by changes in plasma cholesterol precursors. These changes were considered to be due to an off-target effect on cholesterol synthesis. In the middle-dose group, where no lens opacities were observed, there were no changes in plasma cholesterol precursors or other toxicity-related findings. Based on clinical information for drugs with the potential to cause lens opacities, it is possible to estimate the risk in clinical trials of SNG-30 and to set monitoring parameters. Taking the safety margin into account, it was determined that the development of SNG-30 was feasible.

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Background and Purpose: Oligonucleotides (ONTs) are a class of promising therapeutics for the treatment of genetic neuromuscular diseases. However, achieving efficacious concentrations in muscles and central nervous system (CNS) has hindered progress to success in the clinic. To overcome this limitation, we developed the FORCE platform, which consists of an antigen-binding fragment (Fab) highly specific for the human transferrin receptor 1 (TfR1), to which ONT payloads can be conjugated via a cleavable valine-citrulline linker. The platform was designed to allow for conjugation of either neutral or charged payloads to enable the flexibility to match payload to mechanism of disease. TfR1 plays an important role in iron trafficking and erythropoiesis, and anemia has been reported nonclinically and clinically for biologics that leverage TfR1 to enable therapeutic delivery of payloads to muscle and CNS. **Methods:** To understand the impact of FORCE platform on erythropoiesis, cynomolgus monkeys were administered FORCE conjugates harboring either a phosphorodiamidate morpholino (Fab-PMO), a negatively charged gapmer (Fab-ASO), or a small interfering RNA (Fab-siRNA) for 13 weeks at 3- to 4-week intervals followed by 8-week recovery periods. The concentration of the test article in bone marrow was determined at necropsy using a qualified method developed for each test article and red cell mass endpoints were measured 24 to 48 hrs after the first dose and at necropsy. **Results:** In these studies, all Fab conjugates were well-tolerated and exposure of bone marrow to each payload increased in a dose-responsive manner. Reticulocytes were transiently decreased 24-48 hrs after administration of either Fab-PMO or Fab-ASO in a dose-dependent manner and returned to pre-study values prior to the next dose administered while hematologic endpoints were unchanged after administration of Fab-siRNA. Red blood cells, hemoglobin as well as hematocrit remained unchanged for all FORCE conjugates. Given the importance of iron on erythropoiesis, iron trafficking was also characterized. Fab-PMO caused a reversible, minimal, but non-dose responsive increase in serum iron 48 hrs after the fourth dose. Neither transferrin nor ferritin serum levels were changed after repeated administration with Fab-PMO. Fab-ASO, at a similar dose range, did not alter serum iron nor transferrin, although there was a transient increase in circulating ferritin, which was interpreted to be an indication of a minimal inflammatory response, a known effect of phosphorothioate ASOs in non-clinical species. Fab-siRNA had no impact on any iron parameter tested. **Conclusions:** The totality of the data indicates that despite exposure to the bone marrow and minimal transient alterations in serum iron levels and resultant decreased reticulocytes, repeat administration of FORCE conjugates to cynomolgus monkeys does not result in anemia. Further the lack of consistent effects on transferrin or ferritin indicates the FORCE platform does not compete with endogenous TfR1 ligands.

PS 4707 Summary of three toxicity study of repeated intravenous infusion of exatecan derivative-ADCs to cynomolgus monkey

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Background and Purpose: Test articles A, B and C were three ADCs containing different targets of exatecan. This study summarizes the toxic reactions after repeated intravenous infusion of three exatecan ADCs of A, B and C, in cynomolgus monkey conducted in JOINN Laboratories, providing some toxicity references for the development of similar drugs. **Methods:** 40 cynomolgus monkeys (20 animals/sex) were used and divided into 4 groups (5 animals/sex/group) and treated with vehicle control article and low-, middle-, and high-dose group in each study. The dosages of the test article A were 10, 30 and 45 mg/kg. The administration doses of test article B were 7.5, 15 and 30/20 mg/kg (30 mg/kg for the first administration and 20 mg/kg for the second and third administrations). The dosages of the test article C were 10, 30 and 50 mg/kg. All animals were administered by intravenous infusion once every 3 weeks for a total of 2 or 3 times. During the study, clinical signs, body weight, clinical pathology, immune-related indicators, and histopathological examination etc. were performed. **Results:** The highest non-severely toxic dose (HNSTD) was 45 mg/kg for test article A, 15 mg/kg for B, and 50 mg/kg for C. Mortality occurred in 4/10 animals at 30 mg/kg of B, primarily due to malnutrition secondary to persistent diarrhea and general deterioration; no deaths or moribundity were observed with A or C. All compounds caused transient loose stools. Systemic exposure to exatecan was significantly higher for B than A or C, correlating with its lower tolerated dose. Drug-to-antibody ratios (DAR) were 4 for A and 8 for B and C; exposure differences were attributed to linker and target variations rather than DAR. Thus, tolerated dose was inversely related to exatecan exposure. The predominant toxicity for all compounds involved the hematopoietic and immune systems, evidenced by reductions in RBC indices (RBC, HGB, HCT, Retic), thymic lymphocytes, bone marrow hematopoietic cells, and lymphocytes in spleen and lymph nodes. Test article B also induced gastrointestinal, skin, and renal lesions, including intestinal mucosal necrosis, hemorrhage, villous atrophy, lamina propria edema, epithelial detachment; skin inflammation, ulceration, necrosis; and renal tubular casts, dilation, and vacuolation. Exposure to total antibody, ADC, and exatecan increased dose-dependently without accumulation. Anti-drug antibodies (ADA) were detected in A and C without affecting exposure;

none were observed in B. No significant dose-related changes were noted in food consumption, cardiovascular, respiratory, ophthalmic, or other systemic parameters. Consistent with literature, exatecan toxicity primarily affects rapidly proliferating tissues, with noncumulative myelosuppression—especially neutropenia—as the principal dose-limiting effect. Compared to exatecan alone, ADC derivatives markedly reduced hematologic toxicity and eliminated reproductive tissue toxicity. **Conclusions:** The tolerated dose of exatecan derivative ADCs given to cynomolgus monkeys was related to the systemic exposure to exatecan after administration. The administration of exatecan derivative ADCs significantly reduced hematological toxicity. The main toxicities were bone marrow hematopoietic inhibition and immunosuppression.

PS 4708 Safety and Pharmacokinetic Evaluation of MAM01, a Monoclonal Antibody for Malaria infection caused by *Plasmodium falciparum*

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Background and Purpose: Malaria is a life-threatening disease caused by *Plasmodium* parasites, transmitted through bites from infected female *Anopheles* mosquitoes. Of the five species that infect humans, *Plasmodium falciparum* is responsible for over 95% of global malaria cases and 96% of malaria-related deaths in the WHO African Region. In 2022, approximately 608,000 deaths were attributed to malaria, primarily in children in sub-Saharan Africa. MAM01 is an extended half-life IgG1 monoclonal antibody currently in clinical development for passive immunoprevention of malaria in children 3 months to <60 months of age, by targeting *Plasmodium falciparum* circumsporozoite protein. Extensive pharmacokinetics (PK) characterization of MAM01 was conducted in mice and rats, along with safety evaluation in nonhuman primates. These studies were aimed at supporting Phase 1 first in human studies, based on safety and toxicology evaluation. **Methods:** Single intravenous doses up to 100mg/kg were administered in non-GLP studies in rats and monkeys, with long-term post-dose evaluations for 3 months in rats and 6 months in monkeys. A 4-week repeat-dose GLP study in monkeys evaluated systemic toxicity following four weekly administrations of up to 300 mg/kg via intravenous (IV) injection, and 152.1 mg/dose via subcutaneous (SC) or intramuscular (IM) routes. Additionally, a GLP tissue cross-reactivity study was performed using normal adult human tissues to identify potential off-target binding and species selection for the GLP study. A mathematical model was developed using mouse, rat, and monkey pharmacokinetics and mouse efficacy data to project human PK. Simulations were then performed to determine the predicted concentration of MAM01 corresponding to 95% protection in humans. **Results:** In single dose studies conducted in rats and monkeys, MAM01 had an extended half-life of up to 309 hours (~13 days) in rats or 618 hours (~26 days) in monkeys, respectively, following IV administration, and up to 691 hours (~29 days) in monkeys after SC injection, at the low dose of 10 mg/kg. A 4-week repeat-dose GLP study in monkeys identified a no observed adverse effect level of 300 mg/kg IV, providing a 9.3-fold exposure margin over estimated human exposures. A GLP tissue cross-reactivity study in normal adult human tissues revealed no off-target binding. MAM01 demonstrated extended half-life (up to 691 hours SC in monkeys) and consistent pharmacokinetic profiles without evidence of anti-drug antibody interference. No test article-related toxicity, organ-specific effects, or adverse reactions were observed across all doses and routes. The simulations of MAM01 treatment from the mathematical model suggested that prophylactic efficacy may be observed for doses over a range of 1.5 - 40 mg/kg. **Conclusions:** Overall, the nonclinical safety profile of MAM01 supported dosing of humans in a Phase 1 clinical setting.

PS 4709 Non-Clinical Development of the Monoclonal Antibody Zanidatamab for the Treatment of HER2-Expressing Cancers

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Background and Purpose: Zanidatamab is a bispecific antibody that targets human epidermal growth factor receptor 2 (HER2), leading to receptor downregulation and effector-mediated cytotoxicity with consequent antitumor activity in HER2-expressing cancers. A comprehensive non-clinical programme consistent with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) and other jurisdictional requirements was required to support the marketing application of zanidatamab. **Methods:** All *in vivo* toxicology studies were conducted in cynomolgus macaques, since zanidatamab binds with similar sub-nanomolar affinities to both human and macaque HER2, but not other species. Zanidatamab was administered intravenously (1-hour infusion) to monkeys (n ≤ 4/sex/group) at 0, 5, 50, or 150 mg/kg once weekly (QW) for up to 13 weeks and assessed for toxicology, toxicokinetics, immunogenicity (antidrug antibodies) and safety pharmacology endpoints. Additional animals (n ≤ 3/sex/group; 0 and 150 mg/kg QW) were similarly dosed and then maintained for an off-dose period of 8 weeks to evaluate the reversibility or progression of any findings. Doses were selected with reference to a preliminary 4-week, dose range-finding study performed in the same species. Cross-reactivity studies were also performed, in

vitro, to investigate membranous immunostaining of zanidatamab in human tissues. Assessment of mutagenicity and reproductive toxicology studies were not warranted for zanidatamab, in accordance with ICH-S9. An antibody binding to the extracellular domain of HER2 is not expected to interact directly with chromosomal material; thus, zanidatamab was not considered to present a mutagenic liability. On the basis of a weight of evidence analysis, zanidatamab was considered an embryo-fetal toxicant, since the critical role of HER2 in fetal development is well established and the potential for embryo-fetal toxicity by HER2-blocking antibodies has been documented. **Results:** Human tissue cross-reactivity studies demonstrated membranous staining of zanidatamab consistent with HER2 expression reported in the literature (Press, 1990; Liu, 2001). Although the spinal cord also exhibited membrane staining, the existence of the blood-spinal cord barrier should limit *in vivo* exposure of this tissue to zanidatamab (Rossi, 2013). Zanidatamab exposure (in terms of area under the curve) in cynomolgus macaques increased approximately dose-proportionately, with a mean terminal elimination half-life of 2.73 to 8.95 days. Zanidatamab-related effects in monkeys were minimal and included a non-dose-dependent increase in the incidence of diarrhoea. Mild increases in blood urea nitrogen and/or decreases in serum albumin levels were not dose-related and were within historical control ranges but were correlated with an increased incidence of diarrhoea for some animals and considered a consequence of mild subclinical dehydration. The no observed adverse effect level (NOAEL) was thus 150 mg/kg QW, the highest dose evaluated. There were no effects of zanidatamab on neurobehavioral, respiratory and cardiovascular functions in macaques. **Conclusions:** In summary, this programme characterised the non-clinical safety of zanidatamab and successfully supported its marketing application. The NOAEL in cynomolgus macaques was 150 mg/kg QW, the highest dose evaluated. **References:** Liu et al, (2001). Expression of the receptor tyrosine kinases, epidermal growth factor receptor, ErbB2, and ErbB3, in human ocular surface epithelia. *Cornea*, 20(1): 81-85. Press et al, (1990). Expression of HER-2/neu proto-oncogene in normal human adult and fetal tissues. *Oncogene*, 5(7): 953-962. Rossi et al, (2013). Current options for drug delivery to the spinal cord. *Expert Opinion on Drug Delivery*, 10(3), 385-396.

PS 4710 Standardizing Immunomodulatory Dosing of Sirolimus in Non-Human Primates for Gene Therapy: A Data-Driven Review

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Background and Purpose: Gene therapy continues to progress as a viable therapeutic intervention for genetic disorders. These therapeutic interventions often use viral vectors (eg, adeno-associated virus) and lipid nanoparticles as a platform which may illicit severe immunogenic responses. Immunomodulatory agents such as Sirolimus, an mTOR inhibitor, improve tolerability due to the immunosuppressive effects, including modulation of T-cell activity and reduction of inflammatory responses. In clinical practice, recommended human dosages of Sirolimus vary based on indication, body weight, and immunologic risk, with target trough blood concentrations generally ranging from 5 to 15 ng/mL. The purpose of this abstract is to establish a nonclinical reference range for Sirolimus dosing in mg/kg to facilitate therapeutic dose calculations (ease of study conduct and improve reproducibility in nonclinical studies) in non-human primates. **Methods:** Across multiple safety assessment studies conducted over the past two years, a total of 35 cynomolgus monkeys were identified as receiving Sirolimus treatment, either as a monotherapy or in combination with other immunosuppressive agents, as part of the study design, involving either once- or twice-daily administration, with or without weekly dose adjustments. Of these, 21 animals were selected for evaluation of target dose level vs corresponding blood concentrations. Whole blood samples were collected weekly, using K2EDTA tubes, and Sirolimus concentrations were quantified via high-performance liquid chromatography with tandem mass spectrometry (LC-MS/MS). For each animal, the Sirolimus dose was calculated based on initial level (mg/m²), most recent body weight, and body surface area (BSA) conversion methodology. This value was then converted into a practical dosing unit of mg/kg. **Results:** Sirolimus treatment typically began 2 to 3 days prior to the gene therapy administration and continued daily for a duration ranging from 5 days to 2 months following the gene therapy dose. In general, incremental increases in mg/kg dosing were associated with higher trough and peak Sirolimus blood concentrations; however, individual animal responses varied considerably throughout the study periods. A review of non-clinical gene therapy study designs indicated an intended target therapeutic blood concentration between 2 and 10 ng/mL per day. Measured blood concentrations across studies ranged from 1.2 to 8.5 ng/mL, with corresponding calculated dose levels between 0.15 and 0.57 mg/kg/day. Notably, inter-animal variability was observed even at similar levels. Additionally, dose adjustments generally resulted in changes to blood concentrations within $\pm 10\%$ of prior levels. Based on the cumulative data review, a Sirolimus dose range of 0.2 to 0.5 mg/kg/day is proposed to achieve the intended target therapeutic trough blood levels. **Conclusions:** Sirolimus dosing regimens in non-human primate gene therapy studies varied in schedule and duration, with some adjustments along the way requiring blood sample collection to confirm blood concentration levels. While higher mg/kg doses generally led to increased blood concentrations, individual animal variability was significant. The proposed dose range of 0.2 to 0.5 mg/kg/day establishes a foundational framework for achieving therapeutic Sirolimus levels in non-human primates, reduce the frequency of blood collections required for monitoring, while also enabling more consistent comparisons across nonclinical studies.

PS 4711 A Retrospective Study of Nonclinical Safety Data for siRNAs

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Background and Purpose: Small interfering RNA (siRNA) therapeutics represent a promising class of drugs. This poster aims to summarize the toxicological findings from a retrospective analysis to identify common patterns and assess overall safety. **Methods:** A retrospective analysis was conducted on a database of 46 GLP-compliant, repeated-dose toxicity studies. The database encompassed 23 different siRNA molecules in preclinical development. Details on therapeutic areas, experimental design (species, duration, route), and extensive data collections were analyzed. The data included toxicokinetic parameters, clinical and anatomic pathology, immunological profiles (cytokines, complement, immunophenotyping), and immunogenicity assessments. **Results: Study Distribution:** The studies covered therapeutic areas including metabolic (35%), cardiovascular (22%), and liver diseases (17%). Test species were primarily nonhuman primates (NHPs, 48%) and rats (39%), with fewer studies in dogs (2%) and mice (11%). **Administration & Exposure:** Most siRNAs utilized the GalNAc delivery system and were administered subcutaneously at doses of 10-600 mg/kg. The time to maximum concentration (T_{max}) was 0.5-2.0 hours in rodents and 1.0-8.0 hours in NHPs, with no observed accumulation. Higher drug exposure was noted in the liver and followed by the kidneys. **Toxicological Findings:** Mild to moderate changes were common in hematology, hepatic parameters, and pharmacological markers, while immunological effects were rare. The primary target organs were the liver, kidneys, lymph nodes, and injection sites, with occasional involvement of the spleen, thymus, and thyroid. Most findings were minimal to moderate in severity and were either reversible or showed partial recovery. **Immunogenicity:** Anti-drug antibodies (ADAs) were rarely detected and had no impact on systemic exposure. **Conclusions:** This retrospective analysis demonstrates a generally favorable and predictable safety profile for siRNA therapeutics in preclinical development. Toxicological findings were primarily confined to expected target organs, were typically mild to moderate, and often reversible. The establishment of a No Observed Adverse Effect Level (NOAEL) in 98% of the studies, coupled with rare and non-impactful immunogenicity, supports the continued development of siRNA drugs.

PS 4712 Acute Acceptable Exposure Levels for Impurities in an Infant Intrathecal Drug Product

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Background and Purpose: Permitted daily exposure limits (PDEs) represent a dose of a substance that is unlikely to cause adverse effects if an individual is exposed, by specified routes, at or below this dose every day over a lifetime. In certain circumstances, such as in less-than-lifetime exposure scenarios, higher doses of the chemical may be safe. Here, we propose an Acute Acceptable Exposure Level (AAEL) for infant exposure to an impurity present in a drug product administered via the intrathecal route. This is illustrated through two case studies for erucamide and ethanol, both of which have published limits set forth by the International Council for Harmonisation (ICH). **Methods:** AAELs were derived based on the adult parenteral PDE approach following ICH Q3C, Q3D, and draft Q3E guidelines, which generally involves understanding the critical effect, selection of a point-of-departure (POD) from a robust study, and the application of the appropriate adjustment factors. To develop the AAEL for the intrathecal route for a 7.6 kg (6-month-old) infant, modifications for this assessment include specific examination for the potential for central nervous system (CNS) as the critical effect, utilization of an acute study where possible, and the omission of a subchronic-to-chronic extrapolation factor. If necessary, a modifying factor was added to account for sensitivity of the developing infant CNS. The resulting dose was then adjusted to a concentration utilizing a 7.6 kg infant blood volume and cerebrospinal fluid (CSF) volume to ultimately identify a safe acute intrathecal dose. **Results:** AAELs for exposure by the intrathecal route for a 7.6 kg (6-month-old) infant were derived for two impurities. First, based on the ICH Q3E draft PDE for erucamide, a POD was selected from a 13-week rat study in which no effects were noted, and was adjusted for a 7.6 kg patient, a modifying factor for potential direct infant CNS effects, and the CSF volume of a 7.6 kg infant. This resulted in an AAEL for erucamide of 150 mcg. Secondly, the threshold of effect of a predicted embryonic ethanol concentration associated with maternal consumption of one alcoholic beverage per day was selected as the POD, and was adjusted for a 7.6 kg patient, inter-individual variability, extrapolation from a LOAEL to a NOAEL, and the CSF volume of a 7.6 kg infant, resulting in an AAEL for ethanol of 40 mcg/day. **Conclusions:** While PDEs are routinely derived for impurities in many drug products, acute exposures for infants via the intrathecal route are rarely encountered. The scientifically plausible approach presented here demonstrates the use of less-than-lifetime exposure, sensitive population (infant) CNS effects, unusual route extrapolation, and concentration at the sensitive tissue to provide a safe dose for inadvertently exposed infants.

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Background and Purpose: In early 2025, the FDA and NIH announced initiatives to reduce mandatory animal testing and expand the use of new approach methodologies (NAMs) in grant applications and preclinical drug development. Among these NAMs is Simcyp®, a physiology-based pharmacokinetic (PBPK) modeling platform that incorporates both human and animal simulation modules. These modules draw on compound-specific physicochemical properties as well as training datasets generated from validated reference compounds. Although the human simulator has undergone substantial validation across key ADME processes, the animal modules lack similar depth. In 2022, the FDA issued draft clinical guidance on assessing the pressor effects of investigational drugs, explicitly highlighting rofecoxib and torcetrapib as recommended positive controls. Validating model-based predictions of these drugs is essential for reducing animal use by establishing PK modules suitable for quantitative systems pharmacology applications. This study compares PK predictions generated with the Simcyp® rat model against *in vivo* and literature PK data. We hypothesize that the Simcyp® rat model will reliably capture the PK profiles of both compounds. **Methods:** A PK study was conducted on eight Sprague-Dawley (SD) rats (four females and four males), the strain on which the validated rat model is based. Animals received torcetrapib (100 mg/kg, PO), followed by a one-week wash-out period and then were administered rofecoxib (100 mg/kg, PO). Venous blood samples were collected from the lateral tail vein at baseline and at 0.5, 1, 2, 4, 8, and 24 hours after dosing and analyzed using LC-MS. Noncompartmental PK parameters were generated with Phoenix WinNonlin®. Literature-derived physicochemical data were used in the Simcyp® rat simulator to generate predicted PK profiles for both drugs. Simulated results were compared with *in vivo* data and published PK values, and any discrepancies in key parameters were quantified. Experimental PK data were used to refine drug profiles within the simulator as needed. **Results:** *In vivo* PK studies are currently underway. For rofecoxib, which has more comprehensive published physicochemical information, Simcyp® generated reasonably accurate predictions of key pharmacokinetic parameters. In contrast, predictions for torcetrapib were less accurate, particularly for exposure-related metrics, reflecting the limited preclinical data available. For rofecoxib, $t_{1/2}$ and t_{max} were accurately predicted compared to values in the literature (5.2h vs 5.0h and 2.7h vs 2.3h, respectively), AUC was underpredicted by approximately 2-fold (16.00 mg*L/h vs 29.63mg*L/h), and C_{max} was overpredicted by 3.8-fold (1.55mg/L and 0.40mg/L). Available literature PK data for torcetrapib are based on a 20 mg/kg dose, whereas our *in vivo* study uses a 100 mg/kg dose to produce a human-relevant pressor response. Despite this dose difference, both the simulated and literature values demonstrated comparable $t_{1/2}$ and T_{max} . Half-lives were predicted at 3.07h, which approximates the literature $t_{1/2}$ values of 3.50h. Similarly, t_{max} was well predicted at 2.40h compared to the literature value of 2.70h. However, AUC and C_{max} were severely underpredicted. Literature values of AUC and C_{max} were reported as 95.8 mg*L/h and 19.1 mg/L. The simulation predicted an AUC of 25 mg* h and C_{max} of 3.32 mg/L, a 5.75-fold and 3.8-fold underprediction. **Conclusions:** The Simcyp® rat model accurately predicts absorption-related pharmacokinetic parameters such as t_{max} and $t_{1/2}$. However, the lack of complete ADME data limits its ability to forecast exposure-related metrics like C_{max} and AUC, which are key for exposure-related toxicology outcomes. Ongoing *in vivo* studies will help refine these drug profiles, supporting the development of validated PBPK modules for use as NAM-based positive controls in evaluating drug-induced hypertension risk. Better PK predictions in rats will improve model-informed toxicological assessments, particularly for exposure-dependent effects, and reduce the dependence on animal testing.

PS 4714 Off target toxicity and efficacy testing of a novel stress reliever in a multi-organ microphysiological system

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Background and Purpose: *Echinacea purpurea*, a known immunomodulator and pain reliever, has shown potential benefits on anxiety-like behavior in animal models. *E. purpurea* contains alkalimides, which can selectively bind and activate cannabinoid 1 (CB1) and cannabinoid 2 (CB2) receptors. One of the main active alkalimide, dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide (dodeca) is reported to have a significant positive relationship with CB1 receptor, but the safety and efficacy of *E. purpurea* and its components as a stress reliever in humans is not well understood. **Methods:** This study used an *in vitro* single-organ central nervous system (CNS) and a multi-organ microphysiological system (MPS) with human cells-based gut, liver, blood-brain-barrier (BBB) and CNS to investigate the efficacy and potential off-target toxicity of oral delivery of *E. purpurea* extract and dodeca in its pure form to ameliorate cortisol-induced stress on the CNS. Cannabidiol (CBD) and diazepam with known stress relieving properties were used as control drugs. A five-point dose range of pure dodeca (1 uM, 3 uM, 10 uM, 30 uM and 100 uM) was utilized to capture the dose-dependent response. The function and health of the constructs was measured by long-term potential (LTP) maintenance (CNS), albumin synthesis and P450 cytochrome activity (liver), and transendothelial electrical resistance (TEER) (BBB and gut) over a 48-hour exposure period to the drugs followed by a cell viability assess-

ment. The transport kinetics of dodeca across BBB and gut were used to create a pharmacokinetic-pharmacodynamic (PKPD) model to predict efficacy of dodeca *in vivo*. **Results:** *E. purpurea* extract showed a dramatic dose-dependent increase in LTP maintenance within both single- and multi-organ MPS, with the effect sustained for a duration of several hours and a significant induction of CYP1A1 activity in the liver without compromising cellular health. The observed fleeting effect aligned with the clinical dosing of *E. purpurea* tinctures as an anti-inflammatory medication. The multi-organ MPS showed a more enhanced rescue effect on LTP compared to the single organ, suggesting the contribution of drug metabolites in the stress relieving process. The extract showed more enhanced effects than its equivalent concentration of pure dodeca showing the contribution of other components within the extract. The PKPD modeling approach predicted dodeca would produce amelioration of stress-induced LTP deficits *in vivo*, consistent with clinical reports of reduced stress. **Conclusions:** Compared to single-organ CNS, inclusion of barrier tissues and liver in the multi-organ MPS showed contribution of drug transport and metabolism creating a more representative system for studying the pharmacokinetics and pharmacodynamics of compounds and metabolites. With the lack of human relevant data and models to study therapeutics, MPS presents a potentially beneficial platform for studying drug-drug interactions with other common therapeutics for both efficacy and off-target toxicity. Additionally, MPS provides supplementary information on the dosing concentrations and strategy based on human-cell response *in vitro*.

PS 4715 Assessing the data landscape of extractables and leachables (E&L) and implementing strategies to fill data gaps

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Background and Purpose: Extractables and leachables (E&Ls) are chemical substances that can migrate into drug products from closed container systems, packaging materials, or medical devices, under exaggerated, manufacturing or storage conditions. As leachables are observed under normal storage conditions, it is important to assess the safety of those leachables which exceed safety thresholds. Where sufficient data are available to assess safety, a permitted daily exposure (PDE) value is calculated to communicate safe levels. However, where data are lacking for PDE derivation, data from a surrogate compound is assessed for its suitability to fill data gaps using read-across strategies. Read-across includes an assessment of data availability, and data suitability, while also ensuring overall analog fitness through an evaluation of similarity. The objective of the current study was to survey the availability of data to support read-across strategies for E&Ls. Additionally, where data are lacking, we discuss considerations for the derivation of a PDE value through a read-across case study using 1,9-Dimethyl nonanedioate as an example. **Methods:** A dataset of ~1200 E&L chemicals was compiled from ELSIE, PQRI and proprietary sources. Each chemical was assigned to a chemical cluster using hierarchical agglomerative clustering. Manual review was used to refine the chemical class assignments. A two-tier system of clusters was established to define the E&L chemical space. The second tier was based on stricter classification rules than the first tier. Each of the chemicals was mapped to data sources that are commonly referenced for safety assessments including the Leadscape (sub) chronic databases, EFSA OpenFoodTox, ECHA, IRIS, ToxRefDB, CPDB as well as established ICH PDEs. An assessment of data availability across the second tier was performed and reported. A case study involving a data poor member of the Aliphatic esters (Di-esters/Straight chain) cluster was used to highlight considerations for PDE derivation using read-across. **Results:** Approximately half of the E&L chemicals mapped to experimental data, with an additional 10% of chemicals having limited experimental data. Second tier clusters meet stricter structural requirements and therefore availability of data within these clusters provides a preliminary indication of the number of clusters where read-across could be considered. More specifically, 37% of clusters were populated by members, which were all data rich, while 80% of clusters were populated by at least one data rich member. For those clusters populated by data-poor members, it is important to find potential analogs with data to support read-across methodologies. 1,9-Dimethyl nonanedioate is a data-poor member of the Aliphatic esters (Di-esters/Straight chain) class with 6 data-rich and 2 data-poor members. The data-rich members together with additional analogs from the Leadscape database were evaluated as potential sources for read-across. Similarity was assessed across multiple domains including examination of experimental and predicted toxicological profiles. Positive Ames test results for 1,9-dimethyl nonanedioate were a key toxicological difference compared to a potential analog dimethyl decanedioate, which is assessed as negative in the Ames test. Such toxicological differences require resolution prior to reading across data from dimethyl decanedioate (analog) to 1,9-dimethyl nonanedioate (target). In the case of 1,9-dimethyl nonanedioate, the positive Ames result is traceable to a metabolite with antimicrobial activity. Whereas a negative mouse lymphoma study and a negative *in vivo* mouse micronucleus assay support the negative assessment of genotoxicity for the dimethyl decanedioate analog. **Conclusions:** The distribution of data across clusters indicates strong potential for applying read-across to support data-poor members, while recognizing that a case-by-case evaluation would still be required. *In silico* profiling reveals that even structurally close analogs within some second-tier clusters may exhibit divergent toxicological features, and such differences must be resolved to support any read-across rationale. The study provides insights into the data landscape for E&L. It is important to identify those chemical classes for which read-across could be applied, and where defaulting to threshold values would be appropriate.

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Background and Purpose: With the rapid advancement of the medical aesthetics industry, an increasing variety of aesthetic products are being widely utilized for skin beautification. However, this trend has also led to a rising incidence of adverse events, including skin necrosis, nodules, alopecia, and even life-threatening complications. Numerous studies have demonstrated that the injection of hyaluronic acid (HA) or similar products may result in tissue hypoxia or necrosis due to vascular compromise. To facilitate the safety evaluation of such aesthetic products, a study was conducted to establish an induced vascular occlusion model and assess both the potential risk of vascular embolism following a single arterial injection of HA and the efficacy of the recommended HA rescue protocol (HA-HYAL). **Methods:** Eight male rabbits were randomly assigned to 4 groups, with 4 rabbit ears in each group. During the Occlusion Phase: Groups 1-2: The single ear of each rabbit received a single injection of equal amounts of HA via the central ear artery. During the Rescue Phase: Groups 3-4: Rabbit auricular arteries were first occluded with HA and subsequently treated with hyalase, administered either intra-arterially (IA) or subcutaneously (SC). Hyalase was injected at the occlusion site 5 minutes after HA administration, based on observed blood flow interruption. For the SC rescue group, a second hyalase injection was performed 30 minutes after the initial HA injection to facilitate embolus dissolution. The formation of transparent emboli was monitored post-injection, and photographs of vascular embolism in the rabbit ear were taken at various time points under strong illumination. A 14-day observation period was conducted to evaluate vascular recanalization and tissue necrosis. The injection rate was controlled at approximately 0.2 mL/min. All animals were necropsied on Day 15, and gross lesion assessment and histopathological examination were performed. **Results:** **Occlusion Phase:** Immediately after HA injection, all treated rabbit ears exhibited transparent emboli, visible in both the main trunk and branches of the central ear artery. Partial recanalization of the central artery was observed in all HA-injected ears by Day 14. On Day 14, two out of four ears showed mild skin damage, while the remaining two demonstrated moderate to severe skin damage. Focal/multifocal to coalescing scabs (3/4 ears) and locally extensive dark red discoloration (4/4 ears) were noted, corresponding microscopically to crust formation, skin necrosis, and chronic/acute subcutaneous inflammation. Multiple emboli were detected in the vasculature at proximal, middle, and distal injection sites in all ears. In some arteries, the vessel wall was infiltrated by multinucleated giant cells and other inflammatory cells. The mean maximum diameter of embolus-containing vessels in the HA group was 704.54 μm . **Rescue Phase:** Immediately after HA injection, all treated rabbit ears exhibited transparent emboli in the central ear artery. Complete recanalization of the central artery was achieved by 24 hours in the HA/IA rescue group and by Day 7 in the HA/SC rescue group. By Day 14, both ears in each rescue group showed only mild skin damage. Focal/multifocal scabs (1/2 ears in each rescue group) and locally extensive dark red discoloration (2/2 ears in each rescue group) were observed, correlating microscopically with crust, skin necrosis, and chronic/acute subcutaneous inflammation. Multiple emboli were present in the vasculature at all injection sites. The mean maximum diameter of embolus-containing vessels in the rescue groups (512.18 μm) was markedly smaller than in the occlusion phase. In the occlusion phase, pathological findings indicated that HA induced multiple vascular emboli and tissue necrosis at the injection sites. **Conclusions:** This study demonstrates: 1 Successful establishment of a standardized vascular occlusion model. 2 Significant therapeutic efficacy of hyaluronidase rescue therapy. 3 Critical importance of early intervention in mitigating irreversible ischemic injury. 4 Validation of a translatable preclinical platform for evaluating vascular safety profiles of aesthetic products, featuring quantifiable occlusion parameters and clinically relevant rescue endpoints.

PS 4717 Immunogenicity and Toxicity of rAAV Gene Therapies in Non-human Primates in Preclinical Safety Evaluation

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Background and Purpose: Recombinant Adeno-associated virus (rAAV) is among the most widely used vectors for gene delivery, yet its clinical application is challenged by severe immune-mediated toxicities. This study aimed to define a core set of parameters for preclinical assessment of rAAV immunogenicity and toxicity. **Methods:** We conducted a pooled analysis of 16 non-human primate (NHP) toxicity studies over the past five years, evaluating a multidimensional dataset: pre-existing immunity, innate immunity (cytokines, complement), adaptive immunity (antibody/T-cell responses), clinical pathology, and histopathology of key organs. **Results:** A pooled analysis across the 16 studies included a total of 538 NHPs administered with rAAV via subretinal, intravitreal, intravenous, or intrathecal injection. By study count, ocular indication was the majority (68.75%), followed by central nervous system (12.5%), hemophilia (12.5%), and cardiac (6.25%) indications. The vectors of AAV2, 5, 8, and 9 were selected based on their distinct tissue tropism. Toxicity profiles were influenced by administration route and vector tropism. As anticipated, ocular delivery was associated with local inflammation, while systemic administration resulted in hepatotoxicity, consistent with the underlying biology of target tissues. To exclude animals with high pre-existing immunity, all studies implemented rigorous serological screening (87.5% used neutralizing antibody (NAb) assay with titer cut-off $\leq 1:4$ to 1:20 and 12.5% used binding antibody (BAB) assay with an OD value of <0.5). Anti-

AAV2 NAb was most prevalent (28.2%-87.4%), followed by AAV5 and AAV9 (~30%), and AAV8 (~5%). Innate immune activation is a recognized trigger of adverse events in clinical rAAV trials. To investigate this, we monitored early post-dosing biomarkers, including a broad panel of cytokines and complement factors, with sampling within the 24-hour window. Notably, across all studies evaluated, no significant innate immune activation was detected. Adaptive immune response is a pivotal determinant of the safety and efficacy profile. Humoral immunity was assessed by measuring serum and local neutralizing antibody against AAV capsid, as well as binding antibody against transgene product. Cellular immunity was evaluated via IFN- γ ELISpot in response to capsid or transgene product. In these studies, both humoral and cellular immune were evaluated prior to dosing, at 2 to 4 weeks post-dosing, and over prolonged periods (months). After dosing, high-titer serum NAb (1:256-1:8000) developed in 70%-100% of animals, while lower-frequency (20%-40%) and lower-titer (1:4-1:256) local NAb and infrequent anti-transgene antibody (10%-30%) were detected. Critically, cellular immune response was minimal, with positive IFN- γ ELISpot signals detected in only 2 of 13 studies. Clinical trials of rAAV products have reported hepatotoxicity, thrombotic microangiopathy and dorsal root ganglia toxicity. To evaluate these potential toxicities, key parameters were monitored, including liver enzymes (ALT, AST and GGT) and kidney function (UREA and Cre), histopathology of liver, kidney, and dorsal root ganglia. In the present review, most studies showed no evidence of treatment-related liver toxicity. An elevation of ALT without corresponding microscopic findings was observed in only one study, affecting 2 out of 10 animals. And hepatocellular vacuolation was noted in three studies from the same sponsor, but confirmed to be fatty granules via Oil Red O staining. It is noteworthy that the only study to report elevated ALT level was one of the two studies with a positive T-cell response, suggesting a potential association between cellular immunity and hepatocellular injury in that specific context. Kidney function, histopathology of kidney and dorsal root ganglia were normal across all studies. **Conclusions:** This analysis confirms immunogenicity as a critical safety concern for rAAV therapy. While robust anti-capsid antibody responses were prevalent, cellular immunity and transgene-specific responses were minimal. Observed toxicities were mild, including transient ALT elevation and steatosis. A comprehensive panel assessing humoral/cellular immunity, clinical pathology, and histopathology characterized these events, supporting its essential role in the preclinical risk assessment framework to ensure the safe progression of rAAV-based gene therapy.

PS 4718 Multiwalled Carbon Nanotubes Modulate Lipid Rafts Assembly and Hippo Signaling.

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Background and Purpose: Multiwalled carbon nanotubes (MWCNTs) are widely used innovative nanomaterials with potential liver toxicity. This study explores how MWCNTs alter lipid rafts (cholesterol-rich membrane microdomains) that are critical for immune signaling and trigger inflammation and Hippo pathway, offering novel insights into nanotoxicology. **Methods:** Human lung epithelial (A549) cells and full-thickness airway microtissues (MatTek EpiAirwayFT) were exposed to 25 $\mu\text{g}/\text{ml}$ MWCNTs for 24 h to quantify cytokines and chemokines by ELISA, and transcription factors by qRT-PCR and immunoblotting. Isolated lipid raft fractions were characterized using immunoblotting, hybrid ELISA or immunofluorescence imaging. Electron microscopy was used to visualize morphological changes in A549 cells. Lipid rafts were modulated using docosahexaenoic acid (DHA), polyunsaturated fatty acids (PUFA), or caveolin-1 (CAV1) neutralization in our cellular study model. **Results:** MWCNTs increased IL-6, IL-8, CCL2, CCL5 at mRNA and/or protein levels and boosted inflammatory drivers (NF- κB , STAT3), but not receptors (TLR4 and RAGE) protein expression, suggesting post-transcriptional regulation in A549 cells and microtissues. Hippo signaling components (YAP/TAZ) were induced, linking to inflammation. MWCNTs altered CAV-1/2, but not flotillin 1/2 expression. Electron microscopy showed membrane distortion and needle-shaped MWCNT internalization. DHA (omega-3 PUFA) pretreatment abrogated raft markers, while general PUFA enrichment had no significant effect on our cellular model. CAV-1 neutralization altered inflammation and Hippo components. **Conclusions:** This study reveals MWCNTs disrupt lipid rafts driving inflammation and Hippo signaling, advancing mechanistic understanding of nanomaterial toxicity. It informs safer nanomaterial design and toxicology research. Future lipidomic study will map membrane changes to develop protective strategies.

PS 4719 Construction of an *in vitro* TDAR alternative model to mimic T-cell-dependent antibody response (TDAR) assay

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Background and Purpose: T-cell-dependent antibody response (TDAR) assay is a gold standard for assessing the effects of chemicals on adaptive humoral immunity, which usually employed to evaluate immunotoxicity of chemical substances and efficacy of immune adjuvants. However, the classic TDAR is conducted on animal models, which have limitations such as time-consuming (at least 4 weeks) and differ-

ent immune responses between humans and animals. Therefore, it is necessary to develop *in vitro* alternative models. **Methods:** Human peripheral blood mononuclear cells (PBMCs) from healthy donors were stimulated with candidates for 10 consecutive days in an environment containing antigens (Keyhole Limpet Hemocyanin, KLH, 20 µg/mL), IL-2 (100 U/mL), and Staphylococcal protein A (SPA, 10 µg/mL), then the KLH-specific IgM/IgG in the supernatants were measured by ELISA. **Results:** We observed PBMCs stimulated with KLH, IL-2, and SPA were able to produce KLH-specific IgM/IgG antibodies, demonstrating the detectability and sensitivity of the *in vitro* TDAR platform. Furthermore, after adding resiquimod (R848) and the candidate (LG01) in this system, we observed a significant increase in the secretion of anti-KLH IgM/IgG antibodies compared to the KLH-only stimulated group. **Conclusions:** The TDAR alternative model was successfully constructed *in vitro* based on PBMCs, which can be applied to high-throughput adjuvants screening and provide more accurate risk assessments of immunotoxicity.

PS 4720 The effects of direct addition of lead(ii) acetate to a human *in vitro* model of hematopoiesis recapitulates exposure to lead *in utero*

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Background and Purpose: Exposure to heavy metals during pregnancy and early immune development is a significant public health concern. Lead, a known developmental immunotoxicant capable of crossing the placental barrier, remains a persistent environmental threat. Michigan's aging infrastructure has contributed to notable disparities in exposure; although Flint drew national attention, Kent County (Grand Rapids, MI) reports the highest childhood lead levels in the state. While prenatal lead exposure has been linked to adverse outcomes, its impact on the developing human immune system remains poorly characterized. Traditional rodent-based immunotoxicity models often fail to fully recapitulate human immune development, underscoring the need for more human-relevant approaches as regulatory agencies adopt new approach methodologies (NAMs). To address this, we applied a human *in vitro* NAM that differentiates CD34⁺ hematopoietic stem cells toward myeloid and lymphoid lineages to assess the effects of lead on immune cell lineage commitment and compared these results with *ex vivo* umbilical cord PBMCs with documented *in utero* exposure. Together, this approach evaluates developmental immunotoxicity in a human-relevant system and benchmarks outcomes against real-world exposure. **Methods:** **Cell culture:** Human CD34⁺ hematopoietic stem cells isolated from umbilical cord blood were cultured for 28 days in complete RPMI containing human serum and antibiotics. Cells were seeded at 5 × 10⁴ cells/mL and exposed to vehicle or lead (ii) acetate (31.25-250 nM) on days 0, 7, 14, and 21. Hematopoietic differentiation was supported by SCF, FLT3L, and IL-6 supplementation on day 0, followed by SCF, FLT3L, and IL-7 on day 7. Half-media changes were performed on days 14 and 21 using cytokine-free media. **Flow cytometry:** Cells were collected on days 0, 7, 14, 21, and 28 to assess viability and lineage-specific differentiation. Samples were stained with a viability dye, surface markers identifying myeloid and B-cell subsets, and intracellular CD79a. After fixation and permeabilization, samples were analyzed on a Cytex Northern Lights cytometer. Compensation was performed using single-stain controls, and consistent gating strategies were applied across time points in FlowJo to quantify changes in developing immune cell populations. **Results:** Lead exposure during hematopoietic differentiation significantly altered human immune lineage development in a cell type specific manner. *In vitro* treatment of CD34⁺ cord-blood-derived stem cells with increasing concentrations of lead (ii) acetate over 28 days revealed dynamic effects on myeloid populations: CD14⁺CD1c⁺ monocytes were transiently suppressed at early time points, CD14⁺CD66b⁺ granulocytes were consistently reduced, CD206⁺ macrophages trended upward, and CD1c⁺ dendritic cells remained unaffected. Lymphoid lineage analysis demonstrated a potentiation in the development of B-cell precursors, with a significant increase in the pre-B cell marker CD79a at day 21 and trends toward elevated CD10⁺ and CD19⁺ cells. These *in vitro* findings were validated in a clinical cohort, where umbilical cord blood from infants with documented *in utero* lead exposure demonstrated reduced circulating monocytes and a significant enrichment of CD10⁺CD79a⁺ early pre-B cells, while total B-cell precursors were unchanged. Together, these results indicate the utilization of a human-based model of hematopoiesis recapitulates the effects of *in utero* lead exposure on the developing immune system. **Conclusions:** Traditionally, developmental immunotoxicology has relied on animal models due to limited access to human samples and a lack of suitable *in vitro* systems. Here, we demonstrate that a human umbilical cord blood-derived CD34⁺ stem cell assay can model hematopoiesis over four weeks and generate nearly all immune cell types. Lead exposure during differentiation selectively suppressed myeloid populations while promoting B-cell lineage commitment, particularly CD79a⁺ pre-B cells. In summary, our *in vitro* findings were confirmed in umbilical cord blood from infants with documented *in utero* lead exposure, demonstrating reduced circulating monocytes and increased early pre-B cells. (This work was funded by the Michigan State University Corewell Health Alliance Grant).

PS 4721 Δ⁹-tetrahydrocannabinol (THC) and cannabidiol (CBD) attenuate CD16⁺ monocyte-induced inflammatory responses by astrocytes in a primary human co-culture system

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Background and Purpose: CD16⁺ monocytes represent a minor subset of the total monocyte population (~15%) yet play a disproportionate role in driving neuroinflammation related to human immunodeficiency virus (HIV)- associated neurocognitive disorders (HAND), largely due to their enhanced transmigration across the blood-brain barrier. CD16⁺ monocytes can be activated by HIV ssRNAs via toll-like receptor (TLR) 7 and subsequently interact with brain-resident cells, including astrocytes. Interactions between CD16⁺ monocytes and astrocytes can amplify inflammatory signaling in the brain. Previous work from our laboratory has identified monocyte-derived IL-1β as a driving factor for astrocyte production of neuroinflammatory factors. Despite cannabis use among the HIV community, the mechanisms by which known immune-modulating cannabinoids, Δ⁹-tetrahydrocannabinol (THC) and cannabidiol (CBD), affect human immune responses remain elusive. The objective of this study was to determine how THC and CBD influence virally activated CD16⁺ monocyte-driven astrocyte cytokine production, and monocyte chemotaxis in HIV- and HIV+ subjects. **Methods:** For this study, we employed a primary human CD16⁺ monocyte-astrocyte co-culture system. Co-cultures were stimulated with the TLR7 agonist R837 and treated with increasing concentrations of THC or CBD (0.5, 1, 5, 10 µM). Supernatants were quantified for soluble IL-6, MCP-1, IL-8, and IL-1β by ELISA. To further assess functional outcomes, an image-based chemotaxis assay was performed to evaluate how cannabinoid pre-treatments influence HIV- and HIV-monocyte migration towards conditioned media from recombinant IL-1β-treated astrocytes. In addition, flow cytometry was used to determine how THC and CBD modulate the expression of f-actin, open conformation LFA-1, and open conformation MAC-1, key proteins involved in monocyte chemotaxis. **Results:** Production of inflammatory mediators, IL-6, MCP-1, and IL-8 in CD16⁺ monocyte-astrocyte co-cultures were suppressed by THC and CBD in a concentration-dependent manner. Furthermore, THC and CBD reduced IL-1β secretion by CD16⁺ monocytes, providing a putative explanation for the suppression of inflammatory mediator production in co-cultures. Additionally, monocytes from HIV+ subjects exhibited enhanced migration compared to monocytes from HIV- subjects, which was suppressed only by THC treatment and not by CBD. This effect was associated with reduced expression of F-actin, open conformation LFA-1, and open conformation MAC-1. **Conclusions:** THC and CBD attenuate CD16⁺ monocyte-driven astrocyte production of neuroinflammatory mediators. In addition, monocytes isolated from HIV+ subjects exhibit enhanced chemotaxis compared to those from HIV- subjects, an effect that is selectively suppressed by THC. Collectively, these findings suggest that THC and, to a lesser extent, CBD may have therapeutic potential for mitigating inflammation associated with neuroinflammatory conditions involving CD16⁺ monocytes. (Supported in part by NIH R01 DA047180 and NIH R01 DA053047).

PS 4722 Glucocorticoid drugs suppress neutrophil control of the opportunistic pathogen *Aspergillus fumigatus*

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Background and Purpose: Even in the absence of an allergic reaction, pharmaceuticals can elicit adverse immune effects in patients undergoing drug therapy. A prime example of that is glucocorticoid-mediated increase of infection susceptibility. Patients undergo prolonged glucocorticoid therapy after bone marrow or organ transplant surgery to prevent rejection. This immunosuppression increases patient susceptibility to opportunistic pathogens. In healthy individuals, innate immunity consisting primarily of macrophages and neutrophils, is sufficient to prevent opportunistic pathogens causing disease. Glucocorticoids suppress this frontline defense increasing patient susceptibility to opportunistic infections. However, the innate immune mechanisms altered by glucocorticoid exposure causing this adverse effect are not fully understood. **Methods:** To investigate this question, we used a zebrafish larva infection model to visualize how glucocorticoids alter host immune cell-pathogen interactions in a live organism. We injected spores of *Aspergillus fumigatus*, one of the most common fungal pathogens infecting glucocorticoid-treated patients, into the hindbrain ventricle of zebrafish larvae. Immediately after infection, larvae were exposed to a common glucocorticoid drug, dexamethasone. We used high-resolution live imaging to visualize the effects of glucocorticoids on macrophage and neutrophil control of *A. fumigatus* pathogenesis. **Results:** Using this zebrafish larva-*A. fumigatus* infection model, we have previously shown that dexamethasone primarily affects neutrophils by suppressing the pro-inflammatory transcription factor NF-κB. We find that neutrophils fail to control invasive growth of *A. fumigatus* extracellular hyphae when larvae are exposed to dexamethasone as compared to vehicle control. We therefore hypothesized that neutrophil extracellular killing mechanisms that target *A. fumigatus* hyphae are inhibited by dexamethasone exposure. We first tested if neutrophils release granules around *A. fumigatus* hyphae. Through live imaging in combination with Sudan Black B staining of fixed larvae, we find extracellular granular material at the site of hyphal growth. However, dexamethasone treatment of larvae does not significantly inhibit neutrophil degranulation. In

addition to granular content, neutrophils also release nuclear content to form neutrophil extracellular traps (NETs). To visualize extracellular nuclear material in a live organism, we used zebrafish larvae expressing histone H2B tagged with a fluorescent marker in neutrophils, that also express a different fluorescent marker that localizes to the neutrophil cytoplasm. Through confocal fluorescence microscopy of these larvae, we find that extracellular H2B puncta are able to trap and break *A. fumigatus* hyphae. We are currently investigating if dexamethasone treatment of larvae inhibits this process. Thirdly, we tested if neutrophil production of reactive oxygen species (ROS) promotes control of hyphae and is affected by dexamethasone treatment. To test the significance of neutrophil ROS production in *A. fumigatus* pathogenesis, we used CRISPR/Cas9 to target the p22 subunit of the ROS-producing Nox2 complex. Larvae injected with Cas9 and guide RNAs targeting the p22 gene *cyba* succumb to the infection at a similar rate to larvae injected with control gRNAs. This suggests that neutrophil ROS does not play a major protective role against *A. fumigatus* and staining for ROS production with H2DCFDA dye demonstrates that dexamethasone treatment does not affect ROS production by neutrophils in response to *A. fumigatus*. **Conclusions:** Overall, it is still unclear how dexamethasone suppresses neutrophil ability to control *A. fumigatus*. However, we have optimized the techniques to visualize that *in vivo*, neutrophils degranulate, generate extracellular nuclear material, and produce ROS against the fungal pathogen *A. fumigatus*, which can be used to test the effects of other pharmaceutical immunosuppressants and neutrophil defense against other opportunistic pathogens.

PS 4723 Development of an *in vitro* human T-cell activation assay to evaluate the potential hypersensitivity to small molecule drug candidates

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Background and Purpose: Immune-mediated drug hypersensitivity reactions (iDHRs) can be life-threatening, but their idiosyncratic nature and inadequate mechanistic understanding make them challenging to derisk during pre-clinical drug development. Significant gaps exist with respect to the immunoregulatory mechanisms and susceptibility factors that predispose specific individuals to iDHRs and uncovering this information could help prevent these adverse events. **Methods:** To better understand the role of immune cell stimulation in the development of DHRs, we established a dendritic cell/T-cell priming assay using immune cells isolated from four HLA-typed human donors. Immune cells were treated with the allergenic protein reactive metabolite of p-Phenylenediamine (Bandrowski's Base (BB)). We additionally sought to characterize T-cell subsets that may play a critical role in the development of DHRs. Autologous CD14⁺ monocyte-derived dendritic cells were cultured with naïve T-cells (CD3+CD45RA+CD45RO-CD197+) and BB (10 μ M) for 2 weeks. On day 15, cells were restimulated with BB (0-20 μ M) for 48 hours and T-cell activation was assessed by evaluating proliferation (CFSE), activation markers (CD25, CD69 and CD71) and immunoregulatory markers (FoxP3 and CTLA4) via flow cytometry. **Results:** Our results demonstrated that naïve T-cells primed with BB showed significant expansion of a drug-specific CD4⁺ T-cell population expressing CD25, CD69, CD71 and CTLA-4, alongside the secretion of IL-4. In addition, a substantial decrease in non-BB-specific CD8⁺ T-cells was observed. Primed T-cells from all donors proliferated and displayed a concentration-dependent increase in the expression of CD25, CD69, CD71 and CTLA-4. Also, the stimulation of BB-specific T-cells occurred in a time-dependent manner. The optimum T-cell proliferation and activation was observed when primed cells were restimulated for 24-48 hours. Furthermore, priming induced significant expansion of CD4⁺CD25⁺FoxP3⁺CTLA4⁺ Treg population. **Conclusions:** This study identifies potential immuno-biomarkers of drug-specific T-cell activation and provides mechanistic insights into the role of various T-cell subsets involved in DHRs. Investigations are ongoing to characterize the nature of BB antigenic determinants responsible for T-cell activation and to delineate BB-induced changes in T-cell gene expression. In addition, experiments to test an expanded list of commercial drugs implicated in clinical drug hypersensitivity have been planned. Finally, integrating the various endpoints from this study will be vital for the development of *in vitro* and *in silico* human predictive models important for drug hypersensitivity risk assessment during preclinical drug development.

PS 4724 Investigating Potential Immunotoxicity of Understudied PFAS Found In North Carolina Waters (perfluoro-3,5,7,9-butaaxadecanoic acid; perfluoro-3,6,-dioxo-4-methyl-7-octensulfonic acid; 2-[difluoro(trifluoromethoxy)methoxy]-2,2-difluoroacetic acid)

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are a large class of nearly 15,000 synthetic compounds with myriad applications across industry and consumer goods, of which many members are understudied. Perfluorooctanoic acid and perfluorooctane sulfonic acid, two well studied members of the PFAS class, are classified as presumed immune hazards to humans. This raises the

question of the ability of other PFAS to induce immunotoxicity, especially those that are under-examined and thus toxicologically data poor. PFAS are widespread and many of the understudied PFAS are in water sources (drinking, surface, and ground waters) across the country. North Carolina, in particular, has extensive PFAS water contamination. Three compounds found in North Carolina waters are perfluoro-3,5,7,9-butaaxadecanoic acid (PFO4DANA; CAS 1035377-21-9), perfluoro-3,6,-dioxo-4-methyl-7-octensulfonic acid (Nafion byproduct 1; CAS 29311-67-9), and 2-[difluoro(trifluoromethoxy)methoxy]-2,2-difluoroacetic acid (PFO2HxA; CAS 39492-88-1). These three compounds, despite being found in North Carolina waters, have only been studied minimally for their toxicity. **Methods:** Based on previous work from our laboratory, which indicated exposure to individual PFAS can induce effects on the immune system, three immunotoxicity studies were conducted, one for each compound. In each study, adult male and female C57BL/6 mice (6-8 weeks old) were exposed by gavage once/day for 30 days with their respective compound at 0, 0.05, 0.5, or 5 mg/kg. Five days before study terminus, mice were injected with sheep red blood cells (SRBC) to induce T-cell dependent antibody responses (TDAR). Spleens were collected one day after exposure ended and immunophenotyping and evaluation of various markers for B cell maturation and differentiation were conducted through flow cytometry. **Results:** Analysis of B cells for PFO4DANA demonstrated statistical differences in plasmablast and marginal B cell numbers in females between controls (0 mg/kg PFO4DANA) and the 0.5 mg/kg PFO4DANA dose group, and between controls and the 5 mg/kg PFO4DANA dose group. There were also statistical differences in plasmablast, follicular, and memory B cell numbers by sex. The TDAR for PFO4DANA did not vary statistically by dose, however there were statistical differences by sex. Analysis of B cells for Nafion byproduct 1 (NBP1) revealed statistical differences in naïve, follicular, and memory B cell numbers in males between controls (0 mg/kg NBP1) and the 0.05 mg/kg NBP1 dose group. The TDAR did not vary statistically by dose, however there were statistical differences by sex. Analysis of B cells for PFO2HxA demonstrated statistical differences in marginal B cell numbers in females between controls (0 mg/kg PFO2HxA) and all other dose groups (0.05, 0.5, and 5 mg/kg PFO2HxA). There were also statistical differences in memory B cell numbers in females between controls and all other dose groups. The TDAR for PFO2HxA varied statistically by dose, between controls (0 mg/kg PFO2HxA) and the 0.05 mg/kg PFO2HxA dose group in females, and there were also statistical differences by sex. **Conclusions:** These PFAS, chosen for their lack of toxicological data and relevance to the people of North Carolina, showcased shifts in important subpopulations of B cells. Despite that these shifts did not always lead to changes in the TDAR by dose, it is still important to understand potential implications of altered B cell subsets. These three compounds are merely a snapshot of novel PFAS found in North Carolina waters and other water sources, globally. Further investigation is needed to understand potential immunological health risks, to investigate other understudied PFAS, and to confirm these findings.

PS 4725 Effects of indium tin oxide nanoparticles on human T helper cells: mitochondrial dysfunction and suppression of cytokine gene expressions

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Background and Purpose: Indium is a rare metal with high electrical conductivity, which has led to its widespread industrial use. Especially, indium tin oxide (ITO) is used in flat panel displays and solar power generation systems, and its global market is expected to continue expanding, which is making its toxicity of concern. Inhalation exposure to ITO causes pulmonary fibrosis similar to other mineral particles, but is accompanied by characteristic pathologies such as emphysematous lesions and pulmonary alveolar proteinosis (PAP). ITO accumulates in draining lymph nodes as well as in the lungs, but the effects of ITO exposure on lymphocyte function have not received much attention ever. In particular, the effect on CD4⁺ T helper cell function may be involved in ITO toxicity through the homeostatic machinery of lung function with alveolar macrophages. The human CD4⁺ T cell line MT-2 is a useful tool to build an *in vitro* model for immunotoxicological study, by which we previously unveiled much knowledge. Therefore, we performed experiments with MT-2 cells to examine the uptake of ITO nanoparticles into intracellular vesicles and their effects on mitochondrial function and cytokine production. **Methods:** The two types of ITO nanoparticles (Sigma ITO-S, specific surface area A = 33.86 m²g⁻¹ or Thermo ITO-T, A = 2.96 m²g⁻¹) were used. MT-2 cells were cultured for two or three days under exposure to ITO-S, ITO-T or titanium dioxide (TiO₂, A = 47.56 m²g⁻¹). Cell viability after culture was quantified colorimetrically using WST-1 reagent. Total RNA was extracted from the cells recovered after culture, and relative mRNA levels of the genes were quantified by RT-qPCR using the SYBR Green method. After culture, cells were stained with the fluorescent reagent JC-1 to examine changes in mitochondrial membrane potential ($\Delta\psi$ m). Furthermore, SEM-EDX and TEM were used to examine the uptake of ITO particles and abnormalities in mitochondrial morphology. In addition, PMA-induced THP-1 macrophages were cultured with several cytokines produced by Th cells and then analyzed for expression of lipid metabolism genes. **Results:** In MT-2 cells cultured for three days, ITO-S exposure clearly reduced cell viability, with significantly lower levels at concentrations of over 50 and 100 μ g/ml compared to ITO-T and TiO₂ respectively, and then the dose of 100 μ g/ml were used for the subsequent experiments. While no significant differences were observed in mRNA levels of the ER stress response or the autophagy-related genes, ITO-S exposure significantly

increased mRNA levels of several proteostasis genes as well as p-eIF2 α cells an indicator of translational arrest. SEM-EDX confirmed ITO uptake into intracellular vesicles. TEM images revealed abnormal mitochondrial morphology in ITO-S and TiO₂-exposed groups, with numerous cristae with aberrantly enlarged intermembrane spaces, that's more marked in the ITO-S group. Furthermore, when $\Delta\psi_m$ were examined using JC-1 staining, low $\Delta\psi_m$ cells (%) significantly increased only in the ITO-S exposure group, meaning mitochondrial damage. These findings suggested possible dysfunction in energy-driven cytokine production. When the cells isolated from culture with ITO-S were stimulated with PMA and ionomycin, they showed significant decreases in IL-4, IL-13, IFN- γ and TNF- α mRNAs, where that in IL-4 was remarkable, while no changes in IL-10 and TGF- β 1. Moreover, the addition with IL-4 induced increases in PPARY, FABP4 and LIPA mRNAs in the culture of THP-1 macrophages, indicating increase in lipid metabolism. **Conclusions:** These results indicate that 1) ITO nanoparticles are internalized in vesicles by human T helper cells, 2) they interfere with intracellular proteostasis and cause mitochondrial dysfunction and 3) ITO-exposed Th cells show dysfunction in stimulation-induced expression of cytokine genes, in particular IL-4, which functions in lipid metabolism of macrophages. The mitochondrial dysfunction might contribute to impairment in expression of cytokine genes after activation. In addition, it is known that impaired lipid metabolism of alveolar macrophages causes PAP. These findings suggest immunotoxic effects of ITO exposure on Th cell function via mitochondrial dysfunction, which might be a link between ITO exposure and the related disease.

PS 4726 Preclinical De-Risking of Cell Therapy Products

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Background and Purpose: Engineered T cell therapies such as Chimeric Antigen Receptor (CAR) T cells have emerged as promising therapies but also suffer from severe safety risks. One major concern is that (low) target antigen expression in healthy cells or non-specific antigen recognition could potentially result in off-disease toxicities. Another safety concern is that the random integration of the CAR-encoded DNA cassettes in the host genome can potentially cause insertional mutagenesis and may contribute to oncogenic transformation of the T-cells. The lack of pharmacologically relevant animal models emphasizes the need to evaluate the safety of CAR-T cell therapy products *in vitro*. **Methods:** The human epidermal growth factor receptor 2 (HER2) is a well-established therapeutic target for various cancer types. To explore potential off-disease effects of HER2-targeting CAR-T cells, the Retrogenix™ cell microarray screening technology was used to assess off target binding against a panel of >6500 proteins. In addition, HER2 CAR-T cells were co-cultured with primary or iPSC-derived cells to evaluate any unwanted cytotoxicity against healthy cells. To de-risk the CAR-T cells for potential insertional mutagenesis after stably introducing the CAR cassette, HER2 CAR-T cells were assessed for their potential uncontrolled and cytokine-independent growth, and the CAR vector copy number (VCN) was determined by droplet digital PCR (ddPCR). Finally, lipid nanoparticles (LNP) were explored as an alternative and non-integrating delivery method for the generation of mRNA-encoding CAR-T cells. **Results:** Although the Retrogenix™ screen only identified on-target and CAR-independent interactions and no off-target binding, HER2 CAR-T cells showed activation and cytotoxicity towards several primary cell types, most likely due to on-target off-disease effects. A clear decrease in viability and cell numbers was observed when HER2 CAR-T cells were cultured in the absence of cytokines and these results are further supported by the relatively low VCN of 3 (a VCN of less than 5 is recommended to minimize the risk of oncogenesis), indicating low risk of CAR-T cell transformation under the conditions tested. Finally, LNP mRNA CAR-T cells showed HER2 CAR expression in the majority of T cells and cytotoxicity towards a HER2-expressing tumor cell line. **Conclusions:** In conclusion, HER2 CAR-T cells were evaluated using several *in vitro* safety assays. Although no off-targets were identified and no cytokine-independent growth was observed, the HER2 CAR-T cells showed cytotoxicity towards several tested primary cell types, highlighting the importance of evaluating the safety of CAR T-cell therapies and de-risking them early in their development. Furthermore, functional efficacy studies show a huge potential for LNP-mRNA as a non-viral and non-integrating delivery method for CAR-T cell generation, shaping the future of the cell therapy landscape.

PS 4727 Characterizing Effects of Non-Toxic Aryl Hydrocarbon Receptor (AHR) Ligands on IgG1 antibody production in Human Peripheral Blood Mononuclear Cells

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Background and Purpose: Multiple sclerosis (MS) is an autoimmune disorder characterized by the demyelination of neurons in the central nervous system, which is due in part to pathogenic antibodies such as IgGs. Previous epidemiological studies have reported decreased serum IgG1 levels in individuals exposed to the aryl hydrocarbon receptor (AHR) ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Consistent

with this, our lab found that TCDD suppressed IgG1 production in the experimental autoimmune encephalomyelitis (EAE) mouse model of MS. To determine the potential for non-toxic AHR ligands to suppress antibody production in humans and eventually MS, our objective was to examine effects on IgG1 production in human peripheral blood mononuclear cells (PBMCs). **Methods:** PBMCs were isolated from leukopaks obtained from 24 healthy donors using Histopaque 1077 density gradient centrifugation. Cells were pretreated overnight with vehicle (0.01% DMSO) or AHR ligands; TCDD (30 nM), ITE (1 μ M), FICZ (0.1 μ M), or I3C (2 μ M). After overnight treatment, cells were then stimulated with pokeweed mitogen (PWM, 5 μ g/mL) to induce IgG1 production. Cells were incubated for 2 days or 5 days following PWM treatment. Cells after 2-day stimulation were used for *IL6* and *IL1B* gene analysis via RT-PCR and RNA sequencing. Supernatants after 5-day stimulation were analyzed for IgG1 antibody production. **Results:** Our PCR results revealed that PWM significantly upregulated the expression of proinflammatory cytokine genes *IL6* and *IL1B* at day 3. Our ELISA results demonstrated that PWM induced the human PBMC to produce IgG1 after 6 days. IgG1 antibody production was modestly suppressed by pretreatment with AHR ligands, with I3C showing the most consistent inhibitory effect across most of the donors. Transcriptomic analysis revealed a greater number of downregulated genes relative to upregulated ones following I3C exposure. KEGG pathway analysis identified several signaling pathways including oxidative phosphorylation, T cell receptor signaling, Th17 cell differentiation, PI3K-Akt signaling, MAPK signaling, and NF-kappa B signaling, that were suppressed with I3C treatment in PWM-stimulated PBMC, many of which also contribute to the pathogenesis of MS. **Conclusions:** These findings demonstrate that AHR activation, particularly by I3C, suppressed human IgG1 antibody production and downregulated multiple proinflammatory signaling pathways in PBMCs. This study will help us address the gaps in our understanding of the mechanistic pathways involved in immune suppression by AHR ligands in human cells and their possible use for MS treatment.

PS 4728 Investigating how per- and polyfluoroalkyl substances (PFAS) affect neutrophil function

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Background and Purpose: The term per- and polyfluoroalkyl substances (PFAS) denotes a collection of highly stable, synthetic chemicals used in consumer products such as nonstick cookware, food packaging, stain repellent materials, and fire fighting foams. Their resistance to most forms of degradation has allowed them to become nearly omnipresent, posing a threat to environmental and human health. Concerningly, exposure to select PFAS has been shown to impair the immune system, cause developmental delays, increase the risk of cancer, dysregulate hormonal balance, and induce disorders of the liver, kidney, and thyroid in humans. Despite these observations, our knowledge of the health risks posed by PFAS remains lacking due to a limited understanding of how different PFAS alter the cellular functions that culminate in the aforementioned outcomes, as well as the sheer number of PFAS that still require testing. Previous work has shown that certain PFAS suppress the ability of neutrophils to release antimicrobial stores of reactive oxygen species in a process known as the respiratory burst. This work aims to better isolate this interaction and understand how these chemicals may be interfering with neutrophil biology. **Methods:** Neutrophil-like HL-60 cells were stimulated with phorbol myristate acetate (PMA) and a measure of reactive oxygen species (ROS) with dihydrorhodamine (DHR) is used as a proxy of respiratory burst output. To limit excessive sequestration of PFAS by serum albumin, cells underwent a serum-free pulse (SFP) for the first hour in which cells were exposed to PFAS. Cells were exposed to perfluorohexanoic acid (PFHxA) or hexafluoropropylene oxide dimer acid (HFPO-DA or GenX). **Results:** Initial experiments demonstrate a significant reduction in ROS production in the SFP group compared to the control. Importantly, these findings do not correlate with any significant changes in cell viability. **Conclusions:** These results will inform future experimental methodologies designed to uncover the molecular mechanism by which PFAS suppress neutrophil function, and ultimately bridge existing knowledge gaps concerning PFAS exposure and immune function.

PS 4729 Development of rat immunogenicity assays to evaluate the immunostimulatory potential of peptides prior to nonclinical safety studies

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Background and Purpose: Biologics and peptide therapeutics offer enhanced target selectivity over other modalities (ie, small molecules); however, immunogenicity of these therapeutics can present an obstacle to their development. Human-based therapeutics are often highly immunogenic in nonclinical animals, and can have a significant impact on pharmacodynamics, pharmacokinetics, toxicity profiles, and ultimately study interpretation. Screening assays to predict immunogenicity of human-based therapeutics in rats are currently unavailable, and thus impacts to program development may not be known until completion of repeat-dose toxicity studies. To improve screening for immunogenicity risk in rats, we aimed to develop an *in vitro* assay analogous to those established for predicting human immunogenicity. **Methods:** Splenocytes from naive rats were harvested and cultured with test

articles for 7 days, and immune stimulation was evaluated via interferon- γ (IFN γ) fluorospot. **Results:** Our results demonstrate that the assay was sensitive to pan-T cell activating agents Concanavalin A (ConA) and Phorbol 12-Myristate 13-Acetate (PMA) and showed positive correlation to *in vivo* observations of immunogenicity when tested with keyhole limpet hemocyanin (KLH) or 3 internal Merck compounds. **Conclusions:** The assay demonstrated limited sensitivity, reducing its ability to reliably predict *in vivo* immunogenicity outcomes. Further work is ongoing to enhance the assay sensitivity and explore additional endpoints including T cell proliferation, immunophenotyping, serum biomarkers, and transcriptional signatures of immune stimulation.

PS 4730 Antimicrobial cetylpyridinium chloride suppresses mast cell degranulation and cytokine production by targeting tyrosine phosphorylation of Syk kinase and key substrates Linker for Activation of T-Cells (LAT) and Vav1

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Background and Purpose: Cetylpyridinium chloride (CPC) is a quaternary ammonium antimicrobial used in numerous personal care products, human food, cosmetic products, and cleaning solutions. Mast cells (MC) are ubiquitous in the human body and play many roles in the immune and nervous systems. Previously we showed that exposure-relevant, non-cytotoxic doses of CPC inhibit key MC functions, both degranulation (in both rat and human MCs), by inhibiting antigen (Ag)-stimulated Ca²⁺ mobilization and thus microtubule polymerization. With the goal of determining the underlying biochemical mechanisms, we hypothesized that CPC shuts down Ca²⁺ signaling by affecting specific phospho-tyrosines on key early signaling kinases via electrostatic disruption. We also predict that CPC's shut down of the activation of the critical transcription factor Nuclear Factor of Activated T-cells (NFAT) and specific players within the MAPK pathway leads to loss of TNF- α production. **Methods:** Using biochemical techniques such as western blotting and RT-qPCR, as well as confocal microscopy, we demonstrate how CPC affects these specific proteins. **Results:** Here we show that CPC inhibits tyrosine phosphorylation of the specific key players Syk kinase and its substrates Vav1 and LAT, though not all tyrosine-phosphorylated proteins, such as Gab2, are affected by CPC. We also show that CPC inhibits the MC function of cytokine production, specifically the key inflammatory cytokine TNF- α . **Conclusions:** Our findings reveal the mechanisms of action of this ubiquitous chemical, with relevance to human health, and predictions of CPC effects on related cell types like T-cells which share signaling machinery with MC. Work is underway to examine whether CPC directly targets the key activation residues, Y519/520, within Syk kinase. We also are currently investigating the path by which CPC's inhibition of LAT and Vav1 links to its dampening of TNF- α expression, with a focus on NFAT translocation to the nucleus and the MAPK pathway.

PS 4731 Effects of arsenic, cadmium, and lead on macrophage cytokine production

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Background and Purpose: Arsenic, cadmium, and lead are common exposures with immunotoxic effects. Cytokine signaling plays a key role in the tumor microenvironment where it promotes cancer cell death or alternatively, progression and metastasis of the tumor. For example, IL-1 β is a pro-inflammatory cytokine that can regulate both pro- and anti-tumor effects. IL-4 promotes breast cancer cell proliferation. IL-8 attracts other immune cells to sites of tissue damage and promotes angiogenesis. CCL-2 and CXCL-1 both promote breast cancer metastasis, while CXCL-2 also plays a role in the epithelial to mesenchymal transition. MMP-1 is elevated in multiple tumors, is correlated with metastasis, and is necessary for macrophage infiltration. All three heavy metals/metalloids are known to dysregulate cytokine production, but their effects at low doses relevant to human population exposures have not been systematically characterized in human macrophages. **Methods:** THP-1 monocytes were differentiated into macrophages using 100nM phorbol 12-myristate 13-acetate (PMA) for 24 hours then exposed to sodium arsenite (0.12 μ M and 5 μ M), cadmium chloride (35nM and 3.5 μ M), and lead acetate trihydrate (0.17 μ M and 5 μ M) or control for 48 hours in three technical replicates. All doses were confirmed by Hoechst and DEAD green staining to have no impact on cellular viability. To assess migration, macrophages were scratched with a pipette tip, dosed with heavy metals, and imaged for 48 hours. Scratch closure was analyzed using ImageJ and R, comparing percent change in wound area from time zero and 48 hours. Conditioned media was collected and the cytokines IL-1 β , IL-4, IL-8, IL-23, TGF- β , TNF- α , CCL-2, CXCL-1, and MMP-1 were measured by enzyme-linked immunosorbent assay. Cytokine levels were compared by analysis of variance and post-hoc Tukey testing. **Results:** Exposure to the three heavy metals did not impact rates of THP-1 derived macrophage scratch closure. Heavy metal exposure resulted in altered levels in seven out of nine measured cytokines. CCL-2 levels decreased following exposure to 5 μ M arsenic, from 115.0 pg/mL in the control to 64.3 pg/mL ($p=0.002$), but cadmium significantly increased levels from 113.3 pg/mL in the control to 206.7 pg/mL in 3.5 μ M ($p=0.0001$). Levels of IL-8 and CXCL-1 displayed dose-dependent increases in

response to each of the three chemicals except between control and 35nM cadmium ($p<0.05$). Levels of IL-4 increased from 0.3 pg/mL in control and 11.3 pg/mL in 5 μ M lead to 53.0 pg/mL in 0.17 μ M lead ($p<0.001$), while arsenic and cadmium had no effect. Levels of MMP-1 were decreased in the 5 μ M groups compared to control and low doses for arsenic and lead ($p<0.05$). Low dose arsenic and lead had increased TGF- β levels compared to control and high doses ($p<0.05$). Lead increased TNF- α from 2.3 pg/mL in control to 25.3 pg/mL in 0.17 μ M lead ($p=0.02$). **Conclusions:** Arsenic, cadmium, and lead have effects on macrophage cytokine production at physiologically relevant doses. The effects of arsenic show a pattern of decreased CCL-2, increased CXCL-1, decreased MMP-1, and increased IL-8. Increased levels of CCL-2 and CXCL-1 are associated with breast cancer progression and metastasis. MMP-1 helps macrophages migrate through tissue, so lower levels may impair macrophage infiltration into tumors while IL-8 increases angiogenesis, overall leading to worse prognosis. Cadmium exposure resulted in increased CCL-2, CXCL-1, and IL-8 which could promote breast cancer metastasis and angiogenesis. Lead exposure increased CXCL-1, IL-8, and TNF- α while decreasing MMP-1. These results suggest that heavy metal exposure dysregulates cytokine production by macrophages. Further experiments are in progress to assess effects of heavy metals on RNA expression in the macrophages as well as measuring the impact of macrophage-conditioned media on triple negative breast cancer cell migration.

PS 4732 Evaluating the immunotoxicity of a bispecific antibody (glofitamab) using an ex vivo circulating human whole blood assay

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Background and Purpose: Since the advent of targeted antibody therapy, there has been interest in developing bispecific antibodies (BsAbs), but the necessary technology was not available until more recently. Only in the last few years have several BsAbs been approved for clinical use. However, many of the BsAbs are associated with a high risk of cytokine release syndrome and other adverse events. Since many bispecific antibodies target antigens unique to humans and given the need for new approach methodologies (NAMs) that comply with the 3Rs, it would be valuable to have an *in vitro* system for predicting immunotoxicity during preclinical development of BsAbs. **Methods:** ID.Flow is an *ex vivo* system using circulating human whole blood at 37 °C previously evaluated for predicting infusion reactions of monoclonal antibodies and oligonucleotide drugs. The system allows for simultaneous evaluation of multiple immune responses, such as cytokine release, complement activation, activation or depletion of platelets and white blood cells, and coagulation. By using clinically relevant controls, the relative levels of immunotoxicity can be assessed. ID.Flow was used here to evaluate glofitamab, a bispecific T cell engager approved for the treatment of diffuse large B cell lymphoma. Glofitamab is associated with cytokine release syndrome (CRS) including serious or fatal reactions. **Results:** The efficacy and immunotoxicity of glofitamab were investigated in ID.Flow at concentrations similar to the reported C_{max}. As controls, the monoclonal antibodies obinutuzumab and alemtuzumab were used, both of which are associated with manageable CRS in the clinic. Glofitamab resulted in B cell depletion with live B cell counts significantly reduced by 6 hours (60%) and most B cells (90%) depleted by 24 hours. In comparison, B cell depletion using obinutuzumab or alemtuzumab occurred with slightly faster kinetics (80-90% depletion by 6 hours). Correlating with clinical observations, inflammatory cytokines assessed (IFN- γ , IL-2, IL-6, IL-8 and TNF- α) were present at very high levels in circulating whole blood treated with glofitamab for 6 hours. IL-6 and IL-8 were induced to similar levels as those seen using alemtuzumab at C_{max}, and for IFN- γ and TNF- α , the levels were even higher than those seen with alemtuzumab. IL-2 was, as expected, not produced in response to either obinutuzumab or alemtuzumab, but was induced to high levels (similar to an agonistic anti-CD28 antibody) in the presence of glofitamab. Neither obinutuzumab nor glofitamab had any effect on complement activation in ID.Flow, whereas alemtuzumab induced increased levels of C3a and C5a. Interestingly, using flow cytometry, a population of double-positive CD3⁺ CD22⁺ events was observed in samples treated with glofitamab correlating with the mechanism of action of the drug. **Conclusions:** In conclusion, ID.Flow can be used to assess the immunotoxicity and potentially the efficacy of bispecific antibodies with glofitamab as a relevant clinical control.

PS 4733 Detection of circulating immune complexes as a biomarker of immunogenicity

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Background and Purpose: Development of anti-drug antibodies (ADAs) can limit drug efficacy, reduce exposure, and lead to downstream adverse events. ADA binding to drug can lead to the formation of circulating immune complexes (CICs), which can accumulate in blood and slow drug clearance, activate endothelial cells, platelets, and neutrophils, initiate a complement cascade through binding to C1q, or deposit in perivascular tissues of various organs (e.g., kidney, blood vessels, liver, etc) resulting in inflammatory cellular infiltrates. While ADA measurement is a common endpoint in

nonclinical toxicology and clinical studies, measurement of CICs are not commonly examined. **Methods:** Here, we sought to establish methods for detection of CICs in serum or plasma from human, primate, and rat. We established a method for generation of positive control CICs *in vitro* via heat inactivation of serum and show that CICs from these controls, or sera from patients with systemic lupus erythematosus, can be robustly detected using a C1q capture ELISA. **Results:** Moreover, we show that the human C1q ELISA has the capability to bind rhesus CICs, but not rat CICs, with little loss in sensitivity. CIC formation and CIC-C1q assay activity were unaffected by using human or NHP serum or EDTA plasma. Assay performance was also comparable following a single freeze/thaw cycle, suggesting the assay can be used with archived samples. **Conclusions:** We conclude that these assays may be informative as a diagnostic tool of ADA formation and trigger for immunohistochemistry assessments evaluating immune complex deposition, providing additional weight of evidence to understand a drug's immunogenicity risk.

PS 4734 Differential activation of canonical and non-canonical IL-1 β maturation pathways in monocyte subsets in the context of HIV

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Background and Purpose: Despite the success of combination antiretroviral therapy (cART), chronic inflammation persists in people living with HIV (PLWH), contributing to comorbidities such as HIV-associated neurocognitive disorder (HAND). Microbial translocation from the gastrointestinal tract, a major HIV reservoir, exposes peripheral monocytes to toll-like receptor 4 (TLR4) agonists like lipopolysaccharide (LPS), driving the release of proinflammatory cytokines such as interleukin-1 β (IL-1 β). IL-1 β maturation typically depends on inflammasome-mediated caspase-1 activation, which requires the apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). Previous research has shown that ASC-dependent inflammasome formation is significantly reduced in HIV+ monocytes in response to LPS stimulation, despite a 2-fold increase in IL-1 β secretion, suggesting ASC-independent mechanisms of IL-1 β maturation. Alternative ASC-independent mechanisms involving caspases-4/5, caspase-8, and NLR family CARD-containing (NLRC) sensors can also cleave pro-IL-1 β . The contribution of these non-canonical pathways to persistent inflammation in HIV infection remains poorly understood. While pan monocytes were utilized to evaluate ASC-dependent inflammasome formation in the HIV+ population, CD16+ monocytes are considered the more inflammatory phenotype compared to CD16- monocytes and have been shown to be upregulated in HIV+ individuals. The purpose of this study was to define the relative contributions of canonical versus non-canonical IL-1 β activation pathways in monocytes from HIV+ donors and to assess how these mechanisms differ between monocyte subsets. **Methods:** Peripheral blood monocytes from HIV-negative (HIV-) and HIV-positive (HIV+) donors were isolated and stimulated with LPS (TLR4 agonist). IL-1 β secretion was measured by ELISA. Inflammasome activation was evaluated via flow cytometry tracking ASC speck formation. Expression of inflammasome-related genes (NLRC family members) was quantified by qPCR. Pharmacologic inhibitors targeting caspase-1, caspases-4/5, and caspase-8 were used to dissect pathway-specific IL-1 β responses in pan, CD16+, and CD16- monocyte subsets. **Results:** IL-1 β secretion in both HIV- and HIV+ monocytes was comparably suppressed by caspase-1 and caspase-4/5 inhibitors, supporting involvement of both canonical and non-canonical caspases in IL-1 β activation. Among ASC-independent NLRs, *NOD1* mRNA was significantly upregulated in HIV+ monocytes compared to HIV- monocytes, identifying it as a potential alternative sensor protein driving IL-1 β maturation. Both caspase-1 and caspase-4/5 inhibitors suppressed IL-1 β secretion in CD16+ and CD16- monocytes. However, caspase-8 inhibition selectively reduced IL-1 β in CD16- monocytes, indicating subset-specific utilization of caspase-8-dependent signaling. **Conclusions:** These data reveal that monocytes from HIV+ donors rely on both canonical ASC-dependent IL-1 β maturation as well as non-canonical ASC-independent IL-1 β activation pathways. Upregulation of *NOD1* in HIV+ monocytes and differential caspase usage between CD16+ and CD16- monocytes highlight the complexity of inflammasome-independent cytokine processing during HIV infection. Understanding these alternative pathways could identify new targets to mitigate chronic inflammation in PLWH. (Supported by NIH R01 DA053047 and T32 ES007255).

PS 4735 The effects of BPA on murine IL-17A regulation in naïve CD4⁺ T cells *in vitro*.

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Background and Purpose: In a previous study it was reported that prenatal exposure through treatment of dams to BPA increased the frequency of splenic Th17 cells in murine offspring. Based on these findings, the objective of this study was to investigate whether direct addition of BPA to cultured naïve CD4⁺ T cells from mouse spleens promoted IL-17A secretion. **Methods:** Naïve CD4⁺ T cells were puri-

fied from single cell suspensions of splenocytes isolated from female C57BL/6 mice by magnetic assisted cell sorting. Naïve CD4⁺ T cells were then activated with plate bound anti-CD3 and soluble anti-CD28 antibodies under both normal and Th17 polarizing culture conditions for 5 days to establish the kinetics of IL-17A secretion. In addition, IL-22 secretion and intracellular ROR γ t, a transcription factor associated with IL-17 production were also measured. At the time of activation, cells were treated with either 10nM TCDD, increasing concentrations of FICZ, and 10 μ M AHR antagonist, CH223191, as positive and negative controls of IL-17 induction, respectively. For studies examining BPA (0.05, 5, 500, 5000, and 50000nM), naïve CD4⁺ T cells were isolated and activated as above in normal and polarizing conditions with 50nM FICZ and 10 μ M CH223191 as positive and negative controls, respectively, for 4 days. On days 3 and 4 post activation, IL-17A and IL-22 secretion were quantified by ELISA and the frequencies of IL-17⁺, IL-22⁺, and ROR γ t⁺ cells was quantified by flow cytometry. **Results:** The initial studies to establish the kinetics of IL-17A secretion showed that IL-17 concentrations peaked by day 4 post activation in both Th17 polarizing and non-polarizing conditions. Moreover, on day 4, AHR activation by either TCDD or FICZ promoted significant IL-17 and IL-22 secretion compared to vehicle treated controls under non-Th17 polarizing conditions. FICZ also resulted in increased frequency of IL-17⁺ cells, while TCDD-mediated effects were not statistically significant, suggesting that FICZ is an appropriate positive control for IL-17A induction. Treatment of CD4⁺ T cells with BPA did not result in any significant increase in cells positive for IL-17A or increased secretion of IL-17, while 50nM FICZ induced significant increases in IL-17A under polarizing conditions and IL-22 under both polarizing and non-polarizing culture conditions. It is noteworthy that the highest concentration of BPA (50000nM) utilized resulted in altered cellular morphology and decreased cytokine secretion, characteristics of reduced T cell activation. None of the aforementioned changes in IL-17A⁺ cells or IL-17A secretion corresponded to changes in ROR γ t positivity in any treatment group. **Conclusions:** These studies suggest that treatment of mature, naïve CD4⁺ T cells with BPA does not induce IL-17A or IL-22 secretion while AHR ligand FICZ promotes IL-17A and IL-22. Interestingly, FICZ-mediated induction of IL-17A and IL-22 was independent of changes in the percentage of cells expressing ROR γ t. In contrast to exposure to BPA *in utero*, direct addition of BPA to mature CD4⁺ T cells had no effect on IL-17A regulation. These results suggest a need for additional investigations of the effects of BPA on IL-17A.

PS 4736 Developmental Immunotoxicity of Five Understudied Per and Polyfluoroalkyl Substances (PFAS) that Contaminate Drinking Water

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Background and Purpose: Per- and polyfluoroalkyl substances (PFAS) are a large class of environmentally persistent contaminants, characterized by long half-lives in organisms and the potential for bioaccumulation due to their resistance to degradation and metabolism. The PFAS studied are detectable in drinking water across the United States. PFAS exposure has been linked to numerous negative health outcomes, including cancers, thyroid disease, immunotoxicity, and developmental toxicity. Despite the commonality of these chemicals, their effects on the developing immune system are not well understood, which is a critical point for public health. **Methods:** Five PFAS were evaluated for this study; Perfluoro-2-methoxyacetic acid (PFMOAA), Nafion Byproduct 2 (NBP2), Perfluoropentane sulfonic acid (PFPeS), Sodium-Perfluoro-3,5,7,9,11-Pentafluorododecanoic Acid (NA-PFO5DoA), and Hexafluoropropylene Oxide Dimer Acid (HFPO-DA or GenX). C57BL/6 female mice were impregnated by C3H male mice. Dams received daily gavage doses of vehicle control, or one of three concentrations from gestational day (GD) 1 through GD17. Administered doses were based on the ability of legacy PFAS, such as perfluorooctanoic acid, to induce developmental toxicity at 5 mg/kg during this period of exposure. On postnatal day 1 (PND1, day of birth), litters were culled to six offspring and were kept with dams until weaning at PND21. Two subsets of pups had organs and sera collected on PND21 to assess body burden and on PND28 to assess basic immunophenotype. Offspring were not directly dosed to focus on effects of gestational exposure. At PND51, offspring were immunized with sheep red blood cells (SRBCs) to elicit T cell-dependent antibody responses (TDAR). At peak IgM antibody production five days post immunization, offspring were humanely euthanized. Blood, spleen, thymus, kidney, brain, heart, and liver were collected for immunophenotyping, immunohistochemistry, and further analyses. Anti-SRBC IgM antibodies were measured by enzyme-linked immunosorbent assay (ELISA), and liver peroxisome proliferation was measured via Acyl-CoA oxidase activity, the first step in peroxisomal β -oxidation. **Results:** Gestational exposure to NA-PFO5DoA and GenX resulted in statistically elevated liver somatic indices in the higher dose groups. NA-PFO5DoA exposed groups also had statistically decreased spleen weights at PND28 and PND56. At PND 28 PFMOAA exposed groups had statistically increased thymus cellularity in the highest dose group, and statistically decreased spleen cellularity. No statistical alterations in the TDAR were observed in this model for any of the five chemicals. Future analysis will include assessment of peroxisome proliferation across these five PFAS. **Conclusions:** Increased liver somatic indices indicate that liver hypertrophy, a common effect of legacy and novel PFAS, can occur and persist following gestational exposure to NA-PFO5DoA and GenX. Decreased spleen weights from NA-PFO5DoA exposure possibly indicate this as well. The absence of overt effects on the TDAR suggests that mechanisms for developmental suppression of the humoral immune response are not observable in this model. Thus, while the immune system does

not appear to be the most sensitive developmental endpoint in this mouse model, persistent effects of developmental exposure were still evident for some of these understudied PFAS with human exposure potential.

PS 4737 Immune Cell Clearance and Acetaminophen Induced Acute Liver Failure

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Background and Purpose: Acetaminophen (APAP) overdose remains the leading cause of acute liver failure (ALF) in the United States and a major clinical concern worldwide. The pathophysiology of APAP-induced liver injury involves hepatocellular necrosis initiated by the formation of the reactive metabolite N-acetyl-p-benzoquinone imine, which depletes glutathione and causes oxidative stress and mitochondrial dysfunction. While numerous studies have investigated the mechanisms of hepatocyte injury and regeneration, the contribution of immune cell clearance to hepatic recovery is less understood. Following the resolution of necrosis, infiltrating immune cells such as macrophages and neutrophils play essential roles in debris removal, cytokine modulation, and restoration of homeostasis. However, their prolonged retention can perpetuate inflammation and delay tissue repair. Previous work from our laboratory demonstrated that apoptotic signaling in macrophages is critical for their clearance by newly regenerated hepatocytes, facilitating proper termination of inflammation. In contrast, mechanisms governing neutrophil removal from injured liver tissue remain unclear. Given that neutrophils exit through lymphatics in other organs, hepatic lymphatics may similarly support their egress and inflammation resolution after APAP overdose. Understanding this process could identify novel therapeutic targets for enhancing liver repair and preventing progression to ALF. **Methods:** Male C57BL/6J mice (8-10 weeks old) were fasted overnight and administered acetaminophen (300 mg/kg, intraperitoneally) to induce moderate hepatotoxicity. To investigate the role of hepatic lymphangiogenesis in immune cell clearance, a subset of mice received SAR131675 (SAR), a potent and selective inhibitor of VEGFR3-mediated lymphangiogenesis, administered 6 hours post-APAP to target the regenerative phase without affecting the initial injury. Liver injury and recovery were assessed between 12-48 hours post-dose. Serum alanine aminotransferase (ALT) activity was quantified as a marker of hepatocellular damage. Liver sections were examined by hematoxylin and eosin staining for necrosis and regeneration, while immunohistochemistry and immunofluorescence were used to evaluate lymphatic (LYVE-1), neutrophil (Ly6G), and proliferation (PCNA) markers. Immune cell infiltration and hepatocyte proliferation were quantified in QuPath and compared between groups. **Results:** Moderate APAP overdose caused extensive centrilobular necrosis and marked elevation in serum ALT, consistent with acute liver injury at 12-24h. Treatment with SAR did not significantly alter the extent of injury or ALT release, confirming that VEGFR3 inhibition did not influence hepatotoxicity initiation. However, inhibition of lymphangiogenesis markedly altered the inflammatory landscape during recovery. Compared to vehicle controls, SAR-treated livers exhibited persistent, spatially confined neutrophil accumulation, particularly within necrotic and peri-necrotic regions at 24-48 h post-APAP. This retention of neutrophils was accompanied by reduced PCNA expression in peri-necrotic hepatocytes, suggesting impaired proliferative recovery. Vehicle-treated animals showed normal neutrophil clearance and strong hepatocyte proliferation, reflecting a normal shift from inflammation to regeneration. These findings suggest that lymphatic vessel formation and function are essential for neutrophil emigration from the injured parenchyma, likely through VEGFR3-dependent mechanisms. **Conclusions:** Our findings identify hepatic lymphangiogenesis as a previously unrecognized component of the immune resolution process following APAP-induced liver injury. By facilitating neutrophil clearance through hepatic lymphatics, the liver promotes timely resolution of inflammation and supports regeneration. Pharmacologic inhibition of lymphangiogenesis disrupts this process, leading to sustained neutrophil retention, diminished hepatocyte proliferation, and impaired tissue repair. These results provide novel mechanistic insight into how lymphatic remodeling influences immune cell dynamics during drug-induced liver injury. Understanding these interactions may open new therapeutic avenues aimed at promoting immune resolution and preventing the progression from acute injury to acute liver failure in patients with APAP overdose.

PS 4738 Metabolic Reprogramming of CD4⁺ T Cells in Autoimmune-Prone Mice Exposed to Trichloroethylene

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Background and Purpose: Autoimmune disorders have been on the rise, and while the causes are not known, studies suggest that environmental factors contribute largely. One of these environmental factors, Trichloroethylene, an industrial degreaser, has been studied and linked to immunotoxicity and T-cell-driven autoimmunity. Preliminary data have shown that TCE exposure *in vivo* promotes autoimmune disease pathology by increasing the number of proinflammatory cytokine-secreting Th1-like CD4⁺ T cells in autoimmune-prone MRL-MpJ mice (MRL). To better understand the mechanism, RNA sequencing was conducted on CD4⁺ T cells to determine the direct effects of TCE metabolite, trichloroacetaldehyde hydrate (TCAH) *in vitro*, revealing alterations in metabolic pathways, some of which are

related to glycolysis. We hypothesize that TCAH promotes autoimmunity by modulating T-cell imbalance through metabolic reprogramming. **Methods:** RNA was isolated, and sequencing was conducted to determine the direct effects of trichloroacetaldehyde hydrate (TCAH) on gene expression in polarized Th1 and activated CD4⁺ T cells from MRL mice *ex vivo*. Th1 cells were compared with anti-CD3/anti-CD28-activated CD4⁺ cells (activated but non-polarized) to analyze and compare TCAH-altered gene expression in male and female MRL mice between these subsets. Gene expression profiles revealed TCAH-mediated metabolic changes. Consequently, extracellular flux assays (Agilent Seahorse assays) were conducted to confirm and further investigate functional metabolic alterations in TCAH-treated CD4⁺ cells. Naive CD4⁺ T cells were isolated and purified from MRL/MpJ mice and cultured +/- TCAH over time to capture activation (1,2 days) and differentiation (3-4 days). Agilent Seahorse T cell metabolic rate assays were undertaken to assess the metabolic function of these cells in the presence or absence of TCAH across various concentrations to establish a dose response (0, 0.25, 0.50, 0.75 mM). Non-autoimmune C57BL/6 mice were also examined to establish a comparison of genetic susceptibility to TCAH exposure. BCA assays for quantification of protein content were utilized for normalization of Seahorse metabolic assays. **Results:** RNA-sequencing results revealed key sex-dependent differences in activated CD4⁺ T cells treated with TCAH in MRL mice. Females exhibited reduced expression of genes involved in glycolysis, the TCA cycle, and the HIF-1 pathway, alongside upregulation of genes associated with one-carbon metabolism and glutaminolysis. Using Seahorse T cell metabolic assays on C57/BL6 CD4⁺ T cells, 96 hours of TCAH exposure led to decreased OCR at higher TCAH concentrations and an increase in the percentage of ATP produced via glycolysis. In MRL mice, Seahorse glycolytic rate assays showed a trend in a decrease in basal and compensatory glycolysis in TCAH-treated cells at 24 hours, followed by an increase at later time points compared to controls in female MRL mice CD4⁺ T cells. **Conclusions:** Our findings suggest that disruption of metabolic programming by TCE may represent one mechanism by which TCE promotes autoimmunity in human populations. Investigating how TCE affects metabolic pathways mechanistically may enhance our understanding of how environmental factors are associated with autoimmune disease.

PS 4739 Histone Variant-Specific Epigenetic Remodeling in Activated CD4⁺ T Cells Following Exposure to a Trichloroethylene Metabolite

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Background and Purpose: Environmental toxicants such as trichloroethylene (TCE) contribute to immune dysregulation and autoimmune disease, yet the mechanisms of these effects including epigenetic pathways involved remain unclear. Prior work identified DNA methylation changes in CD4⁺ T cells at histone-related genes and Polycomb targets after TCE/ trichloroacetaldehyde hydrate (TCAH) exposure, suggesting additional epigenetic mechanisms may be involved. **Methods:** Naive CD4⁺ T cells from autoimmune-prone MRL/MpJ mice were activated +/- 0.5 mM TCAH for four days. Histone post translational modifications (PTM)s were quantified by high-resolution mass spectrometry, and DNA methylation at heterochromatin-associated repeats (D4Z4, NBL2) was assessed using pyrosequencing. Cell-cycle dynamics and proliferation was measured to determine whether PTM alterations correspond with TCAH-induced checkpoint or replication defects. **Results:** TCAH caused widespread remodeling of histone PTMs. Replication-dependent H3.1 regions maintained repressive stability, whereas replication-independent H3.3 domains showed marked dysregulation, including reduced H3K27me2 and increased H3K36me2/3, patterns consistent with enhanced transcriptional elongation. DNA methylation analysis revealed site-specific hypomethylation at D4Z4 and hypermethylation at NBL2, indicating region-dependent chromatin instability. Preliminary cell-cycle analysis suggest possible S-phase disruption, consistent with altered PTMs affecting replication-associated chromatin states. **Conclusions:** TCAH disrupts multiple epigenetic layers in a histone-variant- and locus-specific manner, potentially priming activated CD4⁺ T cells for transcriptional and cell-cycle dysregulation. These findings highlight the importance of histone variant context in environmental epigenetics and provide mechanistic insight into how chlorinated solvents may influence immune function and autoimmunity risk.

PS 4740 Short Libby Amphibole fibers induce pulmonary inflammation in mice up to 21 days post-exposure

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Background and Purpose: Short asbestos fibers (SAF), <5µm in length and <1µm in width, comprise the majority of commercial and environmental exposures but are assumed to have negligible health impacts due to their rapid clearance from the lungs. However, due to their small size, SAF can penetrate deeply into the lungs and cross into the pleural space, where they can remain for years. Since the pleural cavity is a key site of asbestos related disease development, we assessed the effects of SAF on early immune responses in mouse lungs, comparing responses in the inter-

stitial and pleural spaces. **Methods:** Wild-type C57/Bl6 or Ccr2-null mice (deficient in monocyte recruitment) were exposed to a single small dose of SAF or vehicle control administered via oropharyngeal aspiration. Fibers used in this experiment were elutriated Libby amphibole (LA; mean length $1.9 \pm 2.1 \mu\text{m}$, mean width of $0.39 \pm 0.2 \mu\text{m}$). All groups consisted of equal numbers of male and female mice, and all animal experiments were approved and monitored by IACUC. Immune responses in wild-type mice were assessed at 7-, 14-, and 21-days post-exposure. Bronchoalveolar lavage fluid (BALF) and pleural wash (PW) were collected and immune cells were identified using flow cytometry. Pro-inflammatory and pro-resolving lipid mediators and TGF-beta were quantified in washes and lung tissue, and histological analyses of lung tissues were performed to measure collagen and assess structural changes. **Results:** Compared to vehicle, LA exposure resulted in significantly reduced macrophage and neutrophil numbers but increased B and T cells in both the PW and BALF at early time points. Monocytes were increased in the PW on day 7, suggesting recruitment of these cells. Concentration of the pro-inflammatory mediators leukotriene B4 (LTB4) and prostaglandin E2 (PGE2) were increased in BALF on day 21, as were concentrations of active TGF-beta, a pro-fibrotic cytokine, with no differences were noted in the PW. Interestingly, pro-resolving lipoxin A4 (LXA4) was also significantly elevated on day 21 in both PW and lung, suggesting a shift from inflammation to resolution. No significant sex effects were noted. On day 21, histological analyses showed thickened pleural tissue and increased lung protein citrullination. Since monocytes were a key component of early cell influx and have been associated with pro-inflammatory responses, we repeated the experiment using Ccr2-null mice and examined responses at 21 days post-exposure. Interestingly, LA induced increased LTB4 and LXA4 in these mice at day 21, as seen with wild-type only, but the saline control Ccr2-null mice also showed significant increases in these lipid mediators. Additionally, histological alterations consistent with fibrosis were noted in both the saline and LA treated Ccr2-null mice, similar to LA-treated wild-type mice, suggesting that lung recruitment of monocytes may be critical for resolving fibrotic activity. **Conclusions:** Together, these data suggest that even a single exposure to a small dose of short amphibole fibers induces protracted inflammatory response. Further, measured responses did differ between the pleural and interstitial spaces, though these responses did not appear to be driven by monocyte recruitment. Overall, these findings suggest that the unique cellular composition and immune responses within different pulmonary cavities may drive different responses to inhaled toxicants.

PS 4741 Improved cryopreservation of human Precision-Cut Lung Slices supports tissue integrity

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Background and Purpose: Maintaining the integrity of complex and immunocompetent tissue models poses significant obstacles in biomedical research, particularly in toxicity assessments. The development of effective cryopreservation techniques is crucial for securing a consistent supply of standardized tissue models, decreasing reliance on freshly obtained samples, and facilitating long-term storage for both research and clinical uses. Traditional slow freezing methods frequently result in cellular stress and modifications in immune responses, necessitating the exploration of enhanced preservation strategies. **Methods:** In this investigation, we employed a novel rapid freezing technique for cryopreservation of human precision-cut lung slices (PCLS) and compared it against the established slow freezing method. PCLS were derived from agarose-inflated human lung tissue and were subjected to the respective freezing protocols one day after preparation. Subsequent evaluations included assessments of metabolic activity (WST-1 assay), cytotoxicity (LDH release), live/dead staining, RNA quality and integrity, as well as cytokine production in response to pro-inflammatory stimulation. **Results:** Both freezing methods resulted in diminished viability of PCLS compared to fresh samples. However, the rapid freezing technique exhibited markedly better preservation, with viability rates of approximately 70% immediately post-thaw and 85% after 24 hours, in contrast to 50% and 60% for the slow freezing method. Cytotoxicity levels were also significantly reduced in the rapid freezing group, with 10% and 20% at the respective time points, compared to 40% and 45% observed in slowly frozen slices. RNA quality remained stable across both methods, with RIN values consistently exceeding 7.9. To evaluate immune functionality, PCLS were subjected to lipopolysaccharide (LPS) stimulation for 24 hours, followed by measurements of pro-inflammatory cytokine secretion. The freezing process influenced baseline cytokine production, with increased levels of interleukins, such as IL-8 immediately after thawing. A notable decrease in IL-8 responsiveness was solely present in PCLS subjected to slow freezing. Both methods demonstrated a recovery of cytokine responsiveness over one and three days, with the rapid freezing technique facilitating a quicker and more complete recovery. **Conclusions:** In conclusion, the innovative rapid freezing method significantly enhances the preservation of PCLS viability, RNA integrity, and immune functionality compared to traditional slow freezing. This method represents a promising advance for the long-term storage and standardized use of complex human tissue models in toxicity-focused biomedical research.

PS 4742 Can IGH Variants Drive Antibody Regulation and Environmental Response?

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Background and Purpose: The immunoglobulin heavy chain (IGH) gene encodes all antibody isotypes (IgM, IgD, IgG, IgE, IgA), which are critical for immune defense. Environmental or genetic alterations affecting IGH expression can significantly impact human health. Toxicants that activate the aryl hydrocarbon receptor (AhR), such as TCDD, are known to suppress IGH expression and antibody levels; however, AhR's role in human IGH regulation remains poorly defined. This knowledge gap is compounded by species differences, as rodent-based models do not fully reflect human IGH complexity, including a genetic variation within the hs1.2 enhancer that consists of a one to four ~53 bp repeat. Variation in the hs1.2 enhancer has been associated with hypersensitivity and autoimmune disorders. We hypothesize that hs1.2 variants modulate IGH expression and sensitivity to AhR ligands. **Methods:** Using a human B-cell line (CL-01), CRISPR-Cas9 was used to generate clones with distinct hs1.2 variants (one to four ~53 bp repeats). Sanger sequencing confirmed variant-specific changes in putative transcription factor binding sites. IGH expression and antibody secretion were assessed under basal and stimulated conditions. Preliminary experiments evaluated the effects of the AhR ligand TCDD on these variants. **Results:** The hs1.2 variants exhibited altered IGH expression profiles and antibody secretion compared to wild-type cells. Variant-specific differences in putative transcription factor binding sites correlated with functional changes. Preliminary data indicate differential sensitivity to TCDD among hs1.2 variants. **Conclusions:** Genetic variation in the hs1.2 enhancer influences IGH regulation and antibody production, potentially modifying susceptibility to AhR ligands. Given AhR's role as an environmental sensor for dietary and microbial metabolites, persistent ligands may disrupt homeostatic signaling, increasing risk for immune dysfunction. These findings highlight the importance of human-specific genetic variation in assessing xenobiotic impacts on B-cell function.

PS 4743 Airway Antimicrobial Proteins (AMPs) Orchestrate Defense Against Environmental Fungal Exposures

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Background and Purpose: Humans are continuously exposed to airborne fungal species such as *Alternaria* and *Aspergillus*, and in the endemic regions of the Southwest, *Coccidioides* represents an additional major environmental exposure that is expanding across the western United States. These fungi can induce epithelial injury, dysregulated inflammation, and, in some cases, granuloma formation. However, the epithelial-derived factors that coordinate early antifungal immunity remain poorly defined. Our prior work identified several airway AMPs—particularly BPIFA1—as among the earliest epithelial proteins induced following exposure to environmental fungal spores. Although AMPs are well recognized for their antibacterial activities, their antifungal functions and their roles in shaping granulomatous versus non-granulomatous responses remain largely unexplored. We hypothesized that BPIFA1 and related AMPs act as central epithelial mediators that translate fungal exposure into coordinated innate immune responses required for early fungal containment. **Methods:** BPIFA1-derived peptides (BDPs) were screened for antifungal activity alone or in combination with clinically used antifungal agents. Plasmon-waveguide resonance (PWR) spectroscopy and light/electron microscopy (LM/EM) were used to define the biophysical mechanisms of fungal killing. BPIFA1-deficient (KO) mice were intranasally exposed to fungal spores, and outcomes were assessed by survival, lung fungal burden, histopathology, and granuloma architecture (only in the relevant model). Bronchoalveolar lavage (BAL) fluid was analyzed for cytokines and inflammatory mediators. **Results:** BDPs exhibited potent, broad-spectrum antifungal activity. Notably, BDPs synergized with both first-line (azoles) and second-line (amphotericin B) antifungal drugs, with synergy varying by fungal species. Mechanistically, BDPs may compromise fungal membrane integrity and/or inhibit hyphal growth. *In vivo*, BPIFA1 KO mice were markedly more susceptible to fungal exposure, demonstrating accelerated mortality, increased fungal burden, and exaggerated airway inflammation. **Conclusions:** Airway AMPs—exemplified by BPIFA1—represent previously unrecognized, epithelial-derived regulators of host defense against diverse environmental fungi. These proteins appear essential for shaping early antifungal immunity and influencing host resilience to fungal exposure. Our findings identify a unifying epithelial-innate immune mechanism activated by multiple airborne fungal species and highlight BPI-family-regulated pathways as promising targets for mitigating pulmonary diseases caused or exacerbated by environmental fungal exposures.

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Background and Purpose: Persistent organic pollutants (POPs), including pentachlorophenol (PCP), its metabolite tetrachlorohydroquinone (TCHQ), and hexachlorobenzene (HCB), are widespread environmental toxicants noted for their persistence, hydrophobicity, and bioaccumulation potential. These chemicals disrupt intracellular proteostasis and redox homeostasis processes essential to effective MHC Class I antigen presentation. PCP undergoes metabolic conversion to TCHQ, a reactive hydroquinone that generates reactive oxygen species (ROS) and forms thiol adducts, resulting in oxidative stress and impairment of proteasome function. The protonophoric activity of PCP and mitochondrial dysfunction induced by HCB further deplete cellular ATP pools, compromising the energy-dependent ubiquitin-proteasome system and peptide processing crucial for antigen presentation. **Methods:** To explore potential molecular-level interactions, *in silico* molecular docking studies were performed using AutoDock Vina to evaluate the binding of PCP, TCHQ, and HCB with key components of the MHC Class I pathway. The crystal structures of HLA-A (PDB ID: 1AKJ), HLA-B (PDB ID: 1HSA), and the transporter associated with antigen processing TAP1/2 (PDB ID: 5U1D) were selected as receptor models. Ligand-protein docking was conducted to estimate binding affinities, identify active site interactions, and assess the likelihood of each pollutant interfering with peptide binding or transport. **Results:** All three POPs exhibited moderate binding affinities with MHC I and TAP complexes. For HLA-A, the lowest binding energies were PCP -5.9, TCHQ -6.0, and HCB -5.6 kcal/mol; for HLA-B, PCP -5.6, TCHQ -5.3, and HCB -5.3 kcal/mol; and for TAP1/2, PCP -5.5, TCHQ -5.8, and HCB -5.4 kcal/mol. Docking poses indicated stable interactions within the peptide-binding and transporter domains, suggesting that these pollutants may directly interfere with antigen processing and presentation, an effect that may compromise immune function and contribute to the toxicological risk of POP exposure. **Conclusions:** The observed molecular interactions provide strong evidence that persistent organic pollutants (POPs) such as PCP, TCHQ, and HCB can compromise immune recognition by directly modulating MHC Class I molecules and TAP transporters. These alterations may disrupt peptide loading, trafficking, and antigen presentation, potentially weakening cellular immune surveillance and increasing susceptibility to infection or immune dysregulation. Our findings emphasize the toxicological significance of these compounds in altering adaptive and innate immune pathways, warranting further *in vitro* validation studies using *in vitro* models to confirm these effects at the cellular level.

PS 4745 Comparative Autoimmune and Neuronal Zoomer Antibody Profiling across three Heavy-Metal-Exposed Cohorts from Italy: Adolescents, Ferroalloy Workers, and Elderly Patients with Parkinsonism

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Background and Purpose: Heavy-metal exposure (e.g., Mn, As, Cr) contributes to both neurodevelopmental and neurodegenerative outcomes, yet biomarkers that bridge early-life vulnerability to later-life disease remain poorly defined. While systemic immune activation is increasingly recognized as a mechanistic contributor to metal-associated neurotoxicity, comparative immune-neural antibody dysregulation across the lifespan has not been evaluated. Identifying shared immune-neural signatures across developmental stages could elucidate mechanisms consistent with the Developmental Origins of Health and Disease (DOHaD) framework and help uncover biomarkers linking neurodevelopment to neurodegeneration. This study aimed to characterize overlapping autoimmune and neuronal antibody dysregulation across three multi-age metal-exposed cohorts: (1) adolescents exposed to ambient steel emissions, (2) metal-exposed ferroalloy workers, and (3) parkinsonian patients exposed to ambient ferroalloy emissions. **Methods:** Blood samples were analyzed using a combined Protein microarray platform, containing 106 autoimmune and 64 neuronal-associated antibodies in Vibrant Sciences, LLC (Santa Clara, CA, USA). Comparisons were defined as: exposed vs. control (adolescents, n=132), occupationally exposed vs. control (workers, n=11), parkinsonian patients vs. healthy controls (elderly, n=97). In adolescents, cognitive and behavioral function scores and blood metal concentrations differed significantly between exposed and control groups, and similar case-control differences in metal levels were also observed in workers and elderly cohorts. For each antibody, log₂-transformed mean differences were estimated, and significance was assessed using Analysis of Covariance (ANCOVA) adjusted for age and sex. Antibodies dysregulated in at least two of the three cohorts were defined as "shared markers". Directionality (up- or down-regulation) and effect magnitude were compared using standardized mean differences. **Results:** Across 170 antibodies, five markers exhibited consistent and statistically significant dysregulation in all three cohorts, indicating shared immune-neural perturbations across the exposure spectrum. These included AP3B2, Beta-Amyloid (Aβ), DBH, GFAP, and Human Brain Cerebellum Protein. Parkinsonian cases exhibited the strongest elevations, with AP3B2, DBH, GFAP, and Human Brain Cerebellum Protein signifi-

cantly up-regulated (FDR<0.05), while Aβ was significantly decreased (FDR<0.05). The Ferroalloy Workers showed more moderate but directionally similar increases for all five markers, suggesting early immune activation in occupational exposure. In contrast, children displayed significant inverse regulation for these antibodies, highlighting distinct developmental immune responses. Despite differing life stages and exposure sources, the same antibody clusters mapped to neuronal integrity, synaptic trafficking, astrocytic injury, and catecholaminergic signaling, and pathways implicated in mood and depression, indicating biologically convergent pathways. **Conclusions:** This multi-age cross-cohort comparative analysis identifies a consistent set of immune-neural antibody alterations linked to heavy-metal exposure and neurobehavioral dysfunction. The shared dysregulated markers (AP3B2, Aβ, DBH, GFAP, cerebellar proteins) suggest that metal-associated immune activation may operate along a continuum - from early-life vulnerability to occupational exposure to later-life neurodegeneration. These findings highlight the potential translational value of antibody-based signatures for exposure surveillance, early detection of neurotoxicity, and mechanistic understanding of environmentally driven neurodegenerative processes.

PS 4746 Unregulated Alternative Cannabinoid Vaping Products Cause Significant Pulmonary Toxicity and Immune Dysfunction against Respiratory Pathogens

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Background and Purpose: Alternative cannabinoid vaping products (e.g., Δ⁸-THC, Δ¹⁰-THC, HHC, THCP, THCA) have rapidly emerged in the U.S. market, circumventing regulatory oversight. These products are widely available in smoke shops and gas stations, often flavored to appeal to youth. Despite increasing use, little is known about their pulmonary or immunotoxicological risks, and their impact on antiviral host defense remains poorly understood. **Hypothesis:** Vaping products containing alternative THC analogs exhibit greater pulmonary toxicity and impair host immune responses to influenza A virus (IAV) infection. **Methods:** Eight commercial cartridges labeled as Δ⁸-THC, Δ¹⁰-THC, HHC, THCP, and THCA were analyzed via GC-MS for cannabinoid composition. Bubble extracts in DMSO were evaluated for cytotoxicity and cellular ROS in BEAS-2B bronchial epithelial cells. HHC-induced inflammatory response was assessed by measuring IL-8 cytokine levels. Diversity outbred J: DO mice were exposed to whole-body inhalation exposure to HHC aerosols for 30 min/day over 7 days (51 mL, 2 puffs/min), followed by challenge with mouse-adapted influenza IAV/PR8. Three days post-infection, BALF and lung tissues were analyzed via flow cytometry and inflammatory mediator profiling. **Results:** Chemical analysis revealed widespread mislabeling, with only 4/8 cartridges matched labeled contents. Three were labeled as "THCP" but were predominantly Δ⁸-THC or HHC. All HHC-containing cartridges exhibited racemic R/S mixtures. *In vitro* treatments with HHC-containing extracts produced significant ROS generation and cytotoxicity, and HHC elicited a robust, concentration-dependent IL-8 response ($p < 0.001$). Consistently, *n vivo* exposure to HHC increased BALF cellular infiltration but decreased total lung immune cell counts. IAV infection significantly increased BALF infiltrates, but HHC/THC + IAV co-exposure significantly blunted this recruitment, indicating impaired antiviral immune response. **Conclusions:** Misrepresented alternative cannabinoid vape products, such as those of HHC, elicit significant pulmonary toxicity and suppress host antiviral defenses. These findings suggest that repeated exposure may heighten susceptibility to respiratory pathogens and underscore the urgent need for inhalation toxicology assessment and regulatory oversight of emerging cannabinoid vaping products. This study was supported by the Ralph W. and Grace M. Showalter Research Trust Award at Purdue University.

PS 4747 Redefining Developmental Immunotoxicity Testing: Transitioning From Animal Models to Human-Relevant Frameworks

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Background and Purpose: The developing immune system is uniquely vulnerable to environmental and pharmacological perturbations that can lead to lifelong immune dysfunction. Despite growing evidence linking early-life exposures to allergy, autoimmunity, and impaired host defense, developmental immunotoxicity (DIT) testing remains largely dependent on animal models with limited translatability to human immune ontogeny. The *International Working Group towards Alternatives to In Vivo Developmental Immunotoxicity Testing* is advancing a roadmap to modernize DIT assessment through human-relevant, mechanistic approaches aligned with regulatory needs. **Methods:** We integrated current evidence on age-specific immune vulnerabilities with advances in developmental immunology and systems toxicology. New Approach Methodologies (NAMs)—including iPSC-derived immune models, organoid and microphysiological systems, and single-cell and spatial transcriptomics—were assessed for their applicability to early immune development. A tiered testing framework was constructed that incorporates reference chemicals, defined validation criteria, and key mechanistic markers to support assay qualification. In parallel, initial efforts were made to outline a putative adverse outcome pathway (AOP) linking

early perturbations in immune cell differentiation to impaired immune competence. **Results:** Recent advances in high-dimensional profiling have revealed conserved and human-specific molecular programs driving immune maturation and their disruption by environmental agents. *In vitro* systems now recapitulate essential developmental transitions of hematopoietic and lymphoid lineages, enabling functional evaluation of immune perturbation with improved mechanistic clarity. Integration of these models into tiered testing strategies supports the identification of early key events predictive of adverse immune outcomes, providing a scalable and ethically sound alternative to current *in vivo* methods. **Conclusions:** DIT testing is entering a new era defined by human relevance, mechanistic precision, and translational value. The development and validation of NAM-based DIT frameworks—anchored by reference chemicals, clear qualification criteria, and mechanistically grounded AOPs—will enable more predictive, ethical, and policy-relevant assessment of immune developmental hazards. These advances redefine how we identify, characterize, and ultimately prevent immune disruption across the life course.

PS 4748 Sex-Dependent Immune Reprogramming Following Developmental PFOS Exposure in an ASD mouse model

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Background and Purpose: Perfluorooctane sulfonate (PFOS) is a globally persistent perfluoroalkyl substance (PFAS) with immunomodulatory and endocrine-disrupting effects. However, its long-term impact on immune development and homeostasis in autism spectrum disorder (ASD) remains unclear. As immune dysregulation plays a critical role in the pathogenesis of ASD, it was hypothesized that the combined gestational and early postnatal PFOS exposure at environmental relevant levels could have dose- and sex-dependent effects on splenic leukocyte profiles in adult BTBR-mt⁸⁶ offspring, an ASD mouse model. **Methods:** Timely mated pregnant dams received vehicle or PFOS (0.2 or 1.0 mg/kg/day; *n* = 6) by gavage from conception to postnatal day 21. Adult male and female offspring were euthanized at 14-15 weeks of age, and single-cell splenocyte suspensions were analyzed by flow cytometry for B-cell, T-cell, myeloid, and regulatory markers populations (%) as well as mean fluorescence intensity (MFI). **Results:** In males, PFOS exposure increased both terminal body and spleen weights, with significant effects observed at the low dose. Immunologically, PFOS induced heightened B-cell activations at both doses, as evidenced by elevated CD40L expression (B220⁺CD40L⁺), and showed a trend towards decreased populations of T-cell associated activation markers (CD40⁺CD44⁺CD4⁺). The expressions of myeloid activation markers (GR1⁺F4/80⁺) were elevated at low dose, while cytotoxic T-cell markers (CD3⁺CD8⁺) and regulatory markers (CD25⁺) primarily elevated at high dose, suggesting heightened inflammatory priming and immune responsiveness in males. In contrast, females exhibited predominantly immunosuppressive profiles, particularly at high dose, with decreased myeloid (Mac-3⁺) population and reduced expression of B-cell (B220⁺), T-cell (CD4⁺, CD8⁺), myeloid (F4/80⁺) and regulatory markers (CD25⁺), reflecting compromised adaptive and innate immune cell activation. **Conclusions:** These findings demonstrate that developmental PFOS exposure durably reprograms immune homeostasis in a sex- and dose-dependent manner. The bias of males toward immune activation while females toward immunosuppression highlights the vulnerability of the developing immune system to PFAS exposure and underscores the importance of considering sex-specific risk in assessing long-term immunotoxic outcomes.

PS 4749 Cytokine Profiling for Immunotoxicity Assessment: Comparison of MSD®, Luminex®, and CBA Multiplex Platforms in Preclinical Matrices

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Background and Purpose: Cytokines mediate critical immunological activities, reflect immune activation, and indicate disease status. Cytokine profiling is central to immunotoxicity assessment during drug development. Ligand binding assays are routinely employed to detect cytokine release at the protein level. Multiplex cytokine analysis methods are widely adopted for capturing complex cytokine profiles, offering advantages over singleplex methods by reducing sample handling and minimizing total sample volume requirements. Among these platforms, cytometric bead array (CBA), Meso Scale Discovery (MSD®) and Luminex® provide high-throughput analysis for preclinical risk assessment. Despite their popularity, choosing the right platform and interpreting results across different platforms remains challenging. In this study, we directly compare cytokine profiles generated by three multiplex platforms (MSD®, CBA, and Luminex®) using identical sample sets. **Methods:** Cytokine production was stimulated with in-house positive control mixes. Cytokine release was subsequently measured in serum samples from non-human primates (NHPs) and in supernatants from stimulated human peripheral blood mononuclear cells (PBMCs). The cytokines IL-2, IL-6, TNF-α, and IFN-γ were quantified in all samples using three multiplex platforms: ECL platform (MSD® U-plex), Luminex® platform (ThermoFisher ProcartaPlex) and CBA platform (Biolegend LEGENDplex for NHP serum and BD Flex set for human supernatant). Additional analytes were measured on specific platforms: IP-10 was quantified in NHP serum in MSD® and Luminex® panel. IL-10 was quantified in human PBMC supernatant using MSD® and CBA. All animal research was approved

by the Institutional Animal Care and Use Committee. All assays were performed according to the manufacturers' protocols. Selectivity/ parallelism was performed to determine MRD. Accuracy and precision run was also evaluated. Results from the same sample group were compared across the three platforms. Data values below the lower limit of quantification (LLOQ) were substituted with zero for comparative analysis. Statistical comparisons were performed using ANOVA followed by pairwise t-tests. **Results:** Our results demonstrated a relatively consistent cytokine trend following stimulation as assessed across all three platforms. In pre-stimulation samples, baseline was comparable across all three platforms (MSD®, Luminex®, CBA), with analytes IL-2, IL-6, TNF-α, IFN-γ below the LLOQ. Platform-specific differences was observed during IL-2 and IL-10 detection in NHP serum and during IL-10 detection in human PBMC supernatant. All platforms detected the peak IL-2 release post-stimulation at comparable levels. However, only the MSD® platform consistently detected IL-2 in serum samples at the 6-hour time point, despite the results exceeding the LLOQ of all three assays, suggesting potential differences in the antibody binding of the respective IL-2 assays. Additionally, IP-10 in stimulated NHP serum was detectable only by MSD®, not Luminex®. Detected IL-10 levels in stimulated human PBMC supernatant were significantly higher using CBA versus MSD®, though both platforms showed consistent trends. While IL-6, TNF-α, and IFN-γ showed no significant statistical differences across platforms, absolute concentrations varied by over 3-fold in pairwise comparisons. Consequently, these platforms are suitable for relative quantification looking for trends, but direct comparison of data values is not recommended. **Conclusions:** Our data demonstrated that selecting a multiplex cytokine platform (MSD®, Luminex®, or CBA) for preclinical immunotoxicity assessment requires a fit-for-purpose approach. When comparison between data set is expected, trend rather than data point values may be comparable for data generated across platforms. However, when direct comparison of absolute cytokine levels is crucial, data set from same platform may be required.

A

- A. Swanik, K. 4263
Aalati, A. R. 4508
Abadie, S. 4169
Abarca Palma, D. S. 3158*
Abascal, L. 3581, 4201
Abbott, E. 3035
Abdallahman, J. 3470
Abdelfattah, N. 4028*
Abdelmoneim, A. 3130, 3155, 3193
Abe, T. 3054
Abedini, J. 3303*, 4399
Abel, R. 4354
Abele, G. 3497, 4004*
Abioye, T. 3364
Abou Choucha, F. 4311a
AbouGharbia, M. 3862
Abozaid, L. 4298
Abraham, C. 3225
Abraham, M. J. 3785
Abramova, E. 3239, 3241, 3450, 3925, 3939, 3940
Abramova, E. V. 3934
Abrar, S. 4493
Abreu, A. 3788
Abreu, N. A. V. 3288
Abu Salah, A. 3046*
Abu-Salah, A. 3093, 3566, 3570
Acevedo Reyes, R. 3947*
Achanta, S. 3249*
Acquaah-Mensah, G. 3467
Acra, S. 3133
Adak, S. 4465
Adamcakova-Dodd, A. 4145, 4284
Adamec, J. 4228
Adams, M. 3279
Adams, S. 3413, 4247*
Adamson, G. 3181, 3841
Adebambo, O. A. 3836, 4686
Adeleke, A. 3096
Adeleye, Y. 4119
Adelman, N. E. 4416*
Adeniji, A. 4085
Adepoju, L. A. 4430*
Adesina, K. E. 4588*
Adeyanju, O. Z. 4469*
Adgate, J. L. 4602
Adhikari, A. 3960, 4188, 4194
Adhikari, U. 4740
Adil, S. 4370
Adjei, S. 3408
Adjimatera, N. 3060
Adkins, K. 4111
Adler, D. 4247
Adongo, E. 3167
Adragna, J. 3321, 3987
Advani, I. 4065
Afaghpour-Becklund, M. 4600
Afgah, M. 3220*, 4452
Afsharinejad, Z. 3913
Afzal, A. B. 3990
Agarwal, A. 3230
Agarwal, C. 3246
Agarwal, R. 3246*, 3263
Aggarwal, S. 3463
Aggarwal, A. A. 3069*
Aghaei, Y. 3924
Agina, C. 3426
Agrawal, S. 3263*
Agu, O. A. 4439
Agudelo Areiza, J. 2026, 4505*, 4511, 4523
Aguiar, L. M. 3102*
Aguiar Jr., D. 4603*
Ahabchane, H.-E. 3922
Ahamed, F. 3011*
Aherrera, A. 3914
Ahmad, A. 3248
Ahmad, O. D. 3621*
Ahmad, S. 3248, 3262, 3367, 3807*, 4344
Ahmed, A. 4724
Ahmed, M. 3558
Ahn, J.-H. 3187
Ahn, K. 3391
Aho, B. M. 3427
Ahona, A. M. 3528
Aidnik, H. 3371, 3707, 3709
Aillon, K. 4583
Ajayi, I. 3114*, 3117
Ajisafe, O. 3243
Akagi, J. 3976*
Akakpo, J. 3026, 3035*
Akerman, G. 4483
Akers, N. 3750*
Akhbari, R. 4629
Akhtar, N. 3643
Akhter, J. 3230
Akilesh, S. 3034
Akinsanya, K. 3535
Aksu, L. 3594
Aktuna, S. 4213*
Akulian, J. 3045
Al Janabi, S. 4139
Alaaldin, M. M. 3541, 4571, 4629
Alahmadi, H. 3170, 3817
Alam, M. 3087
Alarabi, A. 4078
Albayrak, M. 3200
Albers, W. 3122
Albert, R. M. L. 4673*
Albert, V. 3527*
Albrecher, N. 3027
Albrecht, S. 3531
Albright, J. M. 4288*, 4540
Albright, M. 3933
Aldridge, K. 3558
Aleksunes, L. 2026, 3020, 3022, 3032, 3036
Aleksunes, L. M. 3202, 3410, 3413*, 4271, 4273
Aleo, M. D. 4331, 4332
Alers, D. 4346
Alewell, D. 4311
Alexander, O. 4008
Alexis, N. E. 4660
Alfaro-Moreno, E. 3796
Alford, L.-T. 4089
Alfrén, P. 3072
Alharthy, K. M. 3642*
Ali, H. 4078
Ali, M. 3636, 4745
Alkhadrawi, H. 3470, 3974
Alkhudaydi, S. 3577*, 4212
Alkire, C. 4060*, 4066
Alkufairi, A. 4450
Allard, P. 3221, 4062, 4305, 4388, 4648, 4653
Allaria, G. 4187
Alle, M. 4608
Allegro, J. B. 4428
Allen, R. 3746
Allen, W. 3407*, 4120*
Alli, A. 3037
Allison, M. 4114*, 4412
Allran, J. 4311
Allred, C. 3214
Allred, C. D. 4409
Allred, K. 3214
Allred, K. F. 4409
Alman, B. 3304
Almond-Thynne, J. 3525
Alotaibi, A. 3862
Alotaibi, S. 4549
Alp, O. 3796
Alper, D. 3350
Alshammari, I. F. 3373*
Alshbool, F. Z. 4078
Alsulami, A. 3576, 4212, 4218*
Altintas, L. 3200
Altschuler, S. 4415
Aluru, N. 3176*, 3614, 4435
Alvarado, I. 3909
Alvarez, J. 3853, 4236
Alvarez, L. 4480*
Amano, M. 3024, 3490, 3954
Ambe, K. 3253, 3257, 4378
Amberg-Johnson, K. 3535
Ameduri, B. 3888
Amend, N. 3235
Ameredes, B. T. 4655
Ames, P. 3010
Ames, P. M. 4501
Amessird, H. 3635
Amini, R. 4068
Ammirante, M. 4044
Ampadu, F. A. 4538*
Amralo, J. 4212*
An, L. 4424
An, S. 4246*, 4716
An, S. S. 4427
An, Z. 3393
Anand, S. 4643*
Anantharam, V. 3122, 3569, 3571, 3572, 4636
Anchan, A. 3046, 3093*, 3566, 3570
Anchordoquy, T. 3263
Andaluri, G. 3907
Andar, A. 4140
Andersano, B. 2024, 4746
Andersen, C. 4354
Anderson, D. 4094
Anderson, J. 4515*
Anderson, K. A. 3171, 3386
Anderson, S. M. 3685
Andersson, J. 3403
Andra, S. 4275
Andracki, M. 4697
Andres, A. 4274
Andresano, B. 4692
Andres-Mateos, E. 3305
Andrew, P. M. 3233, 4269*
Aneskievich, B. J. 4106
Anestis, D. K. 3029
Anetor, J. I. 4640
Angelov, H. 4328
Anger, L. 4355
Anger, L. T. 4352
Aniolek, G. 3560
Aniolek, G. D. 4447, 4459*
Ankley, P. 4463*
Ankley, P. J. 4462
Annalora, A. 3121
Annalora, A. J. 4410
Annida, R. 3031
Annunziato, M. 3086
Ansari, R. A. 3702
Anselm, C. 3870, 4040
Anson, B. 4135*
Antaris, A. 4042
Anthony, C. 3262
Anthony, M. 3036*
Anthony, T. G. 3934, 4429
Antic, O. 3390
Antonijevic, T. 4030*
Antonijevico, T. 3456
Antwi-Boasiako, C. 4469, 4470*
Aoki, M. 3163
Aoki, S. 3432, 3438*
Api, A. M. 3251, 3252, 3266, 3328, 3999, 4107
Apic, G. 3021, 3537, 4257
Appleseth, H. J. 4656
Apte, U. 2037, 4294, 4505, 4641
Aquino, J. 3827
Arakawa, H. 3199, 3257
Arakawa, N. 3432
Araki, T. 4333
Araki, Y. 4171
Araujo, J. A. 3485, 3913, 3923, 3924
Araujo, M. N. 4454*
Arava, M. 3393
Araya-Cloutier, C. 2021
Arbore, H. 3012*
Arce Rodriguez, A. N. 4227*
Aregbe, N. K. 3092*
Areiza, J. A. 4577
Arena, A. 3827*
Arena, A. C. 3224
Argenty, J. 4169*
Argenziano, M. 3383
Argikar, U. 4708
Arib, G. 4108
Arimura, Y. 4050
Armento, A. 3255, 4121
Armijo, A. S. 3773*
Armstrong Suthahar, S. 4388
Armstrong, L. 3857
Armstrong, M. 3273*, 3321, 3472
Armstrong, M. J. 3589*
Armstrong, T. 3966
Arndt, T. 3868, 4054
Arnold, M. 4375
Arnold, M. A. 3734*
Arokoyu, D. G. 4532
Arosh, J. 3215
Arosh, J. A. 3210
Arowolo, O. 3195
Arredondo, A. 4669
Arreola Guerra, J. M. 3033
Arriola, S. A. 4669*
Arroyave, W. 4307
Arroyo, A. 3774*
Arroyo-Salgado, B. J. 3666*, 4482
Arshee, M. 3839
Arteaga Leon, A. 4542
Arteel, G. E. 2039, 3012, 3482
Artosky, K. 4447
Arumaren, I. A. 4640
Aryal, A. 4228*
Aryal, S. 3725
Asabere, D. 3422
Asadian, M. 4170
Asakura, S. 3806
Asano, H. 3333
Asaoka, Y. 4705
Aschner, M. 3114, 3117, 3781, 4209, 4594
Aselmann, H. 3686
Asgharian, B. 3477
Ash, D. 3719
Ashikaga, T. 3253*, 3255, 3257, 3441
Ashpole, N. 3179, 4693*
Ashraf, A. 3481*, 3503
Ashton, R. S. 4129*
Aslam, M. N. 4679
Aslamkhan, A. 4698
Aslamkhan, A. G. 3150
Asowata, J. 4634
Aspinall O'Dea, M. 3536, 4726
Assaf Vandecasteele, H. 3518
Assanome, K. 3841*
Asselin, L. 3300
Astafiev-Holmes, E. 3584
Astete, C. E. 3750
Astuhuaman Davila, T. 4431
Atai, S. 4085
Atala, A. 3508
Atanasio, G. D. N. 4187
Athar, M. 3230
Atif, F. 3304, 3335, 3336*
Ator, J. 3310, 3311*
Aubee, C. 4483
Audouze, K. 4009
Auerbach, S. 3620, 4485
Auerbach, S. S. 4374, 4383, 4385
Augello, A. 3292, 3559
Augusta, J. 3857
August, L. 3320
Augustine, K. 3332, 3856, 4044, 4130
Aung, M. 3632, 3897
Austin, D. 3401, 3710, 4170
Authier, S. 4048
Avalos, H. 3829
Avenbuan, O. 3844
Avenbuan, O. N. 3217*
Avila Oropeza, A. 4530*
Avila-Barnard, S. G. 4305*
Avlasevich, S. 4320, 4321, 4323*
Awada, C. 3464
Awali, S. 3434*
Ay, M. 3566, 3570
Ayala-Fierro, F. 3475, 3478*
Ayeunnie, S. 4121*, 4127, 4167, 4187*
Aygun Kocabas, N. 3442
Azaizah, M. 4617*
Aziz, L. 3922
Azmon, S. 3636, 4745*
Azzi, S. 3210, 3215
Ba, S. 3578
Babakanins, P. 3184*
Babayev, M. 3459
Babu, K. G. 4116
Babu, D. 3370
Baca, A. M. 4529
Bacellar-Galdino, M. 3850, 3865*
Bacher, R. 4405
Bachorz, R. 3066
Bachour El Azzi, P. 3687
Badami, M. 3924
Badding, M. 4684*
Badea, A. 2015
Bader, R. 3058*
Badruzzaman, M. 3598*
Bae, H. 3187*

B

Baettig, C.	4463	Basnet, R.	4251	Berrada, H.	3659	Blasco, L.	3687
Baettig, C. G.	4462*	Bassan, A.	4389, 4715	Berry, J.	4446	Blasini Picart, Z.	4518
Bagchi, I. C.	2018, 3839	Bastos Lopes, C. E.	3153*	Berthold, D.	4668	Bledsoe, H.	3420
Bagchi, M. K.	3839	Batchelor, T.	3492, 4231	Bertollo, N.	4169	Blee, M.	4695
Baglole, C. J.	3466	Batchelor, T. P.	3491	Berube, R.	3287	Blee, M. A.	3761*
Bahtiri, S.	3674*, 4387	Bathina, A.	3855*	Besaw, N.	4594	Blendea, A.	3781
Bai, S. V.	4439, 4628*	Batra, R.	4706	Beshiri, M.	3376	Blesa, J.	3658
Bai, W.	3506	Batra, S.	4018, 4718, 4744	Beskrovnaya, O.	4706	Bleske, B. E.	4541
Bailey, A.	4183*	Battalora, M.	3278*	Besteman, E.	2009	Blesso, C.	3936
Bailey, F.	4373	Bauer, A.	3902, 4634	Betat, A.-M.	4701	Blevins, L. K.	4735*
Bailey, M.	4497, 4550	Bauer, A. K.	3234, 3472*	Bettinsoli, V.	3381*	Bline, A. P.	4479*
Bailie, M.	4713	Bauer, B.	3624	Bettmann, A.	3061*, 3361, 4166	Blinova, K.	3363, 4159, 4160, 4582
Baily, J.	4167	Baumeister, J.	3267	Betz, L.	4306, 4625	Block, G.	4629
Baird, B.	3425, 3791*, 4600, 4657	Baumert, J.	4006	Bevan, S.	3697	Blosi, M.	3544
Baird, B. E.	4541	Baumgard, L. H.	4469, 4470	Bever, R. J.	4157	Blossom, S.	3425, 4600
Baird, S.	3622	Baur, S.	3082	Beyer, S. E.	3919	Blossom, S. J.	4739
Bajaj, P.	4111	Bautista, J.	4064	Beyl, S.	4248	Bluemlein, K.	3326
Bajaj, P. B.	3718	Bavoux, C.	3288	Bhadra, D.	3001*	Bly, A. R.	4414*
Bajard, L.	3673	Baynes, R. E.	3649	Bhagwan, J. R.	3277*	Blystone, C.	4625
Baker, C.	4442*	Baze, A.	3300*	Bhaker, S.	3530	Bo, X.	4453
Baker, E. S.	4185, 4491	Bazina, L.	3496, 3961*	Bhakta-Yadav, M.	4742	Boazak, L.	4110
Baker, J.	3522		4546, 4547	Bhali, J. A.	4312*, 4654	Bobadilla, M.	4241
Baker, J. M.	3932	Bazzi, Z.	4428	Bhali, J. S.	3278	Bobiak, M.	3078
Baker, K.	3018	Beachum, C.	4311	Bhandari, R. K.	4643	Bodduluri, S.	3910
Baker, T.	3400, 3891	Beamish, J. A.	4649	Bhanushali, P.	3051, 3268	Boffetta, P.	3990
Baker, T. R.	3133, 3188	Beard, J.	4489	Bhardwaj, B.	4160	Boguszewski, K.	4685
Bakulski, K.	4646	Beardslee, R.	3341	Bhaskar, K.	2012, 4551	Bohannon, M. E.	4624
Bakulski, K. M.	3119, 3123	Beardslee, R. A.	3361	Bhaskaran, A.	4508	Boireau, W.	3635
Balasubramanian, K.	4197	Beardsley, N.	4135	Bhaskaran, V.	2036, 3332, 3856, 3857, 4044*	Bois, F. Y.	3983, 3984
Balazki, P.	4022	Beasley, J.-M.	2001	Bhatia, S.	3602*	Boitel, E.	4311a
Balbo, S.	4326	Beaudoin, J. J.	4607a	Bhatnagar, A.	4255	Boj, S.	3708*, 4113
Baldone, G.	4381	Beaulieu, C.	4048	Bhatt, B.	4723, 4729	Boland, D. J.	3210, 3215
Balik-Meisner, M.	3355, 4358	Beaulieu, S.	3477	Bhattacharya, A.	3209	Boland, M. C.	3220, 4452
Balik-Meisner, M. R.	4374	Becanova, J.	4505, 4511, 4523	Bhattacharya, B.	3294*	Boland, S.	3576, 3912*
Ball, N.	4474	Beck, B. D.	4575	Bhattacharya, B.	3294*	Bolatimi, O. E.	3010, 3616*, 3617, 4501
Ballone, I.	4350	Beck, S.	3218	Bhavsar, C. T.	4429	Bolisetty, S.	3230
Balmes, J.	3914	Beck, S. A.	3219*	Bhimani, V.	3193a	Bolla, S.	3907
Balodis, I. M.	3409	Beckanova, J.	3895	Bhobe, A.	3637	Bollapragada, A.	3807
Baloni, P.	4371, 4375*, 4617	Becker, K.	4026, 4372	Bhowmick, R.	3693*	Bolstad, H. M.	3909, 4133*
Baltazar, M. T.	3373	Becker, N.	4026, 4372	Bhowmik, K.	3862	Bolt, A.	3921, 4600
Bammler, T.	3034, 3206	Becker, R.	3452	Bhowmik, N.	4100, 4102*	Bolt, A. M.	3772, 3773, 4301, 4592
Bammler, T. K.	3485, 3913	Becker, R. E.	3056*, 3453	Bhuiya, S. J.	3088*	Bolt, M. J.	4232
Bandara, S. B.	2034, 4002*	Bedford, R.	3377, 4077	Bhuju, J.	4609	Bolteho, D. J.	3266
Banerjee, A.	4347*, 4638	Beebe, O.	4340	Bhusal, A.	3579, 3922, 4208*	Bombelli, A.	2021
Banerjee, D.	3016*	Beevers, C.	4098	Bhushan, B.	3013, 3014	Bomstein, Z.	3214
Banerjee, M.	3774, 3775*, 4596	Begay, J.	3425, 3953	Bi, Z.	4292	Bomstein, Z. S.	4409
Banu, S. K.	3210, 3215	Begay, J. B.	4551	Bianchi, E.	3294, 3299*, 3380, 3803, 4091, 4126, 4146	Bonanini, F.	3407
Bär, C.	4239	Begay, J. G.	3791, 4541			Bond, S.	3066, 3731
Barad, I. M.	3763	Behrens, S.	4239	Bianco, C.	3178	Bondesson, M.	2028*
Barahona, A. J.	3595*, 3596, 3600	Behrsing, H. P.	3393, 4175	Bib, Y.-A.	4629	Bondesson, M. B.	4146
Barakat, K. H.	3370	Beier, J.	3634, 4630	Bibb, A.	3790*	Bondy, S. C.	4471
Barar, J.	3702	Beier, J. I.	2039, 2041, 3012, 3482	Bibb, A. M.	3599	Boney, S. S.	3225*
Barathon, F.	3635	Bein, K.	3964	Bidaut, A.	3863*, 3867*	Boni, R.	4167
Barbacane, N.	3528	Bein, K. J.	3498, 3579, 3917, 3922, 4208, 4669	Biemel, K.	3300	Bonjour, C.	4311a
Barbazuk, W. B.	4405	Beins, K.	3756*	Biggerstaff, A.	3386, 4412	Bonnar, D.	4478
Barber, C.	2002	Belanger, N.	4652	Billheimer, D.	4629	Bonnar, D. J.	3321*
Barberan, O.	3042, 3721, 4058	Belden, J.	4440	Billington, C.	3534, 4082	Bonner, D. K.	4057
Barbosa-Torres, A.	3295	Bell, A.	3452	Billue, E.	4431*	Bonner, E.	3926*
Barchowsky, A.	4268, 4644	Bell, A. J.	3453	Binazon, O.	3267	Bonner, J.	3975
Bare, J. L.	4604	Bell, J.	3535	Bing, T.	3334, 4238	Boonhower, S. R.	4267, 4269
Barela, L.	4528	Bell, M.	3096	Biomi, N.	4016	Boone, A.	3053
Barela, L. R.	4541	Bell, M. R.	4525	Bird, B.	3390	Boone, L.	4110
Bari, W.	3820	Bell, S.	4384	Bird, C.	4645	Boonpraman, N.	3046, 3104, 3111, 4206*
Baria, C. A.	3443	Bell-Huff, C.	3625	Biro, E.	3429*, 4416	Boos, A.	3516
Baril, C.	4398*	Bellini, C.	3916, 4068, 4254	Birtele, M.	4129	Boos, J.	3601
Barkalow, F.	4493	Bello, A.	3875	Birukova, A.	3941	Boos, J. R.	3600*, 3602
Barkley, J.	3882*	Bello, D.	3875, 4501	Birznieks, G.	2008	Borboa-Pimentel, A.	3722
Barkley-Levenson, A. M.	3118, 3804	Bellocci, M.	4457	Bisesi, J. H.	4266, 4447	Borchers, J.	3668*
Barlow, N.	4185*	Beltman, J.	3673	Bisesi, S.	4668	Bordelon, M.	3872
Barnes, A.	3263	Beltman, J. B.	4404	Bisesi, S. E.	4266	Borghoff, S.	4013
Barnes, J.	3643	Bemis, J.	4321, 4323	Bishop, E.	4081	Borghoff, S. J.	3314
Barone, F.	3868	Benedict, R.	3304	Bishop, E. L.	4077*	Borgström, A.	3682, 4387
Barousse, C. S.	3085	Bennett, B.	4606	Bishop, L.	3132, 4228	Borisenko, N.	4221
Barr, D.	4087	Bennewitz, M.	3470, 3974	Biswal, S.	3637	Borland, M. G.	4296, 4300
Barr, E.	3425, 3480, 3791, 3921, 3953	Bentley, K.	3911	Biswas, A.	4440*	Borrel, A.	4360, 4394, 4395*
Barrera Hernández, A.	4423	Benton, M. L.	4489	Biswas, K.	3875*	Borton, L.	3067, 3733
Barrett, E. S.	4271, 4273	Ben-Yakar, A.	3191, 4223*	Biswas, S.	3280	Bose, P. G.	4656
Barros, G. P.	3468*	Bera, S.	4109*	Bita, A.	3781	Botelho, D.	3510, 3999, 4107
Barrow, S.	3426	Berengere, B.	4034	Bitzer, Z.	3457	Botero, C.	4666
Bartilson, C.	3470*	Berg, M.	3330	Bizon, C.	2001	Bouchard, G.	3865
Bartlett, A.	3328	Berghaus, O.	3139	Bjoras, M.	4163	Bouchard, K.	3999, 4107
Bartlett, C.	4283	Bergin, I. L.	3119	Bjornstad, P.	3897	Bouffard, R.	4037, 4038
Bartman, A.	4092	Bergman, K. L.	2014*	Black, S. R.	4580	Bourgeois, M.	2013*
Bartnikas, T.	4597	Berke, J.	4578	Blackburn, J. E.	3508*	Bouwman, P.	3270, 3357
Bartone, R. D.	3975*	Bermeo Grajales, D.	4429*	Blackshire, L.	3416	Bouwman, P. J.	3688, 4404
Basa, R. C. B.	4168*	Bernard, J.	4295	Blackshire, L. C.	3418	Bowden, A. M.	3510
Basak, S. K.	3055	Bernard, W.	3711, 3809	Blair, R.	3240	Bowden, J.	4266
Basaly, V.	3880	Bernardino, P. N.	3233	Blake, J.	3906	Bowden, J. A.	4447
Bascul, C.	2020, 3318, 3644	Bernstein, M.	3095	Blake, S.	4550	Bowdridge, E.	4252
Basilio, E.	4618			Blanc, E.	3673	Bowdridge, E. C.	3212
Basiri, B.	4706			Blanchette, A. D.	4025	Bowe, B.	3743

Bowman, A. B.	3107, 3109, 3143, 4203, 4209	Buchanan, D.	3065	Campasino, K.	3647	Cato, P.	3530
Bowman, C. J.	3177*, 3213, 3816	Buchanan, W.	3094*	Campbell Jr., J. L.	4021	Cavalleri Sousa, T.	3819*
Boyer, L. N.	4296	Buchanan, W. J.	3124	Campbell, B. C.	4452	Cavallin, J.	3359
Boyer, R.	3041	Bucher, M. L.	3574	Campbell, K. A.	4274*	Cavaneau, E.	3188, 3891
Bozada, T.	4350	Buchholz, B.	3908, 3972*	Campbell, M. J.	3202	Cave, M.	3010, 3631, 4503
Braakhuis, H.	4534	Buchholz, J.	3554	Campbell, S.	2033, 3207, 3209	Cave, M. C.	4501
Brackenbury, L.	4726	Buck, T.	4197	Campbell, S. C.	3935	Cayea, S.	4344
Bradshaw, J. L.	3966	Buckley, B.	4273	Campen, M.	2012, 3425, 3830, 3953, 4528, 4531, 4541, 4657	Cayley, A.	3395
Brady, M.	3069	Buckley, T. J.	4199	Campen, M. J.	2011, 3791, 4536, 4542, 4548, 4551, 4554, 4591, 4633	Celente, M.	3926
Braeken, D.	4170	Budding, K.	3031	Campion, S.	2006	Cellini, C.	3207, 3209
Braeuninger-Weimer, L.	3710, 4170	Buddy, M.	3937	Campion, S. N.	3213*, 3816	Celniker, A.	3527
Bramatti, I.	3143*	Buechlein, A.	3459	Campos Jorge, B.	3224	Centers, A.	4501
Brambila, D.	4415	Buerger, A. N.	3314*, 3324	Campoy, G.	4336*	Centner, H.	2024
Brand, S.	4039, 4331, 4332*	Buesen, R.	3147, 4026, 4372	Cañas-Carrell, J. E.	4408	Cerrato, J.	4600
Braun, E.	3372, 3978*, 4406*	Buford, M.	3489	Canavez, A. D. P. M.	3288	Cerrata, J. M.	3513
Braun, J. M.	3563	Builee, T.	3066	Canchola, A.	2010, 3428, 3549, 3722, 4083*, 4353*, 4362, 4391	Cerveau, C.	3051
Brayman, T.	3280	Bulera, S.	4048	Candelario Gonzalez, M. V.	4520	Cesta, M.	4626, 4675
Breaux Jr, G.	3005	Bundy, J. L.	4153	Canfield, R. C.	3244*	Cevering, C.	3425*, 4541
Breese, N. M.	3613*	Bunger, M.	4110*	Cannon, C. L.	3495	Chada, A.	3948
Breheny, D.	4077, 4081	Bunis, J.	3897, 4504*	Cannon, J.	3590, 3592, 3899, 4096, 4417	Chadha, A.	4080
Breidenbach, J.	4674	Buol, B. M.	4469, 4470	Cannon, J. R.	3567, 3568, 4516, 4524	Chae, H.	3366
Breljak, D.	3025	Burchat, N.	3935	Cannon, T. J.	3462	Chaffin, K. A.	2035, 3751
Bren, C. L.	4554	Burdick, A.	4706	Cantrell, K.	3999	Chagolla, I.	4016*
Brennan, S.	3041	Burel, A.	3635	Cantu Gutierrez, M. E.	4673	Chakraborty, S.	4643
Brenner, D. E.	4679	Burgdorf, T.	3565	Cantu, A.	3823, 4673	Chakraborty, T.	3598
Breslin, W. J.	3177	Burger, C.	3553*, 3554	Cao, J.	3149*	Chakravarti, S.	4012, 4382, 4564*
Brewin, S.	3286*	Burgers, E. J.	4404	Cao, X.	3405*	Chakravarty, J.	4175
Bridges, C. C.	3765	Burk, D.	3483	Capece, G. E.	3018*	Chakravarty, V.	3268
Brigante, L. N.	3109	Burnett, M.	4693	Caporaso, J. G.	2024	Chalupa, D.	3566, 3911, 3965
Briggs, A.	3791, 4216*	Burns, J.	3857	Cappelletti, M.	3055	Chamberlain, C.	3739
Briggs, A. M.	3599, 4222	Burns, K. S.	3386*	Capunitan, D.	3365	Chambers, B.	3364
Brigham, K. A.	4344	Burns, R.	3492, 4231	Cardenas Vasquez, D. E.	4226*	Chambers, J.	3236, 3243*
Brighton, R.	2008, 4142*, 4714	Burrows, A.	4500	Cardoso Valente, L.	3224	Chan, C.	4607
Brindley, S.	3033, 4603	Burt, A.	4652	Caria, M.	3223	Chan, D.-C.	4418
Brinker, A.	4272	Burton, S.	4566	Carignan, C.	3898	Chan, T.	4015*
Brinkman, M. C.	4085	Burton, T.	4120	Carlin, J. L.	2008*	Chand, A.	3977*, 4431
Brito, H. P.	4454	Busby, N.	3236	Carll, A. P.	4253	Chandan, G.	3843*, 4062
Britt, J.	3314	Buser, M. C.	3995*	Carlson, A.	2012	Chandler, H. L.	4334
Brizendine-Ames, P.	3616	Bush, K.	3359	Carlson, C.	3692, 4135, 4144, 4566	Chandola, A.	3839
Brizendine-Ames, P. M.	3631*	Bushdid, P.	3218, 3866	Carlucci, V.	4381	Chandra, N.	3637
Brockman, A.	3408	Bushdid, P. B.	3821	Carman, L. E.	4106	Chandratike, R.	3268
Brockmeyer, H.	4328	Busler, D.	3682	Carmona, A.	4589	Chandrayan, G.	3075*
Brocksmith, D.	3865	Buteica, S. A.	3781	Carney, R. P.	4552	Chanel, C.	3300
Brooks, A.	3053, 4309, 4310	Buti, L.	3810	Carolina Ferreira, M.	4668	Chang, C.-e. A.	4024
Brooks, C.	4660	Butler, L. P.	4138*	Caron, S.	3279	Chang, D.-C.	3738
Brooks, C. A.	3521	Butruille, L.	3142	Caron, S. E.	2018*	Chang, F.-Y.	4418
Brooks, J.	3643	Butzke-Souza, N.	3101, 3113, 3769, 3778	Carpenter, J.	4220	Chang, H.	4065
Brooks, M.	4332	Buxton, S.	3736	Carpenter, J. M.	3794	Chang, H.-p.	4673
Brouwer, K. L.	4607a	Bybee, G.	4430	Carr, K.	3536	Chang, N.	3539
Brown, A. P.	4642	Byers, L.	3367	Carrell, S. J.	3171, 3887	Chang, R.	3747*
Brown, B.	3321, 4478	Bylsma, L. C.	3183	Carroll, M.	3446, 4724*, 4736	Chang, R. Y.	3732, 3753
Brown, C. E.	4249*	Byrd, E.	4014	Carstens, K.	3814*, 3815, 4399	Chang, W.-H.	3539*
Brown, H.	4456*	Byrd, G.	4153, 4155	Carter, B.	3158	Chang, Y.	4352, 4448*
Brown, I.	3841	Byrne, R.	4169	Carter, C.	3280	Chang, Y.-c.	4448
Brown, J.	3061, 3191, 3238, 3293, 4223	C. Iserte, A. M.	3660	Carter, D. T.	3318, 3644*	Chang, Y.-T.	3572*
Brown, J. M.	3033, 3234, 3244, 4500, 4602, 4665	Caballero-Floran, R. N.	3351*	Carter, E.	4693	Changyuk, M.	4140
Brown, K.	3030	Caballero-Gallardo, K.	3645*	Carter, J. R.	3791	Chantler, P.	4060, 4066
Brown, N.	3536	Cable, S.	3373, 4153	Carter, K.	2001	Chao, F.	4385
Brown, T.	3226	Cabrera, T. A. J.	4446	Carter, T. H.	3439	Chapman, F.	3461
Brown, T. K.	4624*	Cabuzu, D.	3350	Cartwright, J. A.	3861	Chapman, L.	3739*
Brownell, A.	3101, 3113, 3769, 3778	Caddie, M.	3534	Caruana, V.	3466*, 3782, 4067	Chapman, W.	3698
Brownell, A. W.	4682	Cahill, T. M.	4692, 4746	Cary, C.	3170	Chappel, J. R.	3620, 3628*
Brown-Leung, J. M.	3567, 3568, 4516, 4524*	Cai, B.	2007	Casciano, G.	3359	Chappell, G.	3420*, 3906, 4307
Bruce, E.	3481, 3503, 3505	Cai, C.	3393	Casciano, G. H.	3302*	Chapple, T. K.	4447
Brücker, L.	4141, 4178	Cai, J.	3210, 3215, 4256	Casey, W.	3364, 4374, 4383	Charest, N.	3562, 4189
Bruckman, M.	3558	Cai, L.	3971, 4256, 4565, 4594	Casillas, F. O.	3834	Charland, B.	4184
Bruer, G.	4328*	Cai, M.	4743	Casillas, G.	3335, 3336	Charles, G.	3741
Bruggemann, T.	3496	Cai, R.	3544	Casillo, A. N.	3155*	Charlton, A.	3135*, 3289
Bruh, C.	4243	Cairns, J.	4026, 4372	Cassee, F. R.	3544*	Charlton-Sevcik, A. K.	3455
Brunasso, L.	4381	Caito, S.	3415, 3417*, 3646	Castaldi, A.	4065	Chartier, R.	4125
Brunel, B.	3635	Calderón, A. I.	3639	Castañeda-Monsalve, V.	3141	Chatani, K.	3063, 3077
Brunetti, L.	2026, 3032, 3036, 4505	Calderon, L.	3961, 4546	Castellanos-Sanchez, N.	3910	Chatman, C.	3085
Brunkhorst, J.	2024	Caldwell, K. S.	4567	Castelli, F.	3260	Chatron, N.	4332
Brunner, J.	4271, 4273	Caliendo, M.	3527	Castilho, A. d.	3205	Chatterjee, S.	3793, 3919, 4228, 4239, 4466, 4471, 4472
Brunner, R.	3338, 3340, 3341, 3361*	Calipari, E. S.	3109	Castillo, K.	4343	Chatz, L.	3632, 3897, 4275
Bruno, G.	4236	Call, F.	3669	Castoldi, A.	3565	Chaudhari, H. S.	3754
Bruns, I.	3983	Call, M. R.	3000*	Castor-Colmanar, I.	4040	Chaudhary, S.	2039, 3012, 3482
Bruns, I. B.	3984	Callahan, C.	3179	Castro, A. L.	4669	Chaudhuri, A.	4089
Bru-Reason, A.	3295	Callegaro, G.	3138, 3674, 3983, 3984, 4370, 4387, 4404, 4486	Castro, R.	3827	Chauhan, V.	4422
Brusoe, G.	3472	Callewaert, E.	4122	Castro, R.	4518	Chauvin, C.	3635*
Bruton, T. A.	4197	Callis, C.	3305, 3306	Castro, R.	4518	Chavanel, B.	4326
Bruun, D.	3247, 3588	Calvimontes Barrientos, L. D.	3033*	Castro, R.	4518	Chavez, D. K.	4737*
Bruun, D. A.	3233, 3237, 3589	Camacho, J.	3970	Catala Catala, A.	3947	Chavez, V.	3522
Bryce, S.	4321*, 4323	Camara Quilez, M.	4163	Catala Catala, A. G.	3969*	Che, J.-H.	3007
Brzoska, M. N.	3378	Camarillo, G.	4638	Catalano, S.	3989, 4139	Cheema, R.	3184
Bsibsi, M.	3810	Campagna, S.	3484	Catalano, S. M.	3293	Chelladurai, K. S. N.	3089
Bu, P.	3283, 3296, 3874	Campagnolo, L.	3178*	Catalan-Perez, F.	4168	Cheminant, J. R.	3937
				Cathey, D. D.	4256*	Chen, A.	3189
				Catlin, N. R.	3816*	Chen, C.	2024, 3177, 3544
						Chen, C. Y.	3049

Chen, C.-M.	3858	Choudhury, S. R.	4739	Cong, G.	4385, 4485*	Cuevas Mora, K.	3426*
Chen, C.-Y.	3557*	Chouinard, C.	3154	Conklin, D. J.	4468	Cui, J.	3121
Chen, D.	4650*	Chow, M.	3452, 3453	Connell, M.	3891	Cui, J. Y.	3108, 3112, 4410, 4614
Chen, F.	4292	Chowdury, A.	3817	Connell, M. L.	3188*	Cui, L.	4073
Chen, H.	2019, 3904, 4073, 4453, 4531	Choy, E. H.	4667*	Connolly, J.	3964	Cui, S.	3953, 4186
Chen, H.-T.	3161	Christen, C.	3225	Conrow, K. D.	4265*	Cui, S.-Y.	4056
Chen, J.	3003, 3008, 3060, 3190, 3385, 3482*, 3685, 4087, 4126, 4244, 4289, 4587, 4618, 4719	Christen, C. H.	4152	Constant, S.	4108	Cui, Z.	3334
Chen, J. Y.	4192*	Christiaens, P.	3057*	Conti, D.	3897	Cuijpers, M.	4117
Chen, J.-H.	4448	Christian, B.	4227	Conti, D. V.	3632, 4275	Culbreth, M.	3148*, 3712
Chen, K.	3722, 4083	Christians, U.	3556	Contin Ortega, M.	4018	Culpepper, W.	3901
Chen, L.	4238	Christie, J.	3694	Conto, A.	4381	Cumming, I.	3927, 3943
Chen, M.	3648, 4057, 4317, 4319	Christmann, R.	3280	Cook, A.	4583	Cummings, B.	3038
Chen, N. G.	4051	Chrysostomou, P.	3980*	Cook, J.	3213, 3594	Cummings, B. S.	3039
Chen, Q.	3273, 3555, 3725, 4396, 4545	Chu, C.	3050, 3307	Cook, J. C.	2006	Cunefare, D.	4649
Chen, Q. M.	4424	Chu, P.	4339	Cook, M.	3545*	Cunningham, M.	3745*
Chen, R.	4743	Chu, V.	3693, 3710, 4170	Cook, N. E.	3610*	Cunningham, R. L.	3966
Chen, W.	3055, 3299, 3347, 3380, 4126, 4139	Chuang, R.	3924	Cook, T.	3486, 3523	Cunhy, H.	4306, 4307, 4398, 4623
Chen, X.	3555*, 3815, 4150*	Chung, B.	4200	Cooke Bailey, J.	3902	Curbelo-Valiente, A.	3834*
Chen, Y.	3341, 3440, 3656, 4054*, 4385*, 4400*, 4585, 4743*	Chung, B. N. T.	4197*	Cooke-Bailey, J.	4634*	Curd, J.	3399
Chen, Y.-C.	4539	Chung, H. N.	4562	Coomes, G.	3808	Curran, C. P.	3131, 3132, 3408*
Chen, Y.-H.	4324	Chung, H.-S.	4335	Cooper, K. L.	4190	Currie, R.	3347, 4008
Chen, Y.-W.	4366*	Chushak, Y.	4011, 4033	Cooper, L.	3305, 3306	Currie, R. A.	4095, 4119*
Chen, Z. T.	3348, 3354, 4620	Chu-Van, E.	3260	Cooper, M. P.	3859*	Currim, F.	3592, 4516, 4524
Cheng, C.	3266	Chvatal, S.	3529*	Cooper, S.	4588	Currim, F. M.	3567*, 3568
Cheng, M.-Y.	3498, 3917, 4208	Ciccimaro, E.	4044	Coppens-Exandier, H.	3687*	Cushenan, P.	3465
Cheng, S.-Y.	3788, 4587	Cimon, K.	4623	Cora, M.	4626	Cushman, J.	3594
Cheng, W.	2020	Cinquino, S.	3812	Cordoba Munoz, M.	4720	Cvejkus, R. K.	4268
Cheng, X.	3874, 4100, 4303	Cioffi, G.	4564	Córdova Alarcón, E. J.	4423	Czechanski, A.	3273
Chepurny, O. G.	4601*	Cirillo, P. M.	3597, 4280*	Corey, L.	4180	Czepiel, T.	4347
Chereul, E.	4561	Cisneros, J.	4193	Corley, C. N.	3474		
Cherrington, N.	3540, 4629	Citrowske, S.	3080, 3084	Corley, R.	4108		
Cherrington, N. J.	3541, 4571	Clabaugh, G.	3571*	Cormier, S. A.	3483, 3484	da Costa, J. L.	3047
Cherukuri, S.	4292	Clair, G.	3249	Cornelius, V.	3711	da Silva, F. C.	3767
Chesné, C.	3261	Clapp, P.	4029	Corrêa, G. O. P.	3288*	Dada, O.	4473*
Chetta, K. E.	2040	Clark, B. W.	4437	Corsini, E.	3381, 3435	Dagher, M.	4174
Chiang, C.-K.	4448	Clark, J.	4527	Corson, M. T.	3568*	Dahlberg, S.	3442
Chiarugi, D.	4605	Clark, K. L.	3838, 3840	Cortes, R.	3053, 3683	Dahlmann, F.	3686
Chiba, K.	3431	Clark, K. M.	3109*	Cortese, K.	4187	Dai, N.	3990
Chicoine, C.	3547	Clark, R. M.	4541	Corton, C.	4311	Dai, X.	3062*, 3084*
Chien, J.	4269, 4575*	Clarke, E.	3830	Corton, J. C.	4641	Dai, X. S.	3325, 3329
Chien, Y.-C.	4445*	Clarke, J. D.	3000	Corvaro, M.	3293	Dail, M.	3243
Childers, W.	3862	Clavijo, C.	4118, 4120	Cory-Slechta, D.	3144, 3911	Dail, K.	3266
Chilton, M. L.	4027	Clayton, A.	4125	Cory-Slechta, D. A.	3134, 3566	Dalaid, A.	4120
Chimino, G.	3201*	Clayton, C. R.	3879*	Coskun, D.	4354	Daley, M. C.	4251*
Chinala, A.	2011, 3695	Clayton, M.	3179	Costa, A.	3330, 3926	Daley, S.	3359
Chinala, A. R.	3771*	Clemens-Sewall, M. V.	4011	Costa, E.	3380	Dalmas, D.	3384
Chitiyala, P.	3041	Clements, M.	3529	Costa, S. S.	4588	Dalton, P.	4181
Chiu, L.	4597	Clewell III, H. J.	4021	Costalonga Rodrigues, L.	3047	Dammann, M.	4026, 4372
Chiu, W. A.	3373, 3495, 4474	Clewell, H.	3562	Costello, E.	3632	Dams, S.	4535
Chivers, D. P.	3826, 4444, 4460	Clewell, R. A.	3337, 3564, 4011, 4033, 4161, 4162*, 4199	Costin, E. G.	3361	D'Andrea, A.	3528
Cho, B. S.	4056	Clippinger, A. J.	3061, 3361, 3516, 4166	Cottier, K.	3719	Daniel, A.	3384
Cho, C. H.	4563*	Cloetens, P.	4589	Cottier, K. E.	3700*	Daniels, C.	3408
Cho, C. S.	3608	Cloteau, C.	3635	Cottrez, F.	4311a	Danilyuk, T.	3357, 4404
Cho, E.	3622	Clouston, S. A.	4221	Coudray, A.	3350*	Dao, L.	3068
Cho, J. L.	4284	Cluzel, C.	3041	Coulombe, K. L.	4251	Daood, N. J.	4377*
Cho, S.	3007, 3019, 3605, 4615	Cnossen, G.	3063*, 3077	Courtney, C.	3484	Dardenne-Ankringa, E.	4593
Cho, S. Y.	3662*	Coarfa, C.	4349*	Cousineau, S. L.	3316*	Darmon, J.	4311a
Cho, Y.	4044	Cobos-Urbe, C.	4656	Covelli, L. T.	3892	Darou, S. L.	3274
Chodvadiya, D. A.	4356, 4363	Cochran, S.	3943	Covington, T.	3982	Darsie, B.	3321, 3991, 4477
Choi, A.	4074	Cochran, S. J.	3932, 3941*	Covington, T. R.	3997	Das, K.	3068
Choi, B.-R.	4251	Cockburn, M.	4276	Covitz, T.	3134, 3144, 3911	Das, M.	4546*, 4549
Choi, C.	3019, 3605, 4615	Coen, K.	4127	Cowan, D. W.	3564	Dasgupta, S.	3154, 3156, 3168, 3182
Choi, D.	4615*	Coen, M.	3376	Cowan, E. M.	3887*	Dashner-Titus, E. J.	3629, 4190*
Choi, D. I.	3019	Coffin, S.	4133	Cowie, D.	3285	Dasmahapatra, A. K.	3851*
Choi, D.-i.	3605	Cohen, J. M.	3742	Cowie, D. E.	3301, 4095	Date, A. A.	3244
Choi, E.-B.	3007	Cohen, S. M.	4291, 4593, 4684	Cowley, M.	3607*	Davenport, L.	3760
Choi, H.	4056	Cohn, B.	3597	Crago, J.	4461, 4537	Davidse, S.	3357*
Choi, J.	4676	Cohn, B. A.	4280, 4689*	Craig, A.	2041	Davidson, S. E.	4072
Choi, J.-H.	4335*	Coker, E.	4266	Craig, Z. R.	4016, 4031	Davies, R.	4709
Choi, Y.	3701, 4512	Colacino, J.	3105, 3123, 4281, 4646	Crawford, R.	3449, 4721	Davis II, J. W.	4706*
Choi, Y. J.	4524	Colacino, J. A.	3119, 3272, 4131, 4603, 4678, 4679, 4731	Crawford, R. B.	3275, 4734, 4735	Davis, A. P.	4493
Choi, Y.-J.	3701	Colbourne, J. K.	3192, 3660	Criner, G.	3907	Davis, B.	3247, 3588*
Choksi, N. Y.	3314	Cole, A. R.	3364*	Cristillo, K.	3911	Davis, B. P.	3233, 3237
Chollet, C.	3260	Cole, C.	2012, 3029	Cronin, B. A.	3732, 3753*	Davis, C.	3514
Chong, G.	4597	Colicino, E.	3636, 4745	Crooks, I.	4077, 4081*	Davis, J. L.	3649
Choo, C.	3492, 3562	Coller, B.	3848	Croom-Pérez, T. J.	3787	Davis, W.	4054, 4710
Choo, E.	3561	Collier, G. E.	3886*	Cross, K.	4715	Davis, W. R.	4438*
Choo, H. J.	3786	Collins, E.-M. S.	3356, 3587*	Cross, K. N.	4720*	Davletov, B.	4703
Chorna, N. E.	3969	Collins, H.	3830	Cross, K. P.	4389*	Dawod, M.	3296*
Chou, C.	3722	Collins, H. E.	4253	Crotty Alexander, L. E.	4065, 4079	Dawson, J. T.	3318*, 3644
Chou, C. K.	3506*	Colombo, M. V.	3532, 3682	Crouse, L.	4624	Day, K.	3470, 3974*
Chou, E.	3422	Colonna, K. J.	4267*, 4269	Croutch, C. R.	3242, 3245	Day, N.	4066
Chou, W.-C.	2010, 3428, 3549, 4083, 4353, 4362, 4391, 4539	Colvin, D. C.	3109	Crowe, W.	3508	De Angelis, G.	4381
Choudhury, M.	3604, 4245	Colvin, V. C.	3500, 4114, 4132	Crozier, K.	3576	de Aquino, A. M.	3205
Choudhury, S.	3393*	Combs, B.	3111	Cruchley-Fuge, L.	3192	de Caro, E.	3135
		Combs, P.	4485	Crudo, F.	3669	De Carvalho E Silva, A.	3660*
		Commodore, S.	3459, 3946, 4061	Cruz, K.	3869, 4040	de Castro e Horta Júnior, J.	3224
		Conder, R. K.	3694, 4137	Cubello, J.	3134, 4557*	de Gandiaga, E.	4458
				Cuddapah, S.	3125	de Hart, G.	3041
						De Jesus Andino, F.	3852

D

De Jesús Andino, F.	3437	Desfosses, E.	3526*	Donegan, M. E.	4144	Ehman, K.	4564
De La Cruz, A.	3527	Deshpande, A. D.	3763, 4116	Donepudi, S. R.	3705	Ehrlich, A. K.	4496
De La Torre, A. I.	3171*	Deshpande, L.	3240, 3593	Doney, A. T.	4344	Ehrmann, B. M.	3521
de Leon, J. D.	3239	Desjardins, C. A.	4706	Dong, J.	3293	Eichenbaum, G.	4005
De Maddalena, L. L.	3027, 4166	Deteaux, S.	4102	Dong, Q.	3321, 3991, 4477, 4478	Eick, S. M.	4274
de Matos Manoel, B.	3224*	Detert, J. A.	3760*	Dong, W.	4453	Eid, A.	3595
De Miranda, B. R.	3574	Dévedec, S. L.	3984	Dong, Y.	4052, 4053	Eidemiller, B. J.	3416
de Munnik, S.	3536, 3810, 4726	Devine, R. D.	4162	Dönmez, A.	4128	Einhorn, A.	4543
de Oliveira Mendes, L.	3819	Deviney, A.	3214	Donnarumma, F.	3101	Eisenbrand, G.	3328
de Oliveira Pinaffi, V.	3819	Devisscher, L.	4122	Donnelly, J.	4041	Eitoku, M.	4725
de Oliveira Souza, K. A.	3047*	DeVito, M.	4152	Donthamsetty, S.	3343*	Eiya, B. O.	4640
De Oliveira-Cordova, C.	3170, 3904	DeVito, M. J.	3906*	Doonan, L. B.	3301	Ejaz, S.	3307, 4005, 4687
de Ruiter, S.	4120	Dewald, Z.	4697	Doorn, J.	4092	Ekins, S.	3723
De Sousa, M.	4428	Dewey, H.	3273	Doorn, J. A.	3795	Ekpruke, C. D.	3459, 3930
de Souza Castilho, A. C.	3819	Dewey, R.	4262	Doshi, U.	3456	Ekuban, A.	3010*, 3616, 3631, 4501
de Tombe, E.	3398	DeWitt, J.	3446, 4724, 4736	Dostie, I. O.	3427	Ekuban, F.	4503
De Vizcaya Ruiz, A.	3515, 3962, 4064, 4250	DeWitt, J. C.	3386	Dotson, R.	3884*, 4452	Ekuban, F. A.	3010, 3616, 4501*
De Vizcaya-Ruiz, A.	4343, 4667	Dey, R. K.	3499	Dou, J.	4646*	El Azzi, P.	3261
Dean, A. E.	3222, 3228a*	Deyssenroth, M. A.	3223	Dovlatova, N.	3530*	El Hayek, E.	2012, 3992, 4528, 4541, 4543, 4548, 4551, 4554
Dean, B.	3561	Dhanda, A.	4137	Dowling, V.	3729, 3738*	El Hellani, A.	4085*
Dean, H.	4578	Dhayade, S.	3720, 4356*, 4357*	Dragicevic, E.	4235	El Muayed, M.	4601
Dean, K. P.	3105	Dhepe, S.	3051, 3268	Dragovic, D.	3021, 3537, 4257*	El-Bayoumy, K.	4302
Dean, L. E.	3795	Dhruv, N. T.	3419	Drelichman, M.	3207, 3208	Eldalal, O.	3370
Dean, M.	3194	Di Giuseppe, E. C.	3223	Drelichman, M. G.	3209*	Eldefrawy, F.	3670*, 4748
Dear, J. W.	3861	Di, Y. P. P.	4743	Dressel, C.	3144	Elder, A.	3965
Deaton, B. R.	4503, 4694	Diamond, G.	3304	Driebe, E.	2024	El-Fawal, H. A.	4211*
Deb, A.	4727*	Dias, A.	4068	Driessen, C.	3671	Elie-Caille, C.	3635
DeBerry, J. J.	3230	Dias, P.	4173	Driu La Rochelle, C.	4701	Elizalde Velazquez, A.	4093*
Debieen, E.	4054	Diaz Agosto, D. L.	3969	Driggers, K. R.	2040, 4503*	El-Kadi, A.	4413
Deckard, B.	3497, 3988, 4004, 4671*	Diaz Flores, A. Y.	4520	Droege, W.	4108	Elkady, H.	3283*
Decker, A. D.	4536	Diaz Hernandez, M.	3422	D'Ruiz, C.	3764	El-Khoury, K.	4226
Dee, R.	4091	Dibao Dina, A.	3071*	Dsouza, K. G.	3957	Elkin, E. R.	3207, 3208, 3209
Deepika, D.	4009	DiBona, E. R.	4509	Du Plessis, A.	4264	Elkins, A. C.	3220, 3822*, 4452
Deere, C. J.	4439	Dicent, J.	3574*	Du, K.	4443	Ellery, S.	3535
Deering-Rice, C.	3959	Dickey, A.	4492	Du, X.	3023, 3538, 4368	Ellingson, P.	3529
Defontis, M.	3863, 3867	Dickey, A. N.	3373, 4491*	Duan, L.	4103*	Ellison, C.	4023
Deford, P.	4091, 4098	Dickinson, S.	3946, 4061	Duarte-Hospital, C. V.	3574	Elmahrouk, S.	4413*
Deford, P. L.	3380*	Dickson, R. P.	3932	Dubin, S.	3080	Elmore, R.	4007
Defourny, S. V.	4455, 4457	Diduff, M.	3151	Dubois, C.	4311a	Elmoustapha, S.	3775
Degenhardt, T.	4039, 4331*, 4332	Diedrich, J. D.	4683	Ducatman, A.	3897	Elsheikh, E.	3452, 3453
DeGeorge, G.	3078	Diemen, M. v.	3984	Dudi, S.	3456	Elston, S.	3971
Degnan, D. J.	4114	Dierolf, B.	4662*, 4666, 4746	Dudum, R.	3707*, 3709	Elston, S. P.	3952, 4642
DeGraw, A.	3731*	Dies, D.	3401	Dugan, M. S., T. W.	3264	Eltokhy, M. A.	4680*
DeGroote, C.	3364	Diesing, A.	4330	Dugas, T. R.	3488, 3750	Emani, S.	3159*, 4527
Dehmel, S.	3686*	Digman, A.	3114	Duggan, B.	3407, 4117	Eminhizer, M.	3981
Dehne, E.-M.	3406	Dilger, M.	4026, 4372	Duineveld, P.	3075	Emran, T. B.	4298
Dehnert, G.	3167	Dillards, N.	3408	Dunaway, B.	4693	Engel, I. T.	3330*
Deisenroth, C.	3385, 4158	Diller, J.	3326	Duncan, C.	3420	Engelke, M.	4328
Dekant, R. H.	3553, 3554*	Dillon, M. M.	4502	Dunigan-Russell, K.	3941	Engelmann, B.	3141
Dekant, W.	3328, 3553	DiNatale, J. A.	4588	Dunn, A.	3833*	Engelthaler, D.	2024
Del Pino, J.	3581*, 4201	Ding, H.	4743	Dunn, A. C.	3212	Engevik, M. A.	2040
Del Razo, L. Mía.	4423	Ding, W.-X.	4619	Dunn, J.	3505	Engin, A.	3796
Del Rincon, S. V.	3785	Ding, X.	3140, 4572	Dupre, G.	4020*	Engin, A. B.	3796*
Delaere, J.	3031	Dinh, K.	4182*	Durai, M.	4041*	Engin, E. D.	3796
Delalibera Hinokuma, K.	3819	Dipalo, M.	4236	Durainath, A.	3462	Engle, S.	4178
Delaunoy, A.	4701	DiPetrillo, K.	3857, 4044	Durango-Villalba, J.	3666	Engled, S.	4023, 4346
Delcher, C.	4266	Diven, S.	4682	Durk, M.	3561	Enos, J.	3965*
Delker, E.	3320	Diven, S. S.	3101, 3113, 3124, 3769, 3778, 3787, 4605*	DuTeaux, S.	3321, 3987, 3991, 4099, 4100, 4477, 4478	Enright, B.	3280
Dellafiora, L.	3668	Dixon, H.	3048	Dutta, S.	3081, 4042	Enright, H.	3908, 3972
Delnicki, M.	3101	Djuari, M.	4328	Dvorakova, M.	4127	Eom, J.	3429
Delnicki, M. E.	3113, 3769*, 3778	Djuric, Z.	4679	Dwyer, S.	4124, 4178	Eppihimer, M.	3085
Deloid, G. M.	3496, 3961, 4546, 4547, 4549	Doak, S.	3395	Dydak, U.	3091, 3098, 3099, 3102, 3103, 3115, 3783	Eppler, N.	3002, 3015
DeLorme, M.	4094	Dobkins, C. F.	3585*	Dye, H. C.	3485	Erdei, E.	3992, 4190, 4603
DeLouise, L.	3049, 4530	Docea, A. O.	3781*	Dye, J. A.	3494	Erdelyi, V.	3399
Delpy, E.	4701	Dockman, R.	4220	Dzielak, L.	4487	Erdmańska, P.	4608
Del Razo, L. M.	2001	Doctor, N.	3902			Erdos, Z.	4622, 4698
Deme, S.	4062, 4305, 4388*	Dodd, C. A.	3882			Erickson, J.	4485
Demokritou, P.	3496*, 3961, 4546, 4547, 4549	Dodd-Butera, T.	3043*			Erlacher, M.	3381
Demuth, P.	3516	Dodder, N.	2016			Erlanki, A.	3426
Denecke, S.	4490, 4622*	Dodds, J. N.	4185			Erol, M. E.	4514
Deng, N.	3146	Dodmane, P.	3321, 3987*, 3991, 4099			Escandor, J.	3037
Denluck, L.	3535*, 4354	Doepker, A.	4096*			Escher, S.	3686
Dennehy, K.	4601	Doerkes, M.	3082			Escher, S. E.	4389
Denslow, N.	3037, 4668	Doherty, A.-C.	4197			Esckridge, K. R.	3284
Deodhar, C.	3266, 3999*, 4107	Doherty, C. L.	4191			Esman, S.	3230
Deoraj, A.	3903	Doherty, J.	4527			Esper, R. M.	4679
DePalma, R.	4582	Doherty-Lyons, S.	4278			Esquibel, M. A.	3773, 4529
Derekshani, A. A.	4392*	Doka, A.	3855			Esselink, J.	3810
Derksen, M.	4113	Dolan, D. G.	2034, 4002			Etemadi, M.	4255
Dertinger, S.	4320*, 4321, 4323	Dolinoy, D. C.	3105, 3119, 3123, 4237, 4646			Etemadi, S.	3276*
Desai, K. R.	3763, 4116	Doll, M. A.	4677			Etemadi, Y.	3026*
Desai, V.	4383	Dolphin, S. V.	4409*			Ethridge, V.	3297
DeSantis, K.	3782, 4214, 4215	Domanico, M. C.	3498, 3917, 4669			Evans, C.	4526
Descargues, P.	4169	Domaol, J. G.	2016			Evans, R. L.	3474
Descher, E.	4528	Domaol, J. G.	3485*			Evans, T. E.	3776
Descher, E. R.	4554*	Dominguez Reyes, A.	4423*			Evansky, P.	3494, 3514
Dese, K.	3470, 3974	Donaldson, J.	4447			Evenburg, T.	3461, 3678
DeSesso, J. M.	3177	Dondero, L.	4187			Everett, L. J.	4153
		Donegan, M.	4566			Everett, T. M.	4274, 4652
						Ewald, J.	4174

F

- Faber, B. 3770, 4588
 Faber, W. 4570
 Fabian, E. 4022
 Fabisiak, J. P. 2041
 Factor-Litvak, P. 3597
 Fahlman, R. P. 3370
 Fairbanks, H. L. 3836, 4686
 Fairbrother, H. 4537
 Faizan, M. I. 4536
 Faizi, K. 3873
 Falagan Lotsch, P. 4553*
 Falcão, A. 3288
 Falkiewicz, K. 3717
 Fallon, J. K. 3202, 4271
 Fan, F. 2005, 4366, 4621
 Fan, L. 4499
 Fan, X. 3708, 4331
 Fan, Y. 4356, 4647*, 4651*
 Fang, T. 3812
 Fangmann, J. 3686
 Faquette, M.-L. 3922
 Faquih, A. E. 3248
 Farcas, M. 3405
 Fares, R. 3863, 3867
 Farhat, N. 3998
 Farias dos Santos, F. C. 3284*
 Farias, A. 3487
 Farmer, D. 4318
 Farooq, Z. 3048
 Farraj, A. 3514
 Farrell, K. 3473
 Farrera, D. 3540, 4629*
 Farrera, D. O. 3541, 4571
 Farsky, S. H. P. 3468
 Farstad, E. 3926
 Fäs, L. 3682, 4387
 Fasih, E. 3704*
 Fasulo, T. 3646*
 Fatima, N. 3248, 3691
 Faulhammer, F. 3442
 Faulk, D. 3064, 3075, 3076
 Faust, A. W. 4296*
 Faustman, E. M. 3391, 4101
 Fautz, R. 3326
 Fávaro, I. 3837
 Favre, K. 3715
 Favez, H. 3069
 Feaster, T. 4582
 Feaster, T. K. 3363, 4159*, 4160
 Fecho, K. 2001
 Fedorchak, N. 4129
 Feinstein, D. 4447
 Felth Tanaka, L. 3609
 Feltham, J. 4119
 Feltner, M. 3131, 3132
 Felton, R. 2016
 Felty, Q. 3903
 Fénaille, F. 3260
 Fencel, E. 3873
 Feng, F. 3942
 Feng, J. 4322*, 4599
 Feng, L. 3426
 Feng, Z. 3684*
 Fenton, S. E. 3632, 4502
 Ferber, E. J. 3158
 Ferdous, S. R. 3154*
 Ferguson, J. 3048
 Ferguson, N. 3547, 4577*
 Ferguson, P. L. 4555
 Ferguson, S. 2022, 3384, 3683, 4259
 Ferguson, S. S. 4495, 4583
 Fernandes dos Reis, M. 4243
 Fernandes-Fonseca, M. T. 3018
 Fernandez Monreal, M. 4589
 Fernandez, X. 4311a
 Fernandez, Y. 3190
 Fernandez-Ballester, L. 4006
 Ferragut Cardoso, A. P. 4596
 Ferrando, S. 4187
 Ferraresso, F. 3016
 Ferrari, E. 4230, 4699
 Ferrari, M. C. 4460
 Ferreira, C. 3979, 4516
 Ferreira, C. R. 3089, 3950
 Ferreira, I. 3809
 Ferrier, A. 3483
 Feshuk, M. 3340, 3814, 4158, 4399*
 Ficken, N. 3928*
 Fiedler, J. 3686, 4239
 Fiedler, N. 4087
 Fields, A. 3203*, 3832
 Fields, T. A. 3026
 Fields, Z. 3912
 Fiene, R. 3692, 4135, 4144, 4566
 Fierro, S. 3283
 Figueroa-Romero, C. 3419*
 Filip, I. 4392
 Filipov, N. 4220
 Filipov, N. M. 3794
 Filipowicz, A. 4648, 4653*
 Filippi, B. 3532*
 Filippi, B. G. 3682
 Filippini, T. 4187
 Fillmore, C. E. 3039*
 Finelli, J. 4121
 Finley, J. E. 3365
 Finn, B. M. 4734*
 Finnema, S. 3280
 Fioretto, M. N. 3837
 Firsick, E. J. 3206
 Firth, K. 3711
 Fischer, F. 3897
 Fischer, F. C. 3547*, 3563, 3895, 4504, 4505, 4511, 4523, 4577
 Fischer, J. 4042
 Fisher, C. 4668
 Fitch, S. 3183*, 3314, 4005, 4279
 Fitsanakis, V. 4224
 Fitzgerald, L. M. 4175
 Fitzpatrick, J. 3380, 4569*
 Flaws, J. 3203
 Flaws, J. A. 2018, 3196, 3214, 3222, 3227, 3228a, 3284, 3416, 3818, 3819, 3831, 3832
 Fleischmann, E. 3667
 Fletcher, E. 4732
 Flores, A. 3581, 4201
 Flores, J. 3096
 Flynn, B. 4487
 Flynn, K. 3359, 3360
 FNU, H. 3511
 Foley, B. E. 4158
 Foley, J. 4176
 Foley, J. F. 4330
 Foley, M. 3390
 Footz, D. 3491, 3492
 Forbes, S. J. 3861
 Ford, L. C. 3373
 Fordahl, S. 4226
 Forero, A. 3932
 Forest, T. 4420
 Forget, J. 4054, 4710*
 Forreryd, A. 3072*, 3403
 Forro, K. 4127
 Forsido, S. 3755
 Fossey, S. 3280
 Fossier, A. 2036
 Fostel, J. 4578
 Foster, M. 3249
 Foster, R. 3986
 Foster, W. 3177
 Foti, D. 3098, 3102, 3103, 3115, 3783, 4512
 Fowkes, A. 3395, 3986
 Fox, D. 3131*, 3132
 Fox, L. 3457
 Fragozo Mesa, V. 4658
 Francis, E. C. 3198
 Franco-Campos, F. 3800*
 Francois, L. 4726
 Franco-Mesa, J. 4482
 Frank, C. 3154
 Franke, K. 3653
 Franzen, A. 3664
 Fras, K. 4059
 Frawley, R. 4623*
 Frazier, B. 3560
 Frazier, B. S. 4447
 Fredrick, N. 3731
 Freed, J. 3420
 Freeman, B. 3241
 Freeman, E. 3442, 3989, 4373, 4557
 Freeman, J. L. 3088
 Freeman, J. L. 3089, 3106, 3137, 3582, 3890, 4202, 4506, 4507
 Freeman, T. S. 4567
 French, B. 4674
 Frerichs, M. 3977
 Frescura, A. 3369*
 Frey, M. 4638
 Frias Aldeguez, J. 3708, 4113
 Friday, F. 3472
 Friedman, A. 3756
 Friend, D. R. 3204*
 Friend, E. 4452
 Friend, S. 4140
 Friesner, R. A. 4354
 Frijns, E. 4166
 Frisch, J. 4010
 Fritzsche, E. 3135, 4128
 Fritz, K. 4519
 Fritzy, L. 3961
 Froger, J.-A. 3635
 Frohbergh, M. 3079
 Froim, D. 4706
 Froment, L. 3027
 Fronczek, F. R. 4627, 4628
 Frost, G. 3729, 3738
 Frost, K. 3541, 4571
 Frost, K. L. 4629
 Froud, A. 3219
 Frounjan, A. 4065
 Fry, R. C. 3217, 3844
 Frye, R. 4224
 Ftaich, N. 3268
 Fu, Q. 3565
 Fu, S. 3983, 3984
 Fu, T. Q. 3448*
 Fuchs, A. 3326
 Fujii, M. 4376
 Fujii, R. 3024, 4700*
 Fujimori, S. 3438
 Fujioka, M. 3507, 4297
 Fujishima, M. 3777
 Fujiwara, A. 4240, 4700
 Fukuda, J. 3402
 Fukunaga, S. 3333
 Fukushima, T. 4705
 Fukuyama, T. 3490*, 3954
 Fulton, N. 3924
 Fung, E. 3993, 4002, 4325
 Fung, E. S. 3305*, 3306
 Funk, C. 4371
 Funk-Weyer, D. 3147, 4026*, 4372
 Furlong, C. E. 3923
 Furmanski, P. 4685
 Fuselier, S. G. 3587
 Fyle, M. 3514, 4626, 4675*
 Gabal, E. 4371*
 Gaboyan, S. 4065
 Gabrielson, K. 3452, 3453, 4635
 Gabrielson, K. L. 3056
 Gaca, M. 3456
 Gaddameedhi, S. 3876
 Gaddi, E. 4549
 Gadol, H. J. 3732
 Gaffney, J. 3700
 Gagnon, N. 3782, 3785
 Gahagan, E. 3558*
 Gaikwad, H. 3263
 Gaikwad, S. 4680
 Gaitens, J. M. 4184*
 Gaither, K. A. 4573
 Galati, M. 3731
 Galbiati, V. 3381, 3435*
 Galdieri, L. 3527, 4499
 Galib, M. 3977
 Galiciani Pedro Fernandes, M. I. 3819
 Galicioli, M. 3765*
 Gallagher, K. 3746
 Gallego, D. F. 2011, 4541
 Gallego, M. P. 3192
 Gallegos, A. 2034
 Galloway, C. 3911
 Gan, J. 3873
 Gane, A. M. 3781
 Ganesh, B. 4076
 Ganglbauer, J. 4505
 Ganne, M. 3270
 Ganta, C. 4626
 Ganta, C. K. 4649
 Gao, F. 3720, 4356, 4357, 4359*, 4363, 4393, 4647, 4651
 Gao, L. 3936, 4317, 4319*
 Gao, P. 2003, 3538
 Gao, X. 3647*, 4053
 Garced Diaz, A. 4518, 4520
 Garcia, A. G. 4547
 Garcia, J. 2025, 3757
 Garcia, J. 3703, 3704
 Garcia, M. 2012, 3830, 4528, 4541
 Garcia, M. A. 2011, 4529, 4536, 4548, 4554
 Garcia, M. G. 4551
 Garcia, M. I. 3363*
 Garcia-Aguilar, M. D. R. 3586*
 Garcia-Mares, M. 4092
 Garcia-Mares, M. A. 3795*
 Garcia-Sanchez, J. I. 4286*
 Gardella, J. M. 4025*
 Gardner, C. 3173
 Gardner, E. M. 3448
 Gardner, J. 3122
 Gardner, K. 3279
 Gargano, M. 3028
 Gargas, N. 3297
 Garikipati, V. 3907
 Garner, A. 3426
 Garnica Acevedo, D. P. 4365*
 Garrison, C. 2036, 4130
 Garry, A. 4230, 4699
 Garry, M. 4180
 Garside, H. 4696
 Garzon-Ramirez, M. 4266*, 4668
 Gassman, N. 3463
 Gassman, N. R. 4316*
 Gatdula, C. 3184
 Gaudenzio, N. 3372
 Gaulke, C. A. 4103, 4104
 Gautam, L. K. 4145
 Gaviria, C. 3262*
 Gawali, V. S. 3278
 Gaxiola, V. 4207
 Gbadegoye, J. 4731
 Gbegbeawu, C. E. 3274
 Gebremariam, B. 4061*
 Gee, R. 4039*
 Gehen, S. 3299
 Gehl, A. M. 3185*
 Gehring, T. 3083*
 Gehrke, H. 3082*, 3083, 3255
 Geier, M. 4478
 Geier, M. C. 3321, 3991
 Geiger, R. 4582
 Geiger, R. M. 3363
 Geisenfelder, L. 3554
 Gelfand-Titiyevskiy, B. 3940*
 Gelhaus, S. L. 3705
 Gelli, A. 3588
 Gendrin, C. 4169
 Genet, V. 3635
 Geng, Y. 3169, 3189
 Gentile, N. E. 3632*
 Gentry, P. R. 4021
 Geoly, F. 2006
 George, A. 3569*
 George, J. 3144
 George, J. A. 3098*, 3099, 3102
 George, J. V. 3134, 3911*
 George, J. W. 3838
 George, S. 4233
 Georgiades, I. 3404*, 3445
 Gerami, M. 3443
 Gerasuto, A. 3535
 Gerberick, F. 3510
 Gerhold, D. 3586, 4260
 Gerhold, D. L. 4261*
 Gerrish, K. 4310
 Gervacio, S. 3371
 Geter, D. 3716*, 4090
 Getz, K. 4271
 Geyer, L. 3895*
 Ghaderi, I. 4629
 Ghaffari, R. 3294
 Ghanem, C. I. 4428*
 Gharaie, S. 3452, 3453*
 Ghatnekar, P. 3443*
 Ghayur, M. N. 4654
 Ghimire, S. 3711
 Ghorab, R. A. 3840
 Ghorab, R. A. B. D. 3838*
 Ghosh, D. 3141
 Ghosh, O. 2041
 Ghuneim, S. M. 3835
 Ghuneim, S. S. 2017*
 Gi, M. 3507*, 4297

G

Giau, A.	3687	Goodman, D.	3770	Groopman, J.	4637	Hall, L.	2023, 4578
Gibb, M.	3234*, 3244, 4665	Goodman, J. E.	3747	Gross, J.	2012	Hall, M.	4259
Gibbons, S. M.	4614	Goodrich, J.	3898	Groux, H.	4311a*	Hall, N.	4464*
Gibson, M.	3292, 3559	Goodrich, J. A.	3632, 3897, 4275, 4504	Gu, E.-Y.	3174	Hall, S. J.	2015
Giddabasappa, A.	3447	Goodson, M. L.	4496	Gu, H.	3108, 3953, 4186	Haller, S. T.	4674
Gietz, A.	4239	Goodwin, L.	3923*	Gu, Q.	4749*	Hama, A.	3024
Giffin, M.	2035, 3749, 3751	Goodyke, A.	4720	Gu, W.	4065	Hamadeh, A.	4023
Gijzen, L.	3031, 4118	Gopala, S.	3428*	Gu, Y.	4139	Hamaji, C.	3993*
Gilbert, A. P.	3006, 4612*, 4616	Gordon, K. L.	4694	Guardini, L.	4311a	Hamdoun, A.	4465
Gilbert, A. P. G. P.	4613	Gordon, T.	3464, 3914	Guarino, A.	3891	Hamida, H.	3009*, 3789, 4287
Gilberto, V.	4212	Gorga, C.	3010	Guenat, O. T.	3469	Hamilton Jr., R. F.	4540
Gilbreth, P.	3966	Goriainova, V.	3125*	Guerra-Menendez, L.	3581	Hamilton, C. M.	4384
Giles, B.	3785	Gorr, M. W.	4069	Guerra-Menéndez, L.	4201	Hamilton, M.	3723
Gillera, S. E. A.	3149	Gorzoni, A. B. R.	3205	Guerrero Arroyo, J.	4423	Hamilton-Brehm, S. D.	4452
Gillette, J.	3480, 3792, 3921	Gossai, D.	3754	Gueye, F.	3769	Hamlin, R.	3519
Gilliland III, S.	3456	Gosse, J. A.	3427, 3429, 4416, 4730	Gueye, S. F.	3101, 3113, 3778	Hamm, J.	3364
Gillio Tos, E.	4381	Gossmann, M.	3277, 4235, 4242*	Guffroy, M.	3280	Hammerlindl, H.	4415
Gillman, I. G.	3475	Gosting, S.	3972	Guicheney, C.	3300	Hammock, B. D.	3237
Gillois, K.	3277	Goswami, D.	3238	Guilarte, T. R.	3120	Hammontree, M.	3802*
Gilmour, I.	3514	Goto, A.	3806*, 4045	Guilbert, C.	3779, 3785*	Hampson, A.	4041
Gilmour, M. I.	3494, 3506	Goto, M.	3431	Guili, L. N.	3527	Han, B.	4209
Ginesta, J.	3372	Gottardo, E.	3323	Guillouzuic, J.	3635	Han, C.	3680*
Gingerich, D. B.	3422	Gottesman, H. L.	4210*	Guilick, P.	3449, 4734	Han, D.	3703
Gioia, R.	4475	Gould, G. G.	3180	Gullapalli, R.	3771	Han, K.	3174
Girand, M.	3524, 3853*, 4236	Gould, J.	4712	Gullapalli, R. R.	2011*, 3695	Han, K.	4386
Giri, R.	4347	Goulett, N.	3636	Gum, G.	4338	Han, K. S.	4350*
Girireddy, M.	4012*, 4564	Gour, A.	3843	Guntur, K.	3255	Han, S.	3766*, 4073, 4275, 4666
Girmay, T. H.	4184	Gow, A.	3450, 3925, 4028	Guo, C.	3450, 3484, 3925, 4028, 4337	Han, S. J.	3197
Givens-Burton, A.	3419	Gow, A. J.	3241, 3410, 3928, 3929, 3934, 3938, 3939, 3940	Guo, G.	3410*	Han, V. X.	3371
Glaab, W.	4698	Gowadia, N.	3996*	Guo, G. L.	3880	Han, W.	4572
Gladwell, L. R.	4245	Gowda, B. S.	3055	Guo, J.	3535	Han, Y.	3046, 3391, 3595, 3596, 3597, 3600, 3601
Glass, M.	4082	Gowdy, K.	3943	Guo, L.	4711	Han, Y.-G.	3499
Glibreth, E.	3416	Gowdy, K. M.	3927, 3932, 3941	Guo, T. L.	3670, 4748	Hancock, R.	3870, 3907, 4040
Glos, E.	3131	Gozalbos, R.	4327	Guo, X.	3334*, 4238*, 4749	Handley, C.	4168
Gluth, T.	4252	Graber, A.	4508	Guo, Z.	3553, 3672, 3758, 4258, 4304*	Haneoka, M.	3258
Gluth, T. D.	3212*	Grace, C.	2009	Gupta, A.	3456, 4162	Hanna, A. M.	3527
Go, D.-M.	4335	Graceli, J. B.	3284	Gupta, D.	3925*	Hanna, N.	3619
Go, M.	4265	Gradin, R.	3072, 3403	Gupta, E.	3446	Hannon, P.	2017
Go, Y.-M.	3595, 3597, 3655, 3786*, 4204, 4280, 4585, 4586, 4689	Grady, K.	3250, 3545, 3676	Gupta, I. R.	3922	Hannon, P. R.	3835
Goddard, Y.	4555	Grady, S. L.	4011	Gupta, R.	3485, 3913, 3923	Hansen, C.	4239
Godin-Cuadrado, M.	4482*	Graf, K.	3678	Gupta, S.	3215	Hansen, T.	3518
Godino, A.	4236	Graham, A. A.	4656	Gurcan, A.	3852	Hao, Y.	4244
Godlewska, K.	3079	Graham, C.	3079*	Gurevich, I.	3692	Haque, F.	4505
Goebel, C.	3326	Graham, D. L.	3578*	Gurkoff, G. G.	3589	Harbison, R.	2013
Goedken, M.	3173, 3202	Graham, M.	3603, 4213	Gurle Albayrak, E. Y.	3200	Harbolic, A.	3170*
Goedkin, M.	3000	Granaldi, K.	3857	Gutierrez, A. M.	3190	Harbollic, A.	3170*
Goel, S.	3263	Grangier, N.	3469	Gutierrez, A. M.	4422, 4618	Harden, L.	3956
Goetz, A.	3344, 3989, 4008*, 4015, 4093	Grant, S.	3095	Gutierrez, M.	3405, 4030	Hardesty, J.	3010
Goff, R. V.	3775	Grasselli, E.	4187	Gutsfeld, S.	2029, 4403*	Hardesty, J. H.	4501
Gogzheyman, C.	3131, 3132*	Graves, A.	3637	Guttenberg, M. A.	3251*	Hardin, S. E.	3120*
Gohil, V. M.	3040	Gravino, F.	3323*	Guzman, D. S.	4496*	Hardonniere, K.	3260
Gokce, N.	4232	Gray, E.	4537	Gwinn, K.	2024	Hardwick, R. N.	2036
Golden-Mason, L.	4275	Gray, G.	4006	Gwinn, W. M.	4626*	Hardy, C.	3536
Goldman, S.	3472	Gray, J.	3415, 3416	Gwon, J.-H.	4335	Hargrove, M. M.	3344*
Goldsmith, T.	4467	Gray, J. P.	3417			Hari, A.	4383*
Goldsmith, W. T.	3491, 3492*, 3958, 4231	Grayon, A. R.	3223*			Hariri, M.	3152*, 3153
Goldstein, K.	3400	Grayson, C.	4089*			Harischandra, D.	3821, 3866
Goldstone, J. V.	4433, 4437	Graziano, M.	4140			Harischandra, D. S.	3218*
Gollamudi, S.	3069	Greco, D.	4402			Harkema, J. R.	3958, 4684
Gollapudi, B. B.	4684	Greco, S.	4389			Harlin Laven, H.	4732*
Gollner, M. J.	3916	Green, A. J.	4017, 4358, 4360*, 4394, 4395			Harmon, A.	3750
Gombar, V. K.	4360, 4394*, 4395	Green, E.	4434*			Harn, D. A.	3794
Gomes, C.	4026, 4372*	Greenbergs, K.	3037			Harney, J.	4106
Gomes, D. F.	4454	Gregg, B.	3611, 3807			Harold, R.	3115, 3783
Gomes, J.	4422*	Gregory, A.	3122			Harper, C.	2017
Gomes, J. A.	3190*, 4618	Greig, D.	4514*			Harper, C. V.	3835
Gomez Jenkins, M.	4429	Gribble, M. O.	4606*			Harper, D.	3492, 4231
Gomez, G.	4498*	Grice, E. A.	4608, 4609			Harrill, J. A.	3362, 4152, 4153, 4155
Gomez-Caballero, D.	4484*	Grieger, K.	4741			Harriman, A. N.	3596*
Goncalves, C.	3785	Griend, D. V.	4109			Harrington, C. F.	4640
Gong, L.	3008, 4289, 4719	Grier, T.	3770*			Harrington, L. M.	3749, 3751*
Gonkowski, S.	4455	Grier, T. R.	4588			Harrington, N. M.	3196
Gonzales, R. A.	3095	Griffen, E. J.	3396			Harris, F.	4152
Gonzalez Alvarez, E.	4470	Griffin, G. C.	3101, 3113, 3769, 3778			Harris, F. R.	3362, 4153, 4155
Gonzalez Fuentes, A.	4520	Griffin, K. L.	3455*			Harris, J. S.	3723*
Gonzalez Pons, R.	4295	Griffith, J.	3700			Harris, N.	4384
Gonzalez Rivera, C. M.	3969	Griffith, S.	3409*			Harris, P.	4519
González Rivera, C. M.	3947	Griffith, S. T.	4265			Harris, R.	3634
Gonzalez, A.	3965	Griffith, W. C.	4101			Harris, S.	3828, 4306, 4678
Gonzalez, G.	3133, 3188	Grimes, M.	4514			Harris, S. M.	4731
Gonzalez, H.	4425	Grimm, S. L.	4349			Harris-Brown, T.	3711, 3809*
Gonzalez, H. C.	4694*	Gripshover, T. C.	4501			Harrison, F.	3092
Gonzalez, K. N.	3773	Gritton, H. J.	4213			Harrison, F. E.	3090, 3109, 3580, 4203
Gonzalez, R.	3150, 4420, 4622, 4698	Grizzle, D.	3765			Harrison, L.	4168
Gonzalez, R. J.	2009, 4490, 4723, 4729, 4733	Grodzki, A. C.	3591*			Harrison, L. K.	3608
González-Alvarez, E. M.	4469	Gromelski, M.	3717			Harry, J.	4625
Gonzalez-Estrella, J.	2012, 4554	Grommes, M.	4692			Hart, K. D.	3000
Goodla, L.	4293	Groom, S.	3517			Hart, R. P.	3595
						Harter, I.	4219
						Hartley, K.	3901

Hartman, J.	3415, 4425	Henson, S. N.	3971*	Hogberg, H.	3815, 4032	Huang, S.	4244, 4349
Hartman, J. H.	2040, 4503, 4694	Heo, S.	3019, 3605*, 4615	Hogberg, H. T.	3364	Huang, X.	3280, 3343, 3857
Hartmann, J.	3417	Herbert, G.	3425, 3953, 4541	Hoh, E.	2016	Huang, Y.-T.	3161
Hartung, T.	2004, 4282	Herbert, G. W.	4592	Hok, S.	3972	Huang, Y.-W.	3471
Harvey, D. J.	3237	Herbert, R.	3594	Hokamura, K.	3024*	Huang, Z.	4487
Harvey, K.	4183	Herbstman, J. B.	3223	Holder, A. L.	3506	Huayta, J.	3575*
Harvison, P.	3684	Hering, S.	4248	Holder, R. E.	4603	Huber, E. A.	4125*, 4148
Hasan, A. K. M. M.	4444*	Herman, D.	3487, 3842, 4207	Holian, A.	3489, 4288, 4456, 4540	Hudak, H. L.	4300
Hasbani, R.	3657	Herman, D. A.	3515*, 4064, 4343, 4667	Hollander, J.	3981, 4467	Hudson, B.	4220
Hasegawa, A.	4574*	Hermetz, K.	4652	Hollanders, K.	4166	Hudson, B. T.	3794
Hasen, I.	3487, 3515, 4207	Hernandez, A.	3463, 4316	Hollings, M.	3377*, 3456, 4077, 4081	Hudson, L. G.	3629, 4190
Hasgall, P.	3079	Hernandez, I.	4038	Hollister, L.	3548	Huebert, S.	3074*
Hashim, N. U. B.	3060	Hernandez, M.	3354*	Hollon, M.	4572	Hueser, A.	4090
Hashimoto, K.	3199	Hernández, M.	3118*, 3804	Holmes, E.	3971, 4642	Hughes, M. F.	3906
Hashsham, S. A.	4019	Hernandez, R. M.	4554	Holmes, W.	3249	Hugi, A.	3027
Haspeslagh, E.	3031	Herr, E.	3290	Holst, M. M.	4447	Hui, J.	3177
Hass, E.	3446, 4736	Herrera, R.	2012, 4528*	Holt, S.	3379*	Hui, X.	4256
Hassan, M. T.-A.	3904	Herrera, R. M.	4548	Holth, A.	3074	Huleatt, J.	4708
Hassan, Y.	4074	Herrmann, M.	4241	Holz, G. G.	4601	Hummel, J.	3070*, 3744
Hasselgren, C.	4352, 4355	Herwig, J.	4149, 4156	Holzer, A.	4212	Humphries, C.	3861*
Hassounah, F.	4414	Herzig, M. X.	4548	Holzmayr, S.	3082	Hundling, J.	3711
Haston, C.	3694	Herzke, D.	2029	Hombink, P.	4117, 4118	Hung, G.	3068*
Hatai, D.	3843, 4062*	Heselton, M.	4452	Honegger, T.	3268	Hunker, J.	3277, 4235
Hatano, K.	3253, 3257	Hess, S. T.	3427	Hong, A.	4587	Hunnerdose, A.	4710
Hattori, K.	3389	Hesse, C.	3686	Hong, C.	3189	Hunt, P. R.	3358*
Haug, L.	2029	Hewitt, N.	3326	Hong, S.	3880	Hunter, M.	4550
Haupt, L. J.	3689	Hewitt, P.	3675, 3690, 3693, 3710, 4112	Hong, S. H.	3351	Hunter, R. P.	3212, 4252*
Havens, L.	3901	Heyward, S.	4141, 4170, 4178	Hood, D. B.	3422*	Hurley, A.	4060, 4066*
Haverfield, J.	3922*	Hickman, E.	3700	Hoque, T.	3539	Husain, H.	4468*
Hawkins, E.	3240, 3593	Hickman, J.	3620, 4660*	Horcas-Nieto, J.	3175	Hüsken, S.	4128
Hawley, D.	4186	Hickman, J.	2008	Horgan, M. J.	4211	Hussain, A.	4468
Haws, L.	3314	Hida, N.	4207*	Horio, E.	3441	Hussain, R.	3965
Haws, L. C.	3324	Hietpas, C.	4537*	Horn, S.	4672*	Hussain, S.	3809, 3859, 3941, 3958, 4231, 4467
Hayakawa, Y.	3432	Higashiyama, G.	3824	Horn, T. L.	4041	Hussein, B.	4341
Hayashi, R.	3216*	Higgins, L. G.	3442*	Hornburg, K.	4703	Hutchens, S.	3095
Hayashida, K.	4333	Higuchi, M.	3494	Hornung, E.	3594, 3911	Hutchins, Z.	4284
Hayden, E. T.	3835	Higuchi, T.	4171	Horvat, J.	4420*	Hutter, V.	3461, 4661
Hayden, P. J.	4175*	Higuchi, Y.	4584	Horzmann, K.	3946	Hutton, G.	3927, 3932
Hayek, E. E.	2011, 4529	Hike, N.	4046	Horzmann, K. A.	3153	Hutton, G. M.	3941
Hayes, M.	3561	Hikida, Y.	3254, 4234	Hoshino, Y.	3152	Huynh, T.	3914, 3992*
Hayes, W.	3802	Hilberer, A.	3266, 3999, 4107	Hosokawa, Y.	3824*	Hvzidak, M.	2027*
Hays, S. M.	3312	Hilditch, J. M.	3451*	Hou, H.	3333	Hwang, G.-W.	3777*
Hazari, M.	3514	Hill, E. M.	4679*	Houle, C.	4073	Hwang, H.	4354
Hazel, B.	3018	Hill, J.	3087*	House, E. L.	2006, 3213	Hwang, J.	4596
Hazemi, M.	3360	Hill, S.	3536	Howard, B.	4097*	Hyland, P.	3363
Hazenoot, M.	4726	Hiller, M.	4726	Howard, B. E.	3355	Hyun, J. Y.	3701
He, B.	3189*	Hiltebrand, O.	3460*	Howard, K.	4007*, 4017, 4307, 4358*, 4360		
He, C.	3725	Hilton, G.	4015	Howard, K. E.	3452		
He, F.	3023, 3387, 3388, 3533, 3538, 3681, 4368	Hilton, J. M.	4168	Howard, T.	3056, 3453	Iakoucheva, L.	4498
He, M.	3760	Himmelfarb, J.	3034	Howard, T.	2012	Iakoucheva, L. M.	4543
He, S.	3660	Himmelstein, M. W.	4559*	Howard, W.	3297	Iam, J.	4498
He, X.	3465*	Hinchcliff, B. J.	2030	Howe, Z.	3594	Ibanez, C.	4009
Head, B.	3734, 4557	Hinckley, C.	4124, 4178	Howes, M.	3534*	Ibarburu, I.	3192
Headrick, K.	3712*, 3713	Hinerman, J.	3664	Hoyer, J.	4231	Ibinson, N.	4060
Healy, S.	3342	Hines III, J. E.	4439*	Hoyo, C.	3607	Ibrahim, M.	4429
Hearon, S.	3442	Hines, D.	4384*	Hoyt, C. J.	3158	Ibrahim, S.	3194
Heck, J. E.	4276	Hines, E.	3871, 4037, 4038	Hoyt, K.	4487	Ibrez, M.	3729
Heck, K.	3639	Hinokuma, K. D.	3205	Hsia, F. K.	3745	Igarashi, K.	4071
Hecker, M.	3767	Hintzsche, H.	3624	Hsiao, P.	4541	Igarashi, M.	3441
Hedge, A.	3218, 3219	Hipps, H.	4498	Hsieh, J.-H.	2023, 3648, 4032*, 4151	Igbinosa, I. H.	4640
Hedlich-Dwyer, J.	3463, 4316	Hirabayashi, Y.	3402, 3860	Hsieh, Y.-C.	3652	Igharo, E. L.	4640
Heijmans, J.	4118	Hirano, S.	4071	Hsu, C.-t.	3091	Igharo, O. G.	4640*
Heikal, D.	4251	Hirao, T.	3432	Hsu, H.-W.	3487, 4207	Ilgwe, C.	3459*, 4061
Heim, K. E.	3736	Hiroaki, S.	3758	Hsu, J.	4499*	Ihmig, F.	4741
Hein, D. W.	4677*	Hirose, N.	3349	Hsu, J.-H.	4445	Iida, M.	4378
Heine, L. K.	3773, 4529*, 4592	Hirota, K.	3054	Hu, A.	4490	Ijichi, N.	4050
Heinig, S. A.	3012, 3705*	Hirway, S.	4161	Hu, C.	3788*	Ikawa, M.	3860
Heintz, M. M.	3324*	Hirway, S. U.	3564*	Hu, C. J.	3830*	Ikeda, A.	3498
Heldman, S.	3287	Hleb, K.	4042	Hu, D.	3018	Ikeda, K.	3216
Heldt, G. H.	4458	Ho, C.	4186	Hu, H.	3223	Ikeuchi, Y.	4314
Helewicz, S. C.	3772, 4301*, 4592	Ho, N.	3720*, 4357	Hu, J.	4172*	Ilieva, N. M.	3574
Hellmann, J.	4468	Hoang, J.	3873*	Hu, J. C.	3392	Ilisley, N. P.	3202, 4271
Helmbreck, J.	2015	Hobbs, C. A.	4330	Hu, K.	4368*	Im, E.	3467*
Helmke, P.	4486	Hobi, N.	3027, 3469*, 4166	Hu, K.	3823	Imaida, K.	3174
Hembury, M.	3810	Hocevar, B. A.	4510	Hu, M.	3538*	Imakura, Y.	4566*
Hemmerich, C.	3459	Hock, B.	3041	Hu, Q.	3450*	Imamura, M.	3254
Henderson, B.	4085	Hodroj, R. K.	4175	Hu, T.	2007	In, J. G.	4541
Henderson, R. G.	3664*	Hoebart, C.	4248*	Hu, W.	3028, 3755*, 3768, 4280, 4414, 4494, 4689	Ingermanson, R. S.	4168
Hendriks, G.	3175, 3398*, 4315*	Hoedlmoser, C.	3663*	Hu, X.	3172*, 3846*	Ingram, C.	3523*
Hendriksen, Y.	4113	Hoer, D.	3339*, 4154, 4579	Hu, Y.	3547, 3563	Inman, Z. C.	3832*
Henn, A. D.	3274*	Hofacre, K. C.	4162	Hu, Z.	3512*	Inomata, A.	3394, 4234
Henriquez, J.	4091*	Hofer, T.	4009	Hua, C.	3010, 3616, 3617, 4501	Inoue, H.	4050
Henry, B. J.	3888*, 3900*	Hoffman, E.	3250*, 3461, 4661	Hua, Y.	3342	Inoue, T.	3809
Henry, C. M.	3038*	Hoffman, J. W.	3352*	Huang, A.	3546*, 4499	Ipaye, I.	3815
Henry, O.	4170	Hoffmann, H.	3201	Huang, B.	3209, 4256, 4632	Iqbal, S.	4342
Henry, Z.	2023*	Hoffmann, N.	4239	Huang, J.	3656, 4197, 4200	Ireland, D.	3356*, 3587
		Hoffmann, S.	3173, 3255	Huang, L.	3586, 3726, 3727, 4150, 4151, 4259, 4419, 4495	Irizarry, A.	3400
		Hoffnagle, G.	3988	Huang, R.		Irshad, M.	4014
		Hofstra, A.	3551				
		Hofstra, A. H.	4095*				
		Hogan, C.	3968				

- Irwan, J. 4389
 Irwin, J. 4464
 Isaacs, K. K. 3562, 4189*
 Ishida, K. 3041*
 Ishida, S. 4171*
 Ishihara, Y. 4179
 Ishikawa, A. 3402
 Ishikawa, T. 3119, 4237, 4646
 Iskandar, B. 4129
 Islam, B. 3357
 Islam, D. T. 4019
 Islam, S. 3907
 Islas-Robles, A. 2020*, 3266*, 4107
 Ismail, R. 3372
 Iso, T. 3349
 Ito, K. 3432, 3438
 Ito, T. 4725
 Ito, Y. 3162, 3166, 3258, 3430
 Ivanov, N. 4072
 Ivanov, N. V. 3475*
 Iwabe, S. 3850*, 3865
 Iwasa, H. 3253, 3257
 Iwashita, K. 3333
 Iwobi, N. 3157*
 Iyer, M. 3010
 Iyer, N. 4129
 Izadi, A. 3589
 Izu, J. M. 4629
 Izumi, M. 3333*
 Izzo, L. 3659
- J**
- Jabba, S. V. 4085
 Jachak, A. C. 4556*
 Jacketti, M. 3477*
 Jackson, B. 3551, 4093
 Jackson, J. 4458, 4476
 Jackson, M. 4578*
 Jackson, P. A. 4072
 Jackson, T. W. 4149*
 Jacob, J. 3127*
 Jacobs, A. 4166
 Jacobs, F. 4326
 Jacobs, J. 4140
 Jacobs, K. 3342
 Jacobson, A. R. 3807
 Jadhav, A. 4044
 Jadhav, G. 3248*
 Jadhav, S. L. 4488*
 Jaeschke, H. 3011, 3026, 4737
 Jafaar, C. 3466
 Jagiello, K. 3717*, 4009
 Jagoo, V. 4067*
 Jahan, N. 4268*
 Jahic, M. 3527
 Jaimes, E. 3020, 3464
 Jain, S. 3013, 3014*, 3798*, 4134, 4342
 Jakaria, M. 4417*
 Jalalahmadi, G. 4037, 4038
 Jamal, H. 3124*
 Jamalpoor, A. 3175
 James, K. A. 4602
 James, N. 3711
 Jamin, A. 3687
 Jan, Y.-H. 3231*
 Jana, A. 4324
 Janarthanam, C. 3569, 4636
 Janeczko, K. 3273
 Jang, M. 4514
 Jang, M. S. 3174
 Janga, S. C. 2028
 Janiga-MacNelly, A. M. 4558*
 Janjetovic, Z. 4608
 Jankovic, K. 3021, 3537*, 4257
 Jannusch, T. 4436
 Janson, L. J. 3977
 Jao, T.-M. 4448
 Jarabek, A. M. 4156
 Jarrell, M. 3029
 Jarrell, Z. 4586
 Jarrell, Z. R. 3655, 3755, 3786, 4204, 4585*
 Jasiek, G. 3390
 Jaspers, I. 3521, 3620, 4029, 4664
 Javed, A. 4654*
 Jaworek, T. 3564
 Jayanth, N. 3536
 Jayaraman, A. 3214, 3604
 Jayasundara, N. 4434
 Jaymay, T. 4009
- Jazari, D. A. 3140
 Jee, A. 4712*
 Jefferis, T. 3455, 3501*, 4489
 Jegede, O. P. 3960, 4188*, 4194
 Jehmlich, N. 3141
 Jen, K. Y. 4649
 Jenkins, G. J. S. 3395
 Jenkins, P. M. 3105
 Jenkins, T. 3737
 Jenness, D. 4634
 Jennings, H. G. 3396, 3397
 Jennings, J. 4039
 Jennings, S. R. 4228
 Jennings, T. 4068
 Jenson, K. K. 3535
 Jeon, J. 3465, 3655, 4204
 Jeon, J. H. 3165
 Jeon, S. B. 3174
 Jeon, Y. 3007
 Jeong, B. 3499*
 Jeong, W. 3164
 Jerred, M. 2014
 Jesse, H. 4112*
 Jewell, C. 3530
 Jewell, P. 3308
 Jeyasingh, P. 4440
 Jhuang, J.-H. 4418
 Ji, B. 4367
 Ji, C. 3648*
 Ji, H. 3952, 3971, 4292, 4642*
 Jia, J. 3679*
 Jia, X. 3626*, 4367
 Jia, Y. 4521*
 Jia, Z. 4226
 Jian, X. 3942*
 Jiang, C. 3202, 4271*
 Jiang, D. 3670, 4748*
 Jiang, G. T. 4016
 Jiang, H. 4305
 Jiang, K. 3447
 Jiang, S. 4319
 Jiang, T. 3953
 Jiang, W. 3050, 4670
 Jilek, J. L. 3522*, 4629
 Jilling, T. 4555*, 4668
 Jima, D. 3975
 Jiménez Córdova, M. I. 4423
 Jimenez, N. 3873
 Jimenez-Chavez, A. 3515
 Jimenez-Chavez, A. 4064
 Jiménez-Chávez, A. 4343*, 4667
 Jimenez-Chavez, J. 3962
 Jin, C. 4650
 Jin, H. 3122, 3165, 3374, 3499, 3569, 3571, 3572, 4636
 Jin, X. 4749
 Jing, L. 3759
 Jing, X. 4145, 4284, 4337*
 Jo, H. 3007, 3187
 Jo, M. 3391
 Jo, W. 3146
 Jo, Y. 3351
 Joel, J. 4661
 Joglekar, R. 3320
 Johansson, H. 3072, 3403*
 Johns, A. J. 3248
 Johnson, A. 3530
 Johnson, B. 3160, 3279, 3956*
 Johnson, C. 4715*
 Johnson, D. 4044
 Johnson, G. A. 3594, 3911
 Johnson, K. 3299, 3380
 Johnson, M. A. 4210
 Johnson, N. 4193, 4290, 4630, 4637
 Johnson, N. M. 3495, 4182
 Johnson, R. B. 4733*
 Johnson, R. J. 3033
 Johnson, T. 2009, 3156
 Johnson, V. J. 3255
 Johnston, E. 3127
 Johnston, K. 4098
 Johnston, S. 3710, 4170
 Jojima, K. 4376*
 Jojy, M. 3170
 Jolliet, O. 4200
 Jolly, R. 3305, 3306*
 Jones, D. 3473, 4586
 Jones, D. P. 3028, 3595, 3597, 3655, 3755, 3786, 4204*, 4280, 4585, 4689
 Jones, E. 3002, 3015*
 Jones, G. 4146
- Jones, K. 3396, 3397, 3504
 Jones, L. 4696
 Jones, L. L. 3952
 Jonkers, D. M. 3671
 Joore, I. 4117*, 4118*
 Jordan, M. 4239
 Jordan, N. 3850, 3865
 Jordan, T. 3145, 3609
 Jordt, S.-E. 3249
 Jorge, B. 3827
 Joseph, J. 4652
 Joseph, L. B. 3231, 3242*, 3245, 3935
 Joshee, L. 3765
 Joshi, A. D. 4538
 Joshi, K. 3328*
 Jovanovic, S. 2019
 Joy, M. 3020, 3022
 Joyce, A. 3931*
 Jozwicka, T. 4608
 Juan, C. 3654, 3658
 Juan-García, A. 3654*, 3658
 Juárez Murguía, R. 4412*
 Jude, J. 3496
 Judzińska, B. 4009
 Juenke, A. R. 3410, 4273*
 Jung, W. 3955, 4538
 Jung, W. K. 4676
 Jung, Y. 3632
 Juran, R. 3466
 Jurczak, M. 2039
 Jureziz, A. N. 3316
 Jurglewicz, A. 4153*, 4155
 Jursa, T. 3095
 Justiniano, R. 3326*
- K**
- Kabir, E. 4070*, 4666, 4746
 Kabirov, K. 4042, 4704
 Kachingwe, B. 4262
 Kaddurah-Daouk, R. 4375
 Kaden, T. 3678*
 Kader, S. 3141
 Kadota, T. 4240, 4700
 Kaehr, A. 3493*
 Kahrizsangi, J. G. 4006
 Kaja, S. 4341*, 4342, 4347
 Kakar, K. 4287
 Kakehashi, A. 3507, 4297
 Kakiuchi-Kiyota, S. 3561, 4355
 Kalafut, D. 3492
 Kalashnikova, A. 3321, 4099, 4478
 Kalashnikova, A. A. 3991, 4477*
 Kalian, A. D. 3660
 Kalimtzis, C. 3078*
 Kalinowski, T. P. 3794*
 Kalkir, F. 3200
 Kalpattu, S. 3975
 Kalstabakken, K. 3740
 Kalter, J. M. 4199
 Kalyan, S. 3079
 Kam, Y. 4164*
 Kamal, A. H. 4349
 Kamberi, M. 3062, 3080, 3084, 3325, 3329
 Kamel, A. 4580
 Kamendulis, L. M. 4510*
 Kamezaki, A. 4725
 Kamineni, V. N. 4396
 Kaminski, N. 3449
 Kaminski, N. E. 3275, 4720, 4721, 4734, 4735
 Kamper, J. 3027
 Kamperschroer, C. 4111
 Kaname, N. 4050*
 Kanchi, K. L. 3053, 4308*, 4309, 4310
 Kaneki, A. 3728
 Kaneki, M. 3490, 3954
 Kanemaru, Y. 3456
 Kang, B.-C. 3187
 Kang, H. 3797*
 Kang, J. 3374*
 Kang, M. J. 4056
 Kang, S. 4390
 Kang, S.-M. 4585
 Kang, T. 3967*
 Kang, W. 3384*
 Kang, Y. 4305, 4388
 Kangro, K. 3017
 Kanie, M. 4071
 Kanina, A. 3788
- Kannan, A. 2018, 3839
 Kannan, R. 3535
 Kannappan, V. 4086*
 Kanno, A. 4700
 Kanno, S. 4071*
 Kant, R. 3246, 3263
 Kanthasamy, A. 3122, 3569, 3571, 3572, 4636
 Kanthasamy, A. G. 3571, 3572
 Kapeghian, J. 4697
 Kapila, Y. L. 4560
 Kapinus, T. 4241
 Kaplan, B. 4727
 Kapraun, D. F. 4581*
 Kapre, R. 3319
 Kapuriya, P. B. 3193a
 Karakoltzidis, A. 4009
 Karaman, O. 4241
 Karaman, S. 4035
 Karandashova, S. 4079
 Karchalla, L. 3367*
 Karchner, S. I. 4433, 4435, 4437
 Karimi, B. 3637
 Karmaus, A. 3289*, 3347, 3551, 4015, 4093, 4373
 Karnwal, S. 3527
 Karschnik, T. 4010
 Kasaraneni, N. 4065, 4079
 Kashiwagi, E. 4705
 Kasparek, A. 3359
 Kassotis, C. D. 3287, 3621, 3623
 Kasteel, E. 4535*
 Kastrup, C. 3016
 Kasuske, Z. A. 4408*
 Katahira, Y. 3441
 Katalinic, M. 4059
 Katchmann, B. 2024
 Katenahalli, N. 4044
 Kato, H. 4071
 Kato, R. 3049*
 Katz, S. A. 3378*
 Katzner, T. 3770
 Katzner, T. E. 4588
 Kaufman, L. E. 3764
 Kaur, G. 4115, 4536*
 Kaur, M. 4546, 4549
 Kaushik, R. 4691*
 Kawabe, M. 3174
 Kawai Puua, K. 4219*
 Kawai-Puua, K. 3576
 Kawamura, Y. 3507
 Kay, J. E. 4688*
 Kaye, E. M. 3742
 Kazama, C. 3431
 Kazaoka, A. 3438
 Kearing, S. 3733*
 Keating, A. F. 4469, 4470
 Keddari, N. 3298*
 Keebaugh, A. J. 3337, 4011, 4161*, 4162
 Keeley, S. 4252
 Kehres, E. M. 4296, 4300
 Keil Stietz, K. 3145
 Keil Stietz, K. P. 3615
 Keith, R. J. 4255
 Kejlova, K. 4127
 Kelesidis, G. A. 3961
 Keliher, N. 3447
 Kelkoul, I. 4589*
 Kellner, H. 4125, 4148
 Kellum, S. 3293
 Kelly, E. 3034
 Kelly, E. J. 3378
 Kelly, G. 4068
 Kelly, Z. 3229*, 3238
 Kelman, B. 4607
 Kelsall, J. 3461
 Kelsey, S. 3433*
 Kelso, S. 4653
 Kendall, R. L. 4288, 4456, 4540*
 Kende, A. 3301, 4095
 Kendrick, D. 3594*
 Keng, H. T. 4175
 Kennedy, C. 3145
 Kennedy, C. L. 3609, 3615
 Kennedy, D. 4674
 Kent, L. 3293, 4559, 4565, 4569
 Kenyon, E. 3906
 Kenyon, N. J. 3952
 Kerdine-Römer, S. 3260

- Kerger, B. D. 3550*, 4001
 Kern, P. 3265
 Kerver, J. M. 3898
 Kesapragada, M. 4490
 Keselman, P. 4121
 Kessler, H. 3352
 Ketskemety, D. K. 3810
 Keyser, B. 3456
 Keyser, B. M. 4076
 Khaled, T. 4498
 Khalid, I. A. 3248
 Khan, A. 3702*
 Khan, D. M. I. O. 3275*
 Khan, J. 3230
 Khan, R. 3619*
 Khan, S. 4497, 4550
 Khanna, A. 3051, 3268*
 Khanum, S. 2016
 Khasanova, D. 2002*
 Khasawneh, F. T. 4078*
 Khatun, M. M. 4506*
 Khazae, E. 3742*, 3747
 Khedher, A. 3051
 Khetani, S. 2019, 3380, 3690
 Khurram, M. 4427
 Kiamehr, M. 3673
 Kida, Y. S. 3672, 3758, 4258
 Kido, T. 3444*
 Kidwell, J. 4154
 Kiefer, A. M. 3765
 Kier, L. 4318
 Kilbourn, E. A. 4508*
 Kilburg-Basnyat, B. 3927
 Kilian, J. 4207
 Killius, A. 3892*
 Kilpatrick, K. 3943
 Kim, A. 4235
 Kim, C. 3020, 3022, 3032, 3036, 3600, 3601*, 3602, 4137
 Kim, C. H. 4405*
 Kim, D. 3164, 3805*
 Kim, D. I. 4676*
 Kim, D. M. 3197
 Kim, D.-W. 3046, 3104*, 3111, 4206
 Kim, E. 3238, 4235, 4505
 Kim, G. H. 3641
 Kim, H. 3107, 4203*, 4209
 Kim, H. R. 4562
 Kim, H. Y. 4562
 Kim, I. J. 3856, 3857
 Kim, J. 3086, 3391, 4562
 Kim, J. H. 3164, 3393
 Kim, J. S. 3919, 4145, 4415
 Kim, J. W. 4562
 Kim, J.-B. 3955
 Kim, J.-S. 3698*
 Kim, J.-Y. 4335
 Kim, K. 3165, 3786, 3894, 3905, 3977, 3978, 4406, 4431, 4451*
 Kim, K. B. 4562*
 Kim, K. H. 3374
 Kim, K. H. 4585
 Kim, K.-T. 3374, 4522
 Kim, N. 3007
 Kim, N.-H. 3187
 Kim, R. 3487
 Kim, R. Y. 4560*
 Kim, S. 3114, 3560
 Kim, S. L. 4447
 Kim, S.-D. 4056*
 Kim, S.-T. 2025*, 3757*
 Kim, Y. 3174
 Kim, Y. 3502, 3945
 Kim, Y. H. 3506, 3620
 Kimura, S. 3345*
 Kin, K. 4705
 Kincaid, B. 3637
 King, A. J. 3527, 4499
 King, R. 3895
 King-Heiden, T. 3167*
 Kingston, J. 2012, 4528
 Kingston, J. D. 4548, 4554
 Kinkade, C. 4273
 Kinoshita, K. 3253, 3257
 Kinra, M. 3549
 Kinsky, O. 3074
 Kintzele, E. 3133, 3188
 Kipen, H. 3925
 Kirchoff, R. W. 3409, 4265
 Kirman, C. R. 3312*
 Kiroglu, S. 4263*
 Kissell, K. 3762, 3988, 4663*
 Kitajima, S. 3860
 Kitano, N. 3012
 Kitano, S. 3389
 Kitazawa, M. 3487, 3842, 4207
 Kitta, K. 4240
 Klaren, W. 4005
 Klaren, W. D. 3653, 3982*
 Klarup, K. E. 4199
 Klausner, M. 3255, 4127, 4167, 4563
 Klein, J.-L. 3385, 4126
 Klein, L. 3514
 Kleinman, M. 3962, 4207
 Kleinman, M. T. 3485, 3487, 3515, 3842, 3913, 3923, 4064, 4250, 4343, 4667
 Kleinstreuer, N. C. 3364
 Kleinstreuer, N. 3815, 4400
 Klimas, N. 3055
 Klimkiewicz, S. M. 4011
 Kline, C. J. 4415*
 Kline, O. 3943*
 Klocke, C. 3146*
 Klotzbach, J. 3304
 Kloukinioti, M. 4534*
 Kluaiphannang, S. 3210, 3215
 Kluxen, F. M. 4026, 4372
 Knapp, M. B. 3753
 Knight, C. 3091, 3098, 3102, 3103, 3115
 Knight, C. B. 3099
 Knight, G. 4129
 Knights, A. 3711
 Knipp, G. 3808
 Knox, R. J. 3269*
 Knudsen, T. 3815
 Knutson, A. 3745
 Ko, J. 3366
 Ko, R. 3643
 Ko, Y.-U. 4335
 Kobayashi, R. 3672, 3758*, 4258
 Kobiro, K. 4725
 Kobs, Z. A. 4270*
 Kocarek, T. A. 3287
 Koch, K. 3135, 4128*
 Koch, R. 3078
 Kocks, G. 3313, 3986, 4027
 Kodali, M. 4245
 Koeini, F. 3916
 Koenig, C. 3289, 3291*, 3803, 4373
 Koenig, L. 3406
 Koepke, J. 3383, 3798
 Kohr, M. 3637, 4598
 Koirala, A. 4349
 Kojima, H. 3255, 3402
 Kojima, K. 3255
 Koker, A. 3200
 Kolaja, C. 3901
 Kolla, V. 3210
 Kolla, V. V. 3215*
 Koll, A. R. 3456
 Kolodzieyski, L. 3517
 Kolovou, M.-A. 3671
 Komarova, Y. 4704
 Komizu, Y. 4171
 Kondati, R. 4020, 4744
 Kondatti, R. 4018*
 Kondo, K. 3004*, 4574
 Kondoh, M. 3216, 3345
 Kong, B. 3880
 Kong, E. 3189
 Kong, M. 3619
 Koning, R. 3063, 3077
 Konkol, J. 4546, 4547
 Konrad, K. S. 4306*
 Koo, E. 3041
 Kools, W. 3693, 3710
 Kooter, I. 4534
 Kopec, A. K. 4106
 Korenjak, M. 4326
 Koriyama, C. 4179
 Korn, J. 3768*
 Korte, S. 3869*, 3870*, 3871*, 4037*, 4038*, 4040*, 4049*
 Koshlukova, S. 3987, 3991, 4099, 4477, 4478
 Koshlukova, S. E. 3321
 Koshman, Y. 3390
 Kosicka-Noworzyn, K. 2026
 Kosim, K. 3407, 4117
 Kosmider, B. 3907
 Kostal, J. 4365
 Kostrzewski, T. 3404, 3445, 3706
 Kosugi, M. 3024
 Kothalawala, N. 3741
 Kotlarz, N. 3876
 Kotok, K. 4124, 4141*, 4178
 Kotthru, S. 3842
 Kotulkar, M. 2037, 4294, 4641
 Koturbash, I. 3643
 Kougias, D. G. 3307*, 3736, 4687
 Kouokam, J. C. 3101, 3113, 3769, 3778, 4682
 Kovacs, S. 3646
 Kovochich, M. 3307, 3736, 4687*
 Kowash, Z. 3121*
 Kowcz, A. 3764
 Kowis, G. A. 3583*
 Kozak, A. 3737*
 Kozal, J. S. 4325*
 Kramer, A. T. 3090, 3580*
 Kramer, B. 4118
 Kramer, N. I. 2021
 Kramer, S. 3214
 Kratochvil, M. J. 3734
 Krebs, T. 3469
 Kreider, M. L. 3836*, 4458, 4686
 Krewski, D. 3317*
 Krieg, A. J. 4016, 4031
 Krieg, S. 3710, 4170*
 Krigbaum, N. 3597
 Krigbaum, N. Y. 4280, 4689
 Krisher, L. 4602
 Krishna, S. 3411
 Krishnakumar, A. E. 3565
 Krishnamurthy, H. 3636, 3903, 4745
 Krishnan, R. 3393
 Krivokhizhina, T. 4192
 Krivokhizhina, T. V. 4255
 Kroll, K. 4668
 Krothapalli, S. 3210, 3215
 Krsmanovic, T. 3021*, 3537, 4257
 Krucki, K. 4129
 Kruger, L. 4495*
 Krumholz, J. 3069
 Krupa, E. G. 4341, 4342*
 Kruse, P. 4399
 Kruse, P. M. 4196*
 Krutsch, K. 3363
 Krzykwa, J. 3811
 Krzykwa, J. C. 4242
 Ku, P. 4438
 Kubota, T. 4240
 Kuczaj, A. K. 3456
 Kuds, M. 4074
 Kuenzinger, W. 3499
 Kuhn, J. 3783*
 Kuhn, N. 3104, 4206
 Kuipers, A. L. 4268
 Kukolj, N. 3460, 3466, 3782, 4067
 Kulasena Fernando, V. 4397
 Kulkarni, M. 3408, 4255
 Kulkarni, S. 3240, 3593*
 Kumagai, K. 3431
 Kumar, A. 3005, 3483, 3484*, 4079
 Kumar, A. S. 4635*
 Kumar, K. 3618*
 Kumar, P. 3280
 Kumar, R. 2011, 3003*, 3695*, 3771, 3793, 4466, 4472
 Kumar, S. 3447*, 4009, 4338
 Kumar, V. 3862, 4009, 4091
 Kumari, S. A. 3973
 Kumer, S. 4505
 Kunnen, S. 3138, 3357, 3674
 Kunnen, S. J. 4387*
 Kuno, A. 3672, 3758
 Kunz, K. A. 4245
 Kuo, D. 4260, 4261
 Kuo, H.-L. 3640
 Kupsco, A. 3782
 Kurashiki, H. 4147
 Kurek, D. 3407
 Kurhade, S. 3535
 Kuroda, E. 3441
 Kusmider, B. 3177
 Kuwagata, M. 3402, 3860
 Kuwata, C. 3202*
 Kuzu, T. 3345
 Kwak, T. 3259
 Kwak, T. W. 3641
 Kwakye, G. F. 3584
 Kwiatek, J. 3999
 Kwok, E. 3991
 Kwok, R. 4704
 Kwon, E. 3187
 Kwon, M. 3366*, 3641
 Kwon, S. 3060*
 Kwon, S. J. 3662
 Kwon, Y. 3391
 Kwon, Y. G. 3641
 Kyakulaga, A. H. 4709
 Kyung, S. Y. 3366
 L
 L, S.-H. 3640
 La Du, J. K. 4435
 La Merrill, M. A. 4496
 Labib, R. 3456
 Labibi, M. T. 3055
 Laccioni, V. 3178
 Lachapelle, A. 3883*, 4513, 4527
 Laddha, A. P. 2038*, 3699, 4428
 Ladics, G. 3343
 Laffan, S. 3177
 LaFollette, M. R. 3384
 Lagonell, J. 3291
 Lagrutta, A. 4034
 Lai, J. 3680
 Lai, R. 3176
 Laing, A. 3191, 4223
 Laing, S. 4352
 Laird, J. 2004*
 Lake, B. G. 4291
 Lakhani, D. 4060, 4066
 Lallai, E. 3131, 3132
 Lally, O. 3380
 LaLonde, T. 3564, 4161
 LaLonde, T. J. 3337
 LaLone, C. A. 4010
 LaMadrid, C. S. 3541, 4571
 Lamar, P. 3290
 Lambertini, L. 3636, 4745
 Lamca, J. 3146
 Lamer, S. 3668
 Lamon, T. K. 3210, 3215
 Lampe, B. 3308, 3351
 Lampe, B. J. 3309*
 Lampe, J. N. 2027, 4519
 Lampe, O. C. G. 3495*
 Lan, K.-C. 4418
 Lanaro, R. 3047
 Land, K. L. 3835
 Landguth, E. 4277
 Landon, C. D. 3426
 Landry, F. 2036
 Landry, T. 3255, 4127, 4563
 Landsiedel, R. 3516, 4022
 Lane, C. 4224*
 Lane, P. 4698
 Lane, T. R. 3723
 Lang, M. 3578
 Lange, B. 3412
 Langhoff, S. 3326
 Langouët, S. 3673
 Lansberry, J. D. 3528
 Lansita, J. A. 4336
 Lantsman, S. 4257
 Lanz, H. 3407
 Lao, S. 4621*
 Lapehn, S. 3206*
 Larch, S. R. 3264*
 Lerner, C. 4034
 LaRocca, J. 3293, 3294, 3299, 3380, 4098*
 LaRocca, J. R. 4126
 LaRochelle, A. 3451
 Larrañaga Parrado, A. 4423
 Larson, L. Q. 4512
 LaSalle, C. 3084
 Lash, L. H. 3028*
 Laskin, A. 4666
 Laskin, D. 3173, 3413
 Laskin, D. L. 3239, 3241, 3410, 3928, 3929, 3934, 3935, 3938, 3939, 3940
 Laskin, J. D. 3231, 3239, 3241, 3242, 3245, 3934, 3935, 3938, 3939, 3940
 Latimer, C. S. 4218
 Lattard, V. 4332

- Laughinghouse, D. 4668
 Laurent, J. 3961
 Laurent, O. 4009
 Lautermilch, L. R. 3836, 4686*
 Lauver, A. 4227, 4713
 Lavado, R. 3369, 3886, 4558
 Lavelle, M. 3251
 Lavelle, M. M. 3252*
 Lavoie-Perusse, F. 4163*
 Lawate, B. 2019
 Lawless, M. 2022, 3648
 Lawrence, B. 3714
 Lawrence, B. P. 3346, 3630
 Lawrence, M. 3869, 3870, 3871, 4037, 4038, 4040, 4049
 Laws, M. 3832
 Laws, M. J. 3194, 3196, 3203, 3214*, 3222, 3227, 3228a, 3284, 3818, 3831
 Lawson, G. 3228
 Lay, C. 3528
 Layana, A. 4631*
 Layec, G. 4514
 Lazartiques, E. D. 4228
 Lazzaro, S. 4629
 Le Dévédec, S. 3983
 Le Do, J. T. 4175
 Le Ferrec, É. 3635
 Le Lay, S. 3635
 Le Saint Quinio, C. 3261
 Le, H. 4353
 Le, K. 4186
 Le, N. 4431
 Le, P. H. 4100
 Lea, I. A. 3314
 Leary, C. 4277
 Lebarbenchon, K. 4635
 LeBlanc, D. P. 4330
 Lebre, F. 3796
 Leckie, C. 3139
 LeClair, C. A. 4151
 LeCluyse, E. 3293, 3385, 4126
 Ledesma Vicioso, J. 3949*
 Ledezma, O. 3527
 Ledue, E. 4730
 Ledue, E. L. 4416
 Lee, A. 4673
 Lee, B. Q. 3400
 Lee, B.-H. 3701*
 Lee, C. 3391, 3539, 3838, 3840*
 Lee, C. G. 3098, 3099, 3102, 3103, 3115, 3766, 3783
 Lee, C. L. 4499
 Lee, C.-M. 3655, 3786, 4585
 Lee, C.-M. M. 4586*
 Lee, D. 3292, 3919*, 4145
 Lee, E. 3007, 3114, 3117
 Lee, G. 3365
 Lee, H. 3019*, 3391, 3605, 4615
 Lee, H. J. 4056
 Lee, H. Y. 3655*, 3786, 4204, 4585, 4586
 Lee, I. 3251, 3252
 Lee, I. L. 2020
 Lee, J. 3164*, 3310*, 3391*, 4427*, 4527
 Lee, J. A. 3955
 Lee, J. D. 4562
 Lee, J. H. 4056
 Lee, J. T. 3055
 Lee, J.-M. 3641*
 Lee, J.-Y. 3784*
 Lee, K. 2024, 3214, 4056
 Lee, K. C. 4685
 Lee, K.-W. 3662
 Lee, L. S. 4512, 4524
 Lee, N. 4340
 Lee, N.-Y. 3187
 Lee, S. 4050
 Lee, S.-A. 3259*
 Lee, T. 3366
 Lee, W. 3517, 4610
 Lee, Y.-H. 4335
 Leedale, J. 3135, 3347
 LeFauve, M. 3287
 Lehman, A. 3380
 Lehmann, L. 3667
 Lehmler, H. J. 3615, 3795
 Lehmler, H.-J. 3140, 3613, 3618, 4410, 4572
 Lei, M. 3008*
 Lein, P. 3247, 3588
 Lein, P. J. 3140, 3233, 3237, 3498, 3579, 3589, 3591, 3917, 3922, 4208, 4572
 Leist, M. 3138
 Leite, M. 3658
 Lemenze, A. 3939
 Leng, S. 3480, 3792, 3921
 Lentine, A. 3145, 3609
 Lento, J. 4170
 Lenzen-Hammerel, P. 3541, 4571
 Leon Parada, K. N. 3228, 3825*
 Leonard, H. 3491, 3492, 4726
 Leonard, H. D. 4231
 Leonard, J. 4154*, 4579
 Leone, A. 3759
 Leray, E. 3142
 Lertora, E. 4187
 Lesage, R. 4022
 Leslie, K. 4299
 Lessard, A. 3576
 Lesseur, C. 4087
 Lessing, J. K. 4191*
 Lester, C. 4014*
 Letasiova, S. 4121, 4127, 4187
 Letellier, G. 3743
 Lettieri, M. 4381*
 Letzelter, C. 4169
 Leung, D. 4137
 Leung, M. 2024, 4692*
 Leung, M. C. 3409, 3650, 4265
 Leung, M. C. K. 4746
 Leuthold, D. 3638*
 Levasseur, C. 4561
 Levelut, V. 3066
 Levenson, M. 4653
 Levenson, M. T. 4648*
 Leverette, R. 3456, 4076*
 Levesque, P. 3857
 Levi, H. 4316
 Levi, H. J. 3463*
 Levy, B. 3496
 Lew, D. 4462, 4463
 Lewandowski, T. A. 3264
 Lewis, A. 3514, 4555
 Lewis, C. 3700
 Lewis, E. 3630*
 Lewis, F. 4745
 Lewis, F. C. 3636*
 Lewis, J. 4149, 4156
 Lewis, M. 3062, 3080, 3325*, 3329
 Lewis, R. 4566
 Lewsig, K. 4354
 Leyva, N. 4551
 Leyva, N. I. 4548
 Li, A. 4587*
 Li, B. 4407
 Li, D. 3680, 3816, 4319, 4393
 Li, D. X. 4669
 Li, E. 4685
 Li, F. 3844
 Li, G. 4628
 Li, G.-H. 4539*
 Li, H. 3043
 Li, J. 2039, 3012, 3053, 3482, 3720, 3839, 4407*, 4632, 4749
 Li, J.-L. 3053, 4308, 4309, 4310
 Li, K. 3428, 3722*, 4353, 4426
 Li, L. 3190, 3499, 3953, 4186*, 4198, 4377, 4422, 4618
 Li, N. J. 4729
 Li, R. 3678
 Li, S. 3632
 Li, X. 3388, 3512, 3546, 3618, 3680, 3857, 4053*, 4073*, 4130, 4249, 4317, 4319, 4410, 4453, 4572, 4611*
 Li, X. C. 3474
 Li, Y. 3263, 3720, 3859, 3958*, 4006, 4198*, 4238, 4467
 Li, Z. 3632, 3727*, 4052, 4053, 4275
 Liang, Y. 3755
 Lichtler, R. 3607
 Lichtveld, M. Y. 2041
 Lickiss, B. 3277, 4235*
 Liebersohn, K. A. 4300
 Liepiņš, E. 4486
 Liew, Z. 4498
 Lihan, M. 4354
 Lillard, S. 3132
 Lim, A. 3055, 4476*
 Lim, C. 3842
 Lim, C. N. 3967
 Lim, E. 3480, 3792, 3921
 Lim, G. 3655, 3786, 4204
 Lim, J. 3121, 3829, 3842, 3967, 4410
 Lim, J. J. 3108, 4614*
 Lim, K.-M. 3259, 3366, 3641
 Lim, S.-H. 4385
 Lim, S.-L. 3487
 Lin, E. Y. 3924
 Lin, H. 3756
 Lin, H.-C. 3373, 4061
 Lin, J. 3980, 4180
 Lin, L. 3943
 Lin, L.-W. 3355*, 4358
 Lin, M. 4238
 Lin, S.-C. 3640
 Lin, S.-T. 4499
 Lin, T.-A. 4531*, 4545
 Lin, T.-L. 3471*
 Lin, V. 3249
 Lin, X. 3619, 4250*
 Lin, Y. 3698
 Lin, Y.-C. 3652
 Lin, Y.-H. 4083, 4672
 Lin, Z. 2000, 3555, 3557, 3649, 3725, 4036, 4396, 4576*
 Linakis, M. 3562
 Linakis, M. W. 4021*
 Lindberg, P. 3541, 4571, 4629
 Lindeblad, M. 4347, 4638*, 4704
 Linden, J. 3855
 Linder, P. 4235, 4242
 Linderholm, A. L. 3952*
 Lindqvist, A. 4241
 Linfoot, M. 4212
 Ling, G. 3440
 Lingappan, K. 3823, 4673
 Link, B. G. 3597
 Liss, E. 4213
 Liu, B. 4051
 Liu, B.-H. 3161*
 Liu, C. 3424, 4062, 4511
 Liu, C.-N. 3365, 3714*
 Liu, C.-W. 4322
 Liu, H. 3549
 Liu, J. 2034, 3139*, 3450, 3843, 4062, 4144, 4566, 4654
 Liu, J. Y. 4011
 Liu, K. J. 3773, 4292
 Liu, K. Y. 4117, 4118
 Liu, L. 2009*
 Liu, M. 3535, 4354
 Liu, N. 4716*
 Liu, Q. 4249, 4611
 Liu, R. 4528
 Liu, S. 3162*, 3163, 3166, 3376*, 3776, 4486
 Liu, S.-H. 3858, 4418*, 4448
 Liu, X. 2005*, 3390, 3953, 4186, 4366, 4621
 Liu, Y. 2022*, 3648, 3713, 4073, 4398, 4578
 Liu, Z. 3535, 4351, 4401
 Liviero, F. 3435
 Ljubojevic, M. 3025*
 Llewellyn, C. 3661*
 Llewellyn, M. 3133
 Llewellyn, S. 4243
 Llorens, P. 3654, 3658
 Lloyd, M. 3651
 Lo, K. 3810
 Loccisano, A. E. 3550, 4001*
 Locissano, A. 3319
 Lockhart, S. 3370*
 Loeschel, C. A. 3147
 Lofchy, L. 3263
 Lohmann, R. 4505
 Lohstroh, P. 3991, 4478
 Lohstroh, P. N. 3321
 Loiseau, M. 4701
 Loizou, G. D. 3660
 Lombardo, R. 3437*, 3852
 Londenberg, A. 4389
 Long, C. 2008
 Long, E. K. 2035, 3749*, 3751
 Long, M. E. 3932
 Long, N. F. 4175
 Longoria, C. R. 3410, 3935*
 Loomans, C. 3810
 Lopez Tobon, A. 4390
 Lopez Zazueta, C. 3294
 Lopez, B. 3261
 Lopez, E. M. 4429
 Lopez, V. 3913
 Lopez, V. A. 3378
 Lopez-Ramirez, A. 3881, 3893, 3902, 4634
 Lorch, G. 3265
 Lord, C. C. 3285, 3301*, 4095
 Lorenger, M. K. 4730*
 Lorkiewicz, P. 4192
 Lorkiewicz, P. K. 4255
 Lotsch, P. 3153
 Lott, J. 3179
 Loudon, R. 4154, 4483*
 Lough, V. 2020, 3266
 Louie, A. 3485, 3913, 3923, 3924*
 Louis, M. 3731
 Louis, S. A. 3694, 4137
 Louro, P. 3173
 Lovins, H. 3932
 Lovins, H. B. 3927
 Lowe, C. N. 4197
 Lowe, M. R. 4508
 Lowit, A. 3361, 4311
 Lowran, K. 3028
 Loy, J. 3857
 Lu, H. 3094, 3101, 3113, 3124, 3493, 3769, 3778, 3787*, 4051, 4594, 4682
 Lu, J. S. 4191
 Lu, K. 4322, 4599
 Lu, M. 3387
 Lu, Y. 3610, 4424
 Lubetzki, C. 3142
 Lucas, S. 3953
 Lucchesi, C. 4136*, 4140
 Lucchetti, M. 4170
 Lucchini, L. 4541
 Lucchini, R. 3903
 Lucchini, R. G. 3636, 4221, 4745
 Lucci, F. K. 3456
 Lucey, C. M. 3782*
 Luderer, U. 3211, 3228, 3825, 3829, 3842*, 3967
 Ludwig, H. D. 4220
 Luechtefeld, T. 4350, 4373, 4386
 Lui, R. 2012, 4600
 Luisi, J. 4655*
 Lukacova, V. 3066
 Luna, F. 3842
 Luna, F. A. 3829*, 3967
 Lund, A. K. 3944, 3966
 Lund, L. 3996
 Lundback, S. 3232, 3238, 4339
 Lundberg, A. L. 4669
 Lundblad, D. 3485
 Lundin, B. 4129
 Lunn, R. M. 4307
 Luo, J. 3010, 4501
 Luo, O. 3767
 Luo, S. 4051*
 Luo, W. 3458
 Luo, X. 4259*
 Lusk, S. H. 4567
 Luthin, A. 3074
 Luthy, K. 3508
 Lutton, A. 3932
 Lux, M. 4248
 Luyendyk, J. P. 3016, 3017, 3018
 Luz, A. 4311*
 Ly, B. 3266
 Lymperi, E. 3357
 Lynch, C. 4419*
 Lynch, K. 4278
 Lynn, S. 4158, 4483
 Lynn, S. G. 4157, 4580
 Lyons, S. 3167
 Lyu, V. 4644*
 Lyubimov, A. 4042*, 4173*, 4704*

M

- M. Erikson, K. 4226
 Ma, B. 4065
 Ma, C. 3476*, 3512
 Ma, J. 3172, 3846, 4334, 4690, 4707, 4717
 Ma, Q. 4531
 Mac, N. A. 3018
 MacCoss, M. J. 3485
 MacDonald, J. 3034, 3206
 MacDonald, J. W. 3485
 MacDonald, K. 3226*
 MacDonald, K. A. 4624
 Macheri, S. 3503*, 3505
 Mack, L. K. 4487*

MacKenzie, D.	4190	Maretti-Mira, A. C.	4275	Mattingly, C.	4493*	Meade, A.	3477
Mackman, N.	3018	Mari, B.	4311a	Mattingly, E.	3217, 3844	Meadows, E.	3981
MacLaughlin, P.	3548	Marie, S.	3541, 4571	Maunsell, F. P.	3649	Meakin, C.	3067*, 3733
MacMahon, J. A.	3233, 3237	Marinopoulos, A.	3280	Maurin, A.	4701	Meaza, I.	3101, 3113, 3124, 3769, 3778, 3787, 4605, 4682*
MacRenaris, K.	3780	Marinovich, M.	3381, 3435	Maurya, S.	3193a	Meco, E.	3069
Madadgar, O.	3238	Marjan, S.	3903*	Mav, D.	3355, 4358, 4374	Medewar, A.	3191, 4223
Madden, C.	4334	Markle, A.	3663	Mawn, M. P.	4559	Medina, J.	4561*
Madden, S.	3697*	Marko, D.	3669*	Maximov, A.	4703	Medina, S.	3118, 3629, 3773, 3804, 4529, 4592
Madero, M.	3033	Marks, C.	4264	Mayamei, D.	3977	Medina, V.	3179*
Madl, A. K.	3456, 3474, 4025, 4659*	Marks, S.	3232	Mayanovic, R.	3857*	Meek, E.	3236*
Madrigal Nunez, S.	4740	Marks, S. E.	4683*	Maye, J.	3651*	Meeker, J.	3828
Madrigal, S.	4062	Markus, J.	4127, 4187	Mayfield, D. B.	4607a*	Mehrabi, M.	3707, 3709*
Madunic, J.	3025, 4059	Maroun, M.	3625	Mayo, A. K.	4121	Mehrad, B.	4555
Maeda, A.	4240	Marques, C.	4169	Mayo, M.	2012, 3425, 3791, 4528, 4541*	Mehta, A.	3927
Maerten, A.	4122	Marques, C. F.	4302*	MazloumiBakhshayesh, M.	4017	Mehta, J.	3319, 3989
Maertens, A.	2004	Marques, E.	3338, 3341	McAfee, E.	4142, 4714*	Meier, M.	4265
Maes, B. B.	3773, 4529	Marquesdosreis, E.	4127*	McAleer, C.	4565*	Meijer, L. S.	4079*
Magalhães, L.	3827	Marquez, R.	3636, 4745	McAllister, C.	3193*	Meijer, T.	3398
Magdziarz, T.	2002	Marquez, R. N.	4221*	McAtee, D.	4631	Meiklejohn, D. A.	4548
Magerman, J.	3267	Marquez-Lazaro, J.	3295*	McBride, M.	3278	Meindel, M.	3849
Maggs, C.	3395*	Marraschi, G.	3827	McCarren, H.	3803	Meiser, I.	4741
Magnani, D.	3809	Marrugo-Padilla, A. M.	3295	McCarroll, M.	3534	Meister, M.	3465, 3885
Magosso, N.	3837	Marsanskis, O. M.	3813*	McCarthy, M. J.	3762*	Mejia-Garcia, L.	3052*
Magurany, K. A.	3316, 3351	Marsh, G.	3384	McCauley, K.	4501	Melai, V.	4457
Mahadeo, A.	3034*	Marshall, A.	4167	McClain, C. J.	3593	Melching-Kollmuss, S.	4022
Mahajan, G. V.	4488	Marshall, L. P.	3836, 4686	McClay, J.	4225	Melis, I. R.	4237
Mahbubul, R. H.	3649	Marsillach, J.	3485, 3923, 4101	McClenaghan, C.	3807	Melkote, A.	3095*
Mahe, G.	3810	Marsillach, J. L.	3913	McClintic, W.	3908, 3972	Melnikov, F.	4355, 4390
Mahendran, R.	4661*	Marsit, C.	4087	McCloy, S.	3386, 3500, 4132	Meltzer, G.	4278
Mahfouz, I.	4230	Marsit, C. J.	4652	McClure, R. S.	3632	Melville, D.	4568
Mahmoudi, T.	3708	Martel, E.	4701	McConnell, R.	4090*	Melzi, G.	3381, 3435
Mahoney, A. K.	3868*	Martens, M.	4370	McCoole, M.	3411	Mende, U.	4251
Mahoney, M. M.	4213	Martin, A.	3411, 4661	McCormick, M.	3029	Mendenhall, T. T.	4694
Maier, A.	3305, 3306	Martin, D.	4092	McCullough, R.	3556	Mendes, L. O.	3205*
Maimone, T. J.	4426	Martin, E.	3142	McCurdy, C.	4519	Mendes, O.	4420
Maizieres, M.-A.	3396, 3397	Martin, E. M.	3330, 4176	McDermott, A. T.	4176	Mendez, E.	4154, 4579
Majeed, S.	3323	Martin, J. K.	3353*	McDermott, C.	3113	Mendez, L. B.	3947, 3949, 3969, 4518, 4520
Majewski, D.	4566	Martin, K.	3179, 4693	McDermott, J.	4041	Mendieta, R.	3140
Majewski, D. J.	4144	Martin, L.	4645	McDermott, J.	3504	Menezes, A.	3433
Majid, S.	4133	Martin, M.	4588	McDermott, S.	4065	Meng, Q.	4197, 4200
Majidi, S.	4591, 4633	Martin, S.	4081	McDermott, S.	4224	Menghang, X.	3586
Majumder, S.	4546, 4547*, 4549*	Martinez, T.	4074	McDiarmid, M. A.	4184	Menozzi, A.	4455, 4457*
Mak, A.	4137	Martinez-Alonso, C.	3799*	McDonagh, J.	3396	Mensah, J.	3364
Makori, N.	3868, 4710	Martinez-Guerrero, L. J.	3541, 4571	McDonald, D.	3660	Mensah, P. F.	4628
Makowska, K.	4455	Martinez-Sammons, B. A.	3498	McDonald, O. F.	4529	Mensah, M. L.	2039
Malalage, M.	3505*	Martinez-Santos, A.	4064*	McDonald, T.	4185	Mercola, M.	4168
Malave, A.	4508	Martini, A. M.	4146*	McDonough, P. M.	4168	Merenda, A.	4113
Malaviya, R.	3241*	Martin-Martinez, L.	3443	McElroy, M.	4167*	Mereness, J.	3049
Malfatti, M.	3908, 3972	Marty, J.	3449*	McEaney, J. T.	4688	Merit Stark, L.	3135
Malik, T.	3918, 4255	Marty, S.	3289	McFadden, E.	4175	Mermer, S.	3446
Maliki, L.	3181	Marudamuthu, A.	3009, 3439, 4287	McFadden, M. I.	3932	Merola, C.	4455*, 4457
Mally, A.	3553, 3554, 3668	Marudamuthu, A. S.	3511*	McFall, S.	3915, 4468	Merrihew, G.	3485
Malmberg, J.	3585	Maruo, N.	4725	McFall, S. A.	3918*	Merry, C.	3399
Maloney, M. M.	3541*, 4571*, 4629	Marvel, S.	2023, 4032	McGee, D. M.	3960*, 4188, 4194	Meruvu, S.	3604
Malovichko, M.	3915	Marvin, E.	3134, 3144, 3911	McGill, M. R.	3001	Mesaros, C.	2014
Malovichko, M. V.	4255*, 4468	Masjoan-Juncos, J. X.	3463	McGinnis, C. L.	4526*	Mesic, A.	3687
Malty, V.	3817	Masood, S.	3506	McGlothlan, J. L.	3120	Messerlain, C.	4523
Mamrybayev, A. A.	4449	Massarsky, A.	4458*	McGrail, D. R.	3287*	Messier, K. P.	4196
Manautou, J. E.	2038, 3699, 4428	Massie, P.	4541	McGrath, J. L.	3852	Metcalf, C. S.	3970
Manca, D.	4709	Masson, R. N.	3650	McGrath, S.	3576, 4212, 4218, 4219	Metcalf, M.	4406
Mancini, M. A.	4232	Masso-Silva, J.	4079	McGreer, C.	3232, 4339	Metcalf, N.	3970
Mancini, M. G.	4232	Masso-Silva, J. A.	4065	McGuffey, E.	3030	Metry, R.	4465
Mandal, P.	4375	Masten, M.	3144, 3911	McGuire, C. C.	3316	Metzger, S.	4741
Mandin, C.	4009	Masten, M. C.	3134*	McInally, K.	3517, 4610	Meyer, D.	3133, 3188, 3891
Mandl, C.	4433	Masters, A. R.	4487	McKee, N. C.	4300*	Meyer, J. N.	3575
Mandrioli, D.	3053	Mastrogiovanni, G.	3711*	McKenna, J.	3294, 3299, 3380, 4126	Meyer, N.	4135
Mangas, I.	3565	Masuda, A.	4333*	McKenney, H.	4283	Meyers, A.	3891*
Mangsbo, S.	4732	Masumura, K.	3349, 4376	Mckee, D.	4326	Meyhofer, E.	4508
Manhas, D.	4572*	Matei, E.	4646	McKim, J.	3256	Mhatre-Winters, I.	3046, 3595, 3596, 3597*, 3600, 3601, 4745
Mani, G.	3910*, 3957	Matei, E. K.	3119, 3123*	McKim, Jr., J.	3401	Mi, K.	3656, 3725*, 4036
Manilay, J. O.	3443	Mateo, O.	3581, 4201	McLarnan, S.	3877, 3878	Micek, V.	3025
Mann, K. K.	3460, 3466, 3779, 3782, 3785, 4067	Mathews, T.	4438	McMahon, M.	3845*	Michelin, B.	3307
Mann, V.	3992, 4278	Mathialagan, S.	4629	McMahon, T. J.	3941	Michniak-Kohn, B. B.	3231
Manoel, B.	3827	Matsuda, N.	4147	McMorrin, R.	4493	Mickel, J. A.	3006*, 4612, 4613, 4616
Mansouri, K.	4400	Matsui, E.	3393	McMullin, T.	3926	Micovic, N.	3916
Many, G.	3500	Matsumoto, M.	3333, 3349*	McMurrin, H. M.	3795	Midali, M.	4009
Manzano, J.	3498, 3917*, 4669	Matsumura, S.	4376	McNabb-Kelada, N.	4125, 4148	Middleton, A.	4153
Mao, X.	4292	Matsusaki, M.	3389	McNabb-Kelada, N. A.	4176*	Middleton, A. M.	3373
Marathe, R.	2019*	Matsushita, J.	3431*	McNally, K.	3660	Middleton, L.	4281
Marcantonio, F.	4630	Matsushita, K.	3976	McNutt, P.	3262	Middleton, L. Y.	4731*
Marcantontio, F.	4637	Matsushita, M.	3024	McNutt, P. M.	3367, 3807, 4344	Mielke, S.	4352
Marcella, A.	3740	Matsushita, M. T.	3732*, 3753	McOmber, L.	4740	Mier, L.	4165*
Marchetti, F.	4330	Matsushita, T.	4171	McPherson, C.	2023, 3594, 4032	Migliaccio, C.	3489
Marchetti, N.	3907	Matsuura, C.	4574	McRenaris, K.	3111	Mihalchik, A.	4013
Marchini, G.	4658	Matsuura, R.	3402	McVeigh, C.	3921, 4600	Mihalopoulos, N.	3961
Marechal, D.	3139	Matsuyama, R.	3333	McVeigh, C. M.	3772, 4301, 4592*	Mikaelian, I.	3871, 4037, 4038
Mareco, E.	3205	Matteo, R.	3868	McWhirter, K.	3458	Mikami, S.	4378*
Marek, R. F.	3615	Matthews, S. M.	2030*	McWilliams, D.	3170, 3173		

Mikashima, F.	3824	Monazah, O.	3433	Mune, W.	4475*	Nandi, M.	4124, 4141, 4178
Mikhail, J.	4350, 4386	Mondal, S.	3191*, 4223	Mungall, C.	4384	Nandre, R. M.	4703*
Mikołajczyk, S.	4455	Mondini, M.	4658, 4699	Munger, S.	3273	Nandula, H.	2010*, 3549, 4362, 4391
Milde, S.	3711, 3809	Mondon, P.	3372	Munoz, M.	3247	Narayan, M.	3136, 3896
Miles, M.	4037	Moné, M. J.	3983, 3984, 4486*	Muñoz, M.	3588	Narita, K.	3255
Miljkovic, I.	4268	Monecke, P.	3718	Munoz, M. A.	3233	Narke, D.	3050, 4670*
Millard, D.	3529	Monge, C.	3859, 3958, 4467*	Muñoz, M. A.	3237*, 3589	Narukkottil, S.	3899*
Mille, J.	4311a	Monroe, A. M.	2041	Munson-McGee, S. H.	4591	Naruse, M.	3860
Miller, A.	3805	Monroe, J. J.	4723	Munson-McGee, S. H.	4633	Nash, J.	3265
Miller, C. N.	3494	Monsees, M.	3494	Muotri, A.	4498	Nash, J. F.	3764
Miller, D.	3122*, 3572	Monsivais, H.	3091	Murakami, R.	3254*	Nash, M.	3359, 3360*, 4462
Miller, D. H.	4513*	Monteiro Da Rocha, A.	4237, 4242	Murasaki, W.	3253	Nashed-Samuel, Y.	2034
Miller, E.	3041, 3086, 3210, 3215	Monteiro-Riviere, N. A.	3725	Muraski, M.	4149, 4156	Nasim, N.	3579*, 3922
Miller, E. B.	4354*	Montera, M.	3425	Murayama, K.	3402	Nasser, W.	3248
Miller, G. W.	3574	Montgomery, C. B.	3937*	Murga-Zamalloa, C.	3201	Nassiri, V.	3718
Miller, J. L.	3272*	Montrose, L.	3790, 3912	Muriana, A.	3192*	Natoli, T.	4706
Miller, L. A.	4075	Moon, C.	4136	Murphy, K.	3229, 3238, 3707, 3709	Natsch, A.	3181, 3252
Miller, M.	3926	Moon, H.-B.	3374	Murphy, K. E.	3371	Natsume, T.	3024
Miller, M. G.	3489*, 4540	Moon, J.-I.	3174	Murphy, S. P.	2031	Nault, R.	3000, 3006, 4612, 4613, 4616
Miller, P.	3528	Moon, M.	3584	Murphy, S. V.	3375*, 3508	Nautiyal, M.	3433, 3545, 3676*
Miller, R.	4057, 4273	Moon, S.	3259	Murr, A. S.	3225	Navarré, A.	3659
Miller, R. A.	4069	Moon, S. Y.	4056	Murray, B.	3287	Navarro, J.	3443
Miller, R. K.	4271	Moore, B.	4465	Murray, C. S.	3614*	Nealon, C.	3842
Miller, S. L.	3620*, 3628	Moore, C. O.	3422	Murray, E.	3134, 3144*	Nedelman, M.	3848*
Miller, T. A.	3239	Moore, E.	3285, 3286	Murray, J. R.	4149, 4156*	Needham, K.	3364
Millin, L.	4351*	Moore, M.	4680	Murthy, S.	3005	Negi, C. K.	3373, 3675*, 3677
Mills, A.	4233*	Moore, M. L.	3494	Murthy, S.	4145	Negusse, A.	3293
Mills, D.-G. C.	3962*	Moore, S.	3332, 3509*, 3856*, 4130	Murti, A.	3869	Neier, K.	4487
Mills-Goodlett, R.	3255	Moore, T. L.	3198*	Musso, A.	3986*	Nejak-Bowen, K.	2040
Milnes, D. S.	4296	Moorthy, B.	3050*, 3823, 4349, 4670	Musso, S.	4295*	Nekomoto, C.	4705*
Mimche, S.	3080	Moradkhan, T.	4197	Mustafa, S. A.	3274	Nelms, M.	4384
Min, S. E.	3174	Morales, G.	4060	Mustapha, T.	4193	Nelson, J. W.	3897, 4504
Minasenko, B.	3755, 4280, 4414, 4494*, 4689	Morales, R. A.	3488*	Musters, M.	3810	Netzeband, J.	4236*
Minet, A.	4438	Moranan, A.	4554	Muthumalage, T.	4070, 4662, 4666, 4746*	Neubauer, J.	4741
Minghetti, M.	4138, 4440	More, M.	3793, 4466	Mutlu, E.	3341*, 3906, 4626	Neubert, K.	4224
Minich, A.	3743*	More, M. P.	4471, 4472*	Mutter-Rottmayer, E.	4352	Neuhaus, V.	4741
Mirchandani, R.	4146	More, S.	3746	Mutyala, D.	4020	Neupane, R.	3760
Misharin, A.	3933	More, S. L.	3736*	Muzaffar, S.	3230	Newberry, A.	3016
Mishra, N.	3246, 3263	Moreau, M.	4030	Mwibashiye, L. T.	4363	Newell-Caito, J. L.	3415*, 3417, 3646
Mishra, R.	3107*, 3143	Moreira, M. F.	3837	Mya, N.	4531	Newman, B.	3711
Mitchell, A.	3103*	Morelli, M.	4117	Myers, M.	3177	Newman, K.	3396, 3397
Mitchell, C.	2020	Moreno, G.	3413	Myhre, O.	2029, 4009	Newman, L.	4602
Mitchell, C. A.	3811, 4242, 4641*	Moreno, J.	3577, 4212, 4218	Myles, H.	3285*	Ng, C. A.	2041
Mitchell, E.	4079	Moreno, J. A.	3576, 3585, 3912, 4219	Myles, Z.	3486*	Ng, N. L.	3493
Mitchem, E.	4519	Moreno, J. L.	3772, 3791, 4301, 4592, 4600*			Ng, S.	3347*
Mithi, V.	4262*	Moreno-Bustamante, G. M.	3834			Ngan, D. K.	4151*, 4495
Mitra, S.	4350, 4386	Moreshead, M. L.	3171	Na, M.	3251	Ngatikaura, T.	3927
Mitrut, R.	3781	Moretto, J.	4668	Nacci, D.	4437	Ngo, K.	3663
Mittelstadt, S.	4034	Morford-Oberst, N. D.	3118, 3804	Nachman, D. R.	4199*	Nguyen, A.-H. P.	4629
Miwa, K.	3431	Morgan, R. K.	3119*, 3123, 4646	Nachman, K.	3637	Nguyen, H.	3664
Miyakawa, S.	3441	Mori, N.	3672*, 3758, 4258	Nafikova, R.	3933	Nguyen, H. H.	3494
Miyake, M.	4043	Mori, T.	3163	Nagafuku, N.	4147	Nguyen, J.	4154
Miyoshi, H.	3394, 4234*	Morita, A.	4045*	Nagai, Y.	3490, 3954	Nguyen, K.	4517*
Mizoguchi, I.	3441	Moritz, W.	4387	Nagane, M.	3490, 3954	Nguyen, L.	3433, 3676, 3908, 3972
Mizukawa, M.	4043*	Moroz, A.	3924	Nagane, R.	3763*	Nguyen, N.	3885*
Mizumachi, H.	3258, 3430	Morrill, J.	4464	Nagane, R. M.	4116*	Nguyen, R.	3063, 3064, 3065, 3075
Mizuno, T.	3432	Morris, A.	3901, 4274	Nagaoka, L.	3827	Nguyen, R. H.	3076
Mizuta, Y.	3976	Morris, L.	3262	Nagaraj, V.	4225	Nguyen, T.	4347
Mo, M.	3077*	Morrison, B.	4697*	Nagarkatti, M.	3009, 3439, 3511, 3789, 4286, 4287, 4436	Nguyen, T. B.	3474
Mo, S.	4587	Morrison, E. R.	4730	Nagarkatti, P.	3009, 3439, 3511, 3789, 4286, 4287, 4436	Nguyen, T. K. O.	4371
Mobasheran, P.	4228	Morrison, T.	4316	Nagayama, Y.	3806	Nguyen, V.	3491, 3492, 4231*
Mobley, J. A.	3957	Morris-Schaffer, K.	3442, 4557	Nagyai, A.	4386	Nguyen, V. K.	4281*
Mochizuki, N.	4234	Morshead, M. L.	4411	Nagyai, M.	4350, 4373*, 4386	Nguyen-Alley, K.	3944*
Mochizuki, S.	3430*	Morton, M.	3048	Nah, H. R.	3690	Nguyen-Jones, T.	4126*
Modali, A.	3422	Morton, M. J.	3396*, 3397	Nail, A. N.	3774, 4596*	Ni, H.	4517, 4619
Moe, M.	3493	Mosig, A. S.	3678	Nair, A.	3924	Nicasio, A.	4582
Moeller, J.	3201	Mou, H.	4743	Nair, R. R.	3270*	Nichols, K.	3853
Moerland, M.	3983, 3984	Moustakas, H.	3252	Nair, S.	3307	Nicke, T.	4239
Moffat, I.	3998	Moxon, S.	4384	Naito, Y.	3389*	Nicklay, J.	4044
Mogas Barcons, A.	3048	Moyano, P.	3581, 4201*	Najafi-Majid, E.	3852	Nicklisch, S. C. T.	4552
Mogenson, M.	3849	Moyano-López, C.	3654, 3658	Najami, N.	3657	Nicola, K.	4079
Mogus, J. P.	3981*	Moyer, R. A.	4162	Najjar, A.	3307	Nie, L. H.	3583
Mohammad, Y.	3232	Muddasani, P.	2039, 3482	Nakabayashi, T.	4240	Nie, Z.	3334
Mohammadi, M.	3565	Mueller, T. A.	3689*	Nakagawa, S.	3162	Nielsen, A.	3042*, 3721
Mohamoud, S.	3878*	Mughal, Z.	4350, 4386*	Nakajima, Y.	3402	Nielsen, T. T.	4241
Mohapatra, R.	3097*	Muhammad, D. G.	3544	Nakamoto, S.	3402	Nielsen, J.	4740
Mohar, I.	4003*	Muianga, C.	3304	Nakamura, N.	3253, 3257	Niemeijer, M.	3673, 3674, 3983*, 3984
Mohar, T. I.	3264	Mukherjee, N.	4316	Nakamura, Y.	4071	Nihart, A.	3830
Mohr, E.	4239*	Mukherjee, R.	3013, 3014	Nakane, F.	4240*	Nilsson, L.	4732
Mohr, O.	3210, 3215	Mukherjee, S.	3561*	Nakatsuji, S.	4054	Niman, A.	3339
Mohsenin, M.	4272*	Mukhopadhyay, S.	3095, 3100, 3126	Nakaue, T.	3553, 4258, 4304	Nishikawa, S.	4043
Moinet, D. J.	3691*	Mulcahy, M. J.	4072*	Nakayama Wong, L.	4200*	Nishimura, T.	3199
Moiset, G.	4726	Muldoon, J.	3252, 3510*	Nakazono, S.	4050	Nishimura, Y.	4725*
Molero Flores, A. J.	3819	Mulholland, M.	2009	Naksen, W.	4087	Niu, S.	3424, 4390
Molignano, J.	3405	Muliawan, W. F.	3584*	Nalesnik, M.	4660	Niu, X.	4355*
Molina, P.	3872	Müller, M. R.	3083	Nam, J.-H.	3187	Niwa, Y.	3216, 3345
Mollina, A. K.	4512*	Mulligan, M. E.	3220	Nam, S.-H.	3641	Niyogi, S.	3767, 3826, 4444
Mollaneda, J.	4590*	Mullin, E. J.	4268	Namburi, K.	3590, 3592	Noah, C. E.	3689
Momoli, F.	3998*	Mummy, K.	3297			Noan, C.	4058
Monaco-Brown, M. L.	4211					Noda, B.	4147
						Noel, A.	3486, 3963

Noël, A. 3488, 3523, 4061
 Noerenberg, A. 3678
 Nogami, Y. 3806
 Nohara, K. 3854
 Nohoesu, R. 3981
 Nolan, B. 3838
 Nolan, E. 3694
 Noland, E. 3238
 Nomura, D. K. 4426
 Nomura, Y. 3139
 Nooijens, M. G. 4534
 Noor, S. 2012, 3791, 3953, 4657
 Norberg, T. 3794
 Nordgren, T. M. 3504
 Nored, A. 3506
 Norman, C. 4360
 Norman, G. 3895, 4577
 Norman, K. G. 3764*
 Northeast, R. 3809
 Novac, O. 3404, 3706*
 Novakovic, P. 3517*
 Noyes, J. 3729
 Nukada, Y. 3166
 Nunan, R. 4726
 Nunes, A. 3153
 Nunes, A. M. 4553
 Nurkiewicz, T. 3492, 3958, 4467
 Nurkiewicz, T. R. 3491, 4231
 Nyambego, H. 3314, 3324
 Nyffeler, J. 3565

O

O'Connor, M. 3870
 O'Brien, S. W. 3807
 O'Connor, A. 4555
 O'Hallaran, T. 3111
 O'Quinn, M. 4193
 Oakes, J. 3916, 4068
 Oakes, J. M. 4254
 Oany, A. R. 3368*
 O'Banion, M. K. 3346, 3630
 Obeng, B. 3429, 4730
 Oberdorster, G. 3911
 Oberle, C. D. 3730*
 Obernolte, H. 4741*
 Oberoi, K. 3044*
 Oblizajek, N. 4265
 Occhetta, P. 4230, 4658, 4699
 Ochando Descargues, A. 3372
 Ochando, A. 3267*, 4169
 Ochiya, T. 3860
 O'Connell, M. 2014
 O'Connor, A. 3416
 O'Connor, C. 3273
 O'Connor, T. G. 2031, 4271, 4273
 Odanga, J. J. 3685
 Odebamowo, S. O. 3313*
 Odia, O. 3729*
 O'Donnell, B. 3693
 Oechsle, O. 3525*
 Ofori, K. 3114, 3117*
 Ogata, K. 3333
 Ogawa, K. 3976
 Ogawa, T. 3199
 Ogese, M. O. 4723*
 Ogilvie, B. W. 3689
 Ogunda, A. 4149, 4156
 Ogunfeiti, O. O. 4532
 Ogunsuyi, O. 3769
 Ogunyiola, A. 4473
 Ogura, H. 4046
 Oh, G. J. 4676
 Oh, M. 3480, 3792, 3921
 Oh, S. 3174
 Oh, T. G. 4538
 Ohara, H. 3456
 Ohira, C. 3490, 3954*
 Ohman-Strickland, P. 4271
 Ohoka, A. 4378
 Ohta, E. 4045
 Ojha, S. 3238
 Ojo, Y. V. 3227*
 Okada, M. 3728*
 Okamura, K. 3854*
 Okamura, T. 4046
 Okawa, Y. 4050
 Okonkwo, O. P. 2041*
 Okoyeocha, E. 3232, 3238
 Okoyeocha, O. E. 4339*

Okay, A. B. 4017
 Okubo, Y. 3402*
 Okuda, K. 3945
 Okumura, M. 4574
 Oladele, J. O. 3612*
 Oladosu, J. 2018
 Olaharski, A. 4034
 Olaya, I. 3221*
 Oldach, J. 3405
 Oldham, M. 3478
 Oldham, M. J. 3456*, 3475
 Oldson, D. 3477
 Olesen, K. 3499
 Olesik, J. 3932
 Olewine, M. 3791, 4657
 Olfert, I. M. 4060, 4066, 4084*
 Oliva, D. 4065
 Oliveira, C. S. 3765
 Olivero-Verbel, J. 3645, 4484
 Olivier, T. 4120
 Olivo Caubergs, E. 3924
 Oller, A. R. 3736
 Olsen, L. 3297*
 Olson, B. 3968
 Olson, J. 3291, 4139*
 Olson, P. 3707, 3709
 Olvera, N. 3740
 Olverson, Z. A. 4069*
 Omaie, E. 3458*
 Omichi, Y. 3349
 Omid, Y. 3702
 Omoruyi, I. M. 3281*
 Ono, R. 3860*
 Onteniente, B. 3277
 Onukwufor, J. 3630
 Onukwufor, J. O. 3346
 Onuma, R. 3852
 Ooka, M. 3054*, 4150
 Oosterveen, T. 3809
 Opichka, M. 4620*
 Oppong, L. 3020
 Orak, D. 3754
 Orciani, C. 3041
 Orellana, L. C. 4170
 Orlova, V. 3271
 Orłowska, K. 3696, 3780
 Oropallo, M. A. 4723, 4729, 4733
 Orr, A. 3014, 4277*
 Orr, A. V. 3013
 Orr, M. 3755
 Orr, M. L. 3655, 4585
 Ortega, M. M. 3487
 Ortega, R. 4589
 Ortiz, E. 4194
 Ortiz Balsero, A. S. 4006*
 Ortiz, C. 3320
 Ory, B. 3300
 Osada, T. 4171
 Osadolor, H. B. 4640
 Osako, T. 3663
 Osakue, J. O. 4640
 Osborne, K. 3956
 Osborne, O. J. 3660
 Osei-Akyeampong, P. 3364
 Osemwowa, U. E. 3281
 Oshima, T. 4071
 Oshiro, W. 3514
 Oslack, W. 3948
 Osmitt, T. G. 4108*
 Osna, N. 4430
 Ossont, B. 3850, 3865
 Ostrovsky, A. 4634
 Osunbor, J. O. 4640
 Ota, E. 3806
 Ota, Y. 3490, 3954
 Otaki, J. M. 4071
 Otersen, K. 2026*, 3410
 Otieno, M. 4708
 Ouchi, H. 4314*
 Ouyang, J. 4377
 Owen, R. 2029*, 3565, 3638
 Oyarzabal, E. 3870, 4049
 Oyewole, E. O. 3504*
 Oyewole, O. 3859
 Oyshi, M. T. 3977
 Özcan, E. 3778
 Ozthail, L. C. 4241
 Ozkuyumcu, K. 3245*

P

Pácalová, E. 4127
 Pacchione, S. 4622, 4698
 Pace, B. 3828*
 Pacholke, K. L. 2035*, 3749, 3751
 Pack, I. 4226
 Padgett, Z. 3884
 Padgett, Z. J. 3220, 4447, 4452*
 Padhi, P. 3569
 Pagadala, S. 3637
 Pagan, G. 3457
 Pagès, E. 3372*
 Paik, A. 4275
 Paine, M. F. 4629
 Paine-Cabrera, D. 2037*, 4294, 4641
 Pajarillo, E. 3114, 3117
 Pala, T. P. 3005*
 Palazzotti, D. 4389, 4715
 Palermo, C. M. 3314, 3324
 Pallardy, M. 3260
 Palliyaguru, D. L. 4397*
 Palomares, S. 3971
 Palomino-Schätzlein, M. 4327
 Palrasu, M. 3009, 3439, 3511, 4287*
 Palstra, R.-J. 3708
 Pan, F. C. 3693, 3710*, 4170
 Pan, J. 3119, 4531, 4545, 4646
 Pan, P. 3533
 Panchal, D. 3974
 Panchal, D. M. 3470
 Panda, K. 4508
 Pandiri, A. 3053, 3683
 Pandiri, A. R. 4308, 4309, 4310*
 Pandiri, S. 3630
 Paneque, F. 4266
 Panettieri, R. 3496
 Pankow, J. 3458
 Pannala, V. R. 4374*, 4383
 Panousi, N. 3255
 Panuwet, P. 4087
 Paoli, G. 3317, 3998
 Paoliello, M. M. b. 3315*
 Papaioannou, N. 4009
 Pape, M. 4703
 Papikinos, K. 3456
 Papineni, S. 3651
 Pappas-Garton, A. 3380
 Paquette, A. G. 3206
 Paquette, C. 4048
 Parada, K. L. 3211
 Paramasivam, P. 4551*
 Paramasivan, P. 3278
 Parekh, A. 3946*
 Parenti, M. 3206
 Park, A. 3539
 Park, B. 3366, 3641, 3697, 4243
 Park, C. J. 4676
 Park, C. P. 3164
 Park, E.-J. 3955*
 Park, E.-S. 4056
 Park, H. H. 3498*, 3908, 3917, 4208
 Park, H.-J. 3139
 Park, I. 3007*
 Park, I.-Y. 3187
 Park, J. 4676
 Park, J. H. 3098, 3099, 3102, 3103, 3115, 3641, 3766, 3783, 4666*
 Park, J. J. 4492*
 Park, J. W. 4596
 Park, J.-M. 3174*
 Park, M. 2012
 Park, M. E. 4528, 4541, 4548*, 4554
 Park, S. 4522*, 4545*
 Park, S. J. 3351
 Park, S.-H. 4335
 Park, S.-Y. Y. 4514
 Park, W. 3366
 Parker, C. 2033*
 Parker, E. 3946, 4061
 Parker, L. 4534
 Parker, S. 4564
 Parks, D. T. 3685*
 Parrie, L. 3585
 Parsons, P. J. 4268
 Parvez, S. 3081*
 Pascuali, N. 2019
 Pasquier, C. 4311a
 Passereau, P. 3142
 Passini, E. 4695*

Passoni, F. C. 3435
 Pastarnak, D. 3741
 Patabandige, D. 4452
 Patel, A. K. 3018
 Patel, C. 4281
 Patel, E. 3748
 Patel, K. 3086, 3910
 Patel, K. H. 3957
 Patel, M. V. 3193a, 3763, 4116, 4488
 Patel, N. 4687
 Patel, P. 4580
 Patel, S. 4556
 Patel, V. 3393, 4175
 Patel, V. S. 3382*
 Pathak, A. 4691
 Pathak, R. 3005, 3483*
 Pathania, A. 4430
 Patial, S. 3003, 4675
 Patil, I. 3938*
 Patil, N. Y. 4538
 Patil, S. 2012*, 4528, 4541, 4543
 Patil, S. P. 4551
 Patil, S. S. 4548, 4554
 Patisaul, H. B. 3149
 Patlewicz, G. 4013*
 Patlolla, A. 3973*
 Patnaik, M. 3421*, 3421
 Patrick, M. 3639
 Patrono, A. 3903
 Patterson, B. 3738
 Patti, G. J. 4195
 Patwardhan, D. 3085
 Paudel, Y. 3240*, 3593
 Paul Friedman, K. 3317, 3562, 3814, 4189, 4399
 Paul, G. R. 4709*
 Paul, O. 3202, 4176
 Paustenbach, D. 3497, 3762, 3988, 4004, 4481, 4663, 4671
 Pavan, M. 4715
 Pavic Vulinovic, M. 3025
 Pavlovic, I. 3506
 Pavlovic, M. 3919, 4145*
 Pawlowska, A. 3532, 4387
 Pawlowski, C. 3558
 Payne, C. 3975
 Payton, D.V. 3882
 Payvandi, S. 4119
 Paz, H. 3802
 Pearson, B. 3782, 4215
 Pearson, B. L. 4214
 Pearson, K. 2009, 3150
 Pearson, K. J. 4274
 Pearson, S. N. 4162
 Pecquet, A. 3344, 3989*
 Pedersen, A. F. 3898*
 Pedersen, K. 3872
 Padiaditakis, P. 3683
 Pedroni, L. 3668
 Pedrozo, M. 3315
 Pelayo, N. 4393*
 Pellevoisin, C. 3255*
 Pemberton, A. 3869
 Pena, A. 3658
 Penders, J. 3671
 Peng, J. 4322, 4599*
 Pennell, K. 2015
 Penning, T. M. 4302
 Pennington, A. 3475
 Pennington, J. M. 3386, 4114, 4132, 4412
 Pepin, R. 4061
 Pepper, M. 2009
 Pequeno, K. R. 3549
 Pereira, D. R. 3983, 3984
 Pereira, K. E. 4158*
 Perera, T. 4639
 Peres Junior, P. H. 3819
 Périès, M. 3372
 Pernici, C. 4230, 4658, 4699*
 Perone, D. M. 4435*
 Perrera, S. 4065
 Perron, H. 4561
 Perron, M. 4154, 4483
 Perry, Q. 3497*, 3988*, 4004, 4481, 4671
 Perryman, A. 4079
 Perryman, A. A. 4656
 Perryman, A. N. 4065*
 Personius, E. 4447
 Perugino, F. 3668
 Pestka, J. J. 4529
 Peters, J. M. 4296, 4300

- Peterson, M. K. 3066
 Peterson, R. 3651
 Petit, O. 4561
 Petraitis Quay, D. 4420
 Petric, R. 4226
 Petrick, L. 3898
 Petriello, M. 3898, 4500
 Petrini, A. 4455, 4457
 Pettersson, M. 4732
 Petty, T. 4394, 4395
 Peyre-Teisseire, L. 4311a
 Pfeifer, L. M. 4170
 Pfeifer, L. M. A. 3710
 Pfuma Fletcher, E. 3363
 Phadke, D. 3355, 4358
 Phadke, D. P. 4374
 Pham, C. L. 3133*
 Pham, J. 4065
 Pham, N. 4197, 4200, 4476
 Phandthong, R. 4074*
 Phaneuf, M. M. 4175
 Phatak, S. 4551, 4657*
 Philbrook, T. 4226
 Philipp, J. 4239
 Phillips, J. 4107
 Phillips, S. 4312
 Phillips, G. 3569
 Phillips, G. J. 3616, 3617
 Phillips, J. 3266
 Phillips, T. D. 3612
 Phinney, B. 3480
 Picariello, T. 4706
 Picha, G. A. 3118, 3629, 3773, 3804*, 4529, 4592
 Pied, N. 4589
 Pierce, B. L. 4109
 Pierfelice, J. 4626
 Pierre, V. 4561
 Pierrevet, M. 4561
 Pike, R. 3870, 4040
 Pin, C. 3376
 Pinère, C. 4048*
 Pinkerton, K. E. 3462*, 3474
 Pirro, V. 3540
 Piscupescu, R. 3711
 Pitchai, A. 3465, 3950, 3951*, 3979
 Pitchaiyan, S. K. 4687
 Pitke, V. 3051*
 Pittsa, K. 3379, 3798
 Pitta, L. 3636, 4221
 Placidi, D. 3636, 3903, 4745
 Placzek, S. 3350
 Plahovinsak, J. L. 3414*
 Plaza, J. C. 3581, 4201
 Pliegocaballero, M. 3810
 Plummer, S. 3429
 Podechard, N. 3635
 Podtezhnikov, A. 4622
 Podtezhnikov, A. A. 4698*
 Pohjanvirta, R. 3281, 3855
 Pol, H. 4516*, 4524
 Polak, K. 3267
 Polakowski, N. 4679
 Polemi, K. 2016, 4646
 Polemi, K. M. 3119, 3272, 3608*, 4683
 Poling, T. 4060
 Polk, C. 4239
 Polymeropoulos, C. 2008
 Polymeropoulos, M. H. 2008
 Ponandai-Srinivasan, S. 4732
 Ponce Reyes, R. E. 4241
 Pondugula, S. R. 3639
 Ponting, D. J. 3313
 Poole, L. G. 3018
 Popichak, K. 3585
 Pore, A. 3228*
 Porter, T. 2024
 Portugal, C. 4191
 Posanzini, G. 3178
 Postma, B. 3489
 Potter, C. 3660
 Potter, S. 3651
 Potts, C. 3817*, 3820, 4367
 Poudel, A. 3318, 3644
 Poudel, P. 3741
 Poulin, B. A. 3474
 Poulsen, K. L. 3691
 Pour, S. J. 3461
 Pourfarzad, F. 3708, 4113
 Powell, A. 3085*
 Powell, J. 3534, 4082
 Powell, P. C. 3220, 3560*, 3822, 4447, 4452
 Prabhu, S. 3924
 Pradhan, D. 4740
 Prajapati, M. 4597*
 Prapamontol, T. 4087
 Prasad, B. 4568
 Pratte, P. 3323
 Preble, T. 3322
 Presnell, S. C. 3685
 Preston, S. 4092*
 Preston, S. E. 3795
 Price, J. 3332, 3856, 4130*
 Price, J. H. 4168
 Price, N. 3012, 3634
 Price, N. F. 4268
 Price, O. 3477
 Price, P. 3633*, 3998
 Price, S. A. 4604*
 Prince, R. 4697
 Proctor, D. M. 3997*, 4604
 Proctor, S. 3770
 Proctor-Clarke, J. 3218
 Propper, C. R. 2030
 Prossnitz, E. 3480, 3792, 3921
 Prout, N. 2031*
 Pruss, D. 3455
 Pruss, D. T. 4489*
 Pryce, K. 3754
 Pryde, K. 3376, 4178
 Pu, Y. 3866*
 Pudhota, A. 3910
 Punjani, M. 4210
 Punt, A. 3373
 Puri, S. 4338
 Purohit, S. 2013
 Putluri, N. 4349
 Putluri, V. 4349
 Putnal, S. K. 3767*
 Puzyn, T. 3717
 Pyle, L. 3897
- Q**
- Qasem, R. J. 4271
 Qi, G. 4297
 Qian, B. 4644
 Qiao, Z. 3298
 Qiu, J. 3400*
 Qiu, Y. 4292
 Qu, H. 4424
 Qu, M. 3546
 Qu, N. 3441
 Qu, W. 4583
 Quadrato, G. 4129
 Quazi, H. 3725
 Querny, D. 3409
 Quignot, N. 3983, 3984
 Quilez, S. 4048
 Quill, E. 3558
 Quinones, E. 3210, 3215
 Quintanilla-Vega, B. 3834
 Quintero-Rincón, P. 3645
 Quist, B. 3536, 4726*
- R**
- Ra, S. 4352
 Raab, J. 2029, 4403
 Raabe, H. 3361
 Raasch, M. 3678
 Raasmaja, A. 3855
 Rabe, B. 4629
 Rabeler, C. 3587
 Rabino, C. A. R. 4554
 Rabotnick, M. 3611
 Raburn, D. 3225
 Rabussier, G. 4118
 Rachamalla, M. 3767
 Racz, P. I. 3536, 3810*, 4726
 Radbel, J. 3241, 3939*, 3940
 Radfar, A. 4392
 Radtke, L. R. 3541, 4571
 Raetzman, L. T. 3227
 Rager, J. E. 3506, 3620, 3628
 Raggi, G. 3027
 Raghu, R. 4115
 Raghunathan, A. 3682*
 Rahimi, A. O. 4629
 Rahman, H. H. 4542, 4591*, 4633
 Rahman, I. 3520, 4115, 4536
 Rahman, M. 3496, 3693, 3710
 Rahman, M. Z. 4083
 Rahman, S. 4582*
 Rahman, S. M. 3363
 Rahnama, M. 2024
 Raja, A. 3464*
 Rajaram, S. V. 4284
 Rajasundaram, D. 4644
 Rajendran, K. 3051, 3268
 Rajwa, B. 3353
 Rama R. Gullapalli, R. R. 4541
 Rama, R. D. 3722
 Ramachandran, A. 3011, 3026, 3035, 4737
 Ramakrishnan, V. 3946
 Ramalingam, N. 4678*
 Ramanarayanan, T. 3344, 3347, 3989
 Ramar, M. 3936*
 Ramesh, N. 3944
 Ramirez, B. 4074
 Ramirez, E. 4037, 4038
 Ramirez, L. 4124, 4141, 4178
 Ramirez, M. L. 3500*
 Ramirez, S. A. 3526
 Ramjan, S. 3139
 Ramm, R. 4239
 Ramos-Bastidas, R. 3295
 Ramsaroop, S. 3531
 Ranade, J. R. 3763, 4116
 Rancourt, R. 3239, 4685
 Rancourt, R. C. 3241
 Raneri, I. R. 3194*
 Rangel, E. J. 3515
 Rangel, J. 4175
 Ranieri, J. 4644
 Rankin, C. 3933*, 3941, 3943
 Rankin, E. 3084
 Rankin, G. O. 3029*
 Rankovic, Z. 4035
 Ranpara, A. 3491*, 3492, 3958, 4084, 4231
 Ranville, J. 4537
 Rao, K. K. 4011
 Rao, L. 4415
 Rao, M. 3718, 4230*
 Rapicavoli, N. 4124, 4141, 4178
 Rashedul Hasan, M. 4006
 Rashidi, N. 4174
 Rasponi, M. 4699
 Ratcliffe, L. 3536*
 Rathnayake, R. C. 3086
 Rattray, N. 4096
 Rawtani, H. 3426
 Rayala, R. 2019
 Raymond, E. L. 4487
 Rayo, M. 3422
 Raza, A. 3293*, 3294, 4139
 Razazan, A. 3859, 4467
 Rea, M. R. 4731
 Reamer, H. 3743
 Rebok, A. 3457
 Rebuli, M. 3945
 Rebuli, M. E. 3502, 3521, 3620, 4656, 4660
 Recart González, W. 4442
 Reddy, P. 4508
 Reddy, V. 3948*, 4063
 Redis, R. 4726
 Redman, A. D. 4021
 Reeve, N. H. 4548
 Régent, F. 3277
 Rehan, V. 4062
 Rehan, V. K. 3843
 Rehman, A. 3573*
 Reichard, J. F. 3564
 Reid, N. M. 4437
 Reif, D. 4259
 Reif, D. M. 3620, 4151, 4492, 4495
 Reilly, C. 3959, 3970
 Reilly, C. A. 4567
 Rein, K. 4446
 Reina Gonzalez, P. 3570
 Reina-Gonzalez, P. 3046, 3093
 Reina-Gonzalez, P. J. 3566*
 Reinartz, T. M. 3945*
 Reinert, A. 3234
 Reinert, A. N. 4665*
 Reinholdt, L. 3273
 Reinke, E. N. 3364
 Reinke, P. 3406
 Reinsalu, L. 4014
 Reisdorph, N. 3472
 Reisfield, B. 3364
 Reiss, K. 4382*
 Reiss, R. 3319*, 4684
 Remaut, S. 3142
 Remy, S. 4166
 Ren, C. 4238
 Ren, Q. 4051
 Ren, X. 4319
 Renaut, S. 3219, 3285
 Renda, R. P. 3513*
 Rendall, E. 3530
 Rengarajan, A. 3150*
 Renieri, E. 4009
 Rennert, K. 3678
 Renzetti, S. 3636
 Reseland, E. 3145
 Resh, V. E. 4728*
 Resiliac, J. 3452
 Resnik, J. D. 4011
 Reu, M. 4450
 Rex, R. 4243
 Rex-Rabe, S. 3332
 Reynolds, B. R. 4253*
 Reynolds, J. 3373
 Reynolds, M. 3415, 3416*, 3417
 Reynolds, R. 3170, 3817, 3820*
 Rezin, M. 4583
 Rhoden, J. 3183, 4279
 Riaño, A. B. 4101*
 Riaz, Z. 3572
 Ribeiro, A. R. 3051
 Ribeiro-Davis, A. 3593
 Ricardo, M. L. S. 3205
 Riccardi, M. 3335, 3336
 Rice, C. 3970
 Rice, C. D. 4567
 Rice, O. H. 4075*
 Richard, E. 3901
 Richardson, C. L. 4011
 Richardson, E. 3404, 3445, 3706
 Richardson, G. 3122
 Richardson, J. 3452, 3453, 3601
 Richardson, J. R. 3046, 3595, 3596, 3597, 3600, 3602, 4745
 Richardson, R. J. 3587
 Richmond-Bryant, J. 3484
 Richter, R. J. 3923
 Rickabaugh, K. 4659
 Rickard, B. P. 4502*
 Ricker, D. 3741
 Rider, C. 2023, 3620
 Ridlon, M. 3145, 3609*
 Ridlon, M. M. 3615
 Riedel, I. 4239
 Ries, J. 3528
 Riess, B. 3637
 Riffle, B. 3289, 4022*, 4373
 Riffle, M. 3485
 Rigby, S. M. 3423*
 Riggs, D. 4255
 Righter, R. D. 3836
 Rigutto, G. 4283*
 Riley, III, G. L. 3936
 Riley, K. W. 3223
 Rineer, J. 4384
 Rios de Anda, M. 3809
 Rios, A. 4324*
 Rios-Colon, L. 4497*, 4550
 Risotto-Urbancowicz, E. 4541
 Rispo, F. 4187
 Ritacco, G. 3266, 3999, 4107*
 Ritsema, J. H. 2021*
 Ritsma, L. 3810
 Rittchen, S. 4113*
 Ritter, D. 3518
 Ritu, J. R. 3826, 4460*
 Ritz, B. 4276
 Rivas, M. V. 4330
 Rivera Alsina, A. 4518
 Rivera, A. 3901
 Rivera, B. N. 3314, 3653*, 4114
 Rivera, E. 3637
 Rivera, O. 4639*
 Rivera-Núñez, Z. 4191, 4271, 4273
 Riviere, J. E. 3649*, 3725
 Rizvi, I. 4502
 Rizzo, A. 4509*
 Rizzolo, A. 2036*, 3857
 Roberts, D. 2037
 Roberson, J. 3881, 3893, 3902*, 4634

- Robert, J. 3437, 3852*
 Roberts, A. 3651
 Roberts, A. A. 4268
 Roberts, C. 4193
 Roberts, G. 3053, 4398, 4625, 4626, 4675
 Roberts, R. 3048, 4696*
 Roberts, R. A. 3396, 3397
 Roberts, R. M. 4425*
 Robertson, H. 4527
 Robichaud, N. 4174*
 Robinette, C. 3521, 4660
 Robino, F. 4187
 Robinson, J. 4129
 Robinson, J. F. 3190,
 4422, 4618*
 Robison, K. 3951, 3979, 4544*
 Rocca, D. 4726
 Rocha, S. 4216
 Rocha, V. 3181*, 3841
 Rocha, V. A. 3837
 Rock, A. 4169
 Rock, K. 4452, 4459
 Rock, K. D. 3220, 3560, 3822, 4447
 Rucker, N. 4404
 Rockley, K. 3048
 Rockley, K. L. 3396, 3397
 Rockwell, C. E. 3434, 3448
 Rodda, M. 4381
 Rodichkin, A. N. 3120
 Rodrigues, A. 4120
 Rodrigues, A. D. 4629
 Rodriguez Borrero, E. 4518*, 4520*
 Rodriguez Carrasco, Y. 3799
 Rodriguez González, A. 4518
 Rodriguez, G. 4298
 Rodriguez, J. R. 3934*
 Rodriguez, M. J. 3502*, 3945
 Rodriguez, T. 3473*
 Rodriguez-Carrasco, Y. 3659
 Rodriguez-Carrasco, Y. 3800
 Roe, A. 2022
 Roe, A. L. 3643*, 3648
 Roe, H. M. 4474*
 Rogers, C. J. 3239
 Rogers, E. 3385*, 4126
 Rogers, J. 4622
 Rogers, J. D. 4490*
 Rogers, R. D. 4575
 Rogers, S. 3314
 Rogy, K. 3380
 Roh, T. 3454, 4270
 Rohan, J. 3297
 Rohatagi, S. 4685
 Rohlman, D. 4114
 Rojas, A. A. 3182*
 Roldan, N. 3516, 4166
 Rolle-Kampczyk, U. E. 3141
 Roller, S. 3748
 Rollins, B. 2032
 Rolon, E. 4074
 Romero Franco, M. 4200
 Romero, C. 3255
 Romero, E. 3959*
 Romero, E. G. 4567
 Romero, N. 4164
 Romero, P. 2024*
 Romero, S. S. 3215
 Romero, V. 4743
 Romo, G. 3207*, 3208, 3209
 Roncal, C. A. 3033
 Roney, A. 3229, 3238*, 4339
 Roney, N. 3336
 Rong, C. 4410*
 Roni, M. S. R. 4582
 Ronis, M. 3872*
 Roodgar, M. 4124*, 4141, 4178*
 Roof, S. 3519*
 Roof, S. H. 3124
 Rooney, A. A. 4307
 Rooney, J. P. 3301
 Rooseboom, M. 3442
 Roper, C. 3179, 3399*, 3404, 3445, 3706,
 3956
 Roper, C. S. 3763, 4116
 Rosa, H. J. 3949
 Rosalem, G. 3827
 Rosario Ramos, N. 4518
 Rosati, R. 3028
 Rose, J. 4346
 Rose, R. E. 3210, 3215
 Rosen, H. T. 4426*
 Rosenbloom, A. 4174
 Rosenfeld, E. 3202
 Rosenthal, A. C. 3409
 Rosette, C. 3371*, 3707, 3709
 Rosowski, E. E. 4722
 Ross, A. P. 4394, 4395
 Ross, J. 4525
 Rossini, E. L. 3950
 Rossoshek, A. 4151
 Rostami, A. A. 3456
 Roth, J. G. 4390
 Roth, K. 3898, 4500*
 Roth, S. 4170
 Rothwell, E. 3377
 Rousel, J. 3983, 3984
 Rousselle, D. 3459, 3930*, 3931
 Rower, J. E. 4567*
 Rowland, E. 4541
 Rowley, M. J. 3838
 Rowse, G. 4709
 Roy, S. 3793, 4466*, 4471, 4472
 Roychowdhury, T. 3018
 Royer, C. M. 4642
 Rubens, F. 3608
 Rubin, A. L. 3321, 3991*
 Rubio Villa, J. 3665*
 Ruch, C. M. 3059*
 Rudd, K. 4119
 Rude, C. I. 4573
 Rudel, R. 4281
 Rudel, R. A. 4688
 Rufer, E. 3062, 3080, 3084, 3325, 3329
 Ruivo, P. 3964
 Ruiz, M. 3799, 3800, 3801, 4327
 Rull, R. P. 3901*
 Rumbeihai, W. K. 3416, 3418*, 3805
 Runge, D. 4450
 Rupar, M. J. 2008
 Rus, I. 4538
 Rusch, D. B. 3459
 Ruschman, G. 2017
 Rush, M. 3741*
 Russell, B. 3408
 Russell, R. B. 3021, 3537, 4257
 Rust, A. 4215
 Rusyn, I. 3373, 3675, 3677, 3690, 4112,
 4185, 4474
 Rutkiewicz, J. 3327
 Ryan, A. K. 3922
 Ryan, A. L. 4145
 Ryan, C. 3510
 Ryan, K. 4625*, 4626, 4675
 Ryan, N. 4008
 Ryu, S. 3895, 4523, 4629
- S**
- S. Chelladurai, K. 4202*
 Saavedra, D. I. 3318
 Saba, L. 3246
 Sabatini, H. 3154
 Sabliov, C. 3750
 Sabis, S. 4068
 Sabo-Attwood, T. 4266, 4555, 4668*
 Sacher, O. 2002
 Sachetto, A. T. 3018
 Sachs, C. 3718*
 Saddler, T. 3364
 Sadekar, N. 3382, 3510, 4148, 4181*
 Sadiq, O. 4546, 4549
 Sadko, K. J. 4713*
 Sadovsky, Y. 4644
 Sadrieh, N. 3384, 4159
 Sadrpour, P. 4157*
 Safar, A. M. 3196*
 Safe, S. 3197, 3368
 Safe, S. H. 3612
 Saha, E. 3789
 Saha, S. 3238
 Sahu, D. 3393
 Saiakhov, R. 3715*
 Saini, L. 3474
 Saini, Y. 3003
 Saito, H. 4240, 4700
 Saito, K. 3162, 3166, 3258
 Saito, N. H. 3237
 Sakai, N. 4171
 Sakamuru, S. 3586, 4150, 4151, 4259,
 4419
 Sakaue, H. 3672
 Sakdja, R. 4431
 Sakolish, C. 3675, 3677, 3690*, 4112
 Sakurai, R. 3843
 Sala-Hamrick, K. E. 4237*
 Salamova, A. 3619
 Salanga, M. 2030
 Salazar, K. 3338, 3340*, 3341, 3361, 4400
 Salazar, M. R. 3474*
 Salazar-González, R. A. 4677
 Salehi, A. 3456
 Salerno, K. M. 4199
 Salgado, V. A. 3818
 Salim Saker Santos Lima, M. 4278*
 Salinas, K. 3658*
 Salini, R. 4457
 Salman, N. 3268
 Salminen, A. 3256*, 3293, 3401
 Salphati, L. 3561
 Salucci, S. 4457
 Salukuti, S. 4744*
 Samala, V. 3540
 Samano, V. 4197
 Sameni, S. 3419
 Samet, J. 3506
 Samidurai, M. 4636*
 Sammi, S. 3104, 3592
 Sammi, S. R. 3046, 3111*, 4206, 4417
 Sammoura, F. M. 3596
 Sampaio, A. V. 3694
 Sampaio, H. 3935
 Sampei, K. 4700
 Sample, P. A. 3540*, 3541, 4571, 4629
 Samson-Couterie, B. 3798
 Samulevich, M. L. 4106*
 Sanchez, A. 4169
 Sanchez, G. 4554
 Sanchez, I. 3625, 4168
 Sanchez, L. 3917
 Sanchez, M. 3924
 Sánchez, M. 3801*
 Sanchez, N. C. 3957*
 Sanchez, S. 4173
 Sanchez, S. S. 3637
 Sancho, D. 3343
 Sanders, A. P. 3012, 3634, 4268
 Sandhu, H. 3450
 Sandul, T. I. 3894*
 Sang, Y. 3017
 Sanghavi, D. 4207
 Sanikapally, A. 3122
 Sanjuan, J. 3581, 4201
 Sansom, G. 3454
 Sant, K. 3207
 Sant, K. E. 2016, 3608, 4683
 Santacruz Marquez, R. 3203, 3222,
 3227, 3228a, 3831, 3196, 3818*
 Santhosh, I. J. 3725
 Santos, P. 4091
 Santos, P. C. 3803*
 Santos, S. 3827, 4602*
 Santos, S. 3033
 Santos-Medina, L. 3425
 Santos-Medina, L. V. 4739*
 Sanz Serrano, J. 4122
 Sapienza-Lundie, L. A. 4502
 Sapkota, K. 3087
 Saporito, A. F. 3914*
 Sarathy, P. 4068
 Saravanan, I. P. 3210*, 3215
 Sargis, R. M. 3096, 4525
 Sari, Y. 3862*
 Sarkar, S. 3046, 3093, 3566, 3570, 3793,
 4466, 4471*, 4472
 Sarker, N. 3454*
 Sarodaya, N. 3499
 Sarumi, Q. 3484
 Sasaki, D. 3402
 Sasidharan, A. 3342*
 Satake, R. 3253, 3257*
 Satbhai, K. 3159, 4513
 Satbhai, K. M. 3883, 4527*
 Sathisaran, U. 4506, 4507*
 Sathyanarayana, S. 3206
 Sato, J. 4046
 Sato, K. 3402
 Sato, M. 3854
 Sato, T. 3199
 Satoda, Y. 3430
 Satoh, M. 3784
 Sattler, A. 4450
 Satyal, P. 3318, 3644
 Satybaldiyeva, U. A. 4449*
 Sau, S. 3193a
 Saunders, B. 3941
 Sausen, M. 4326*
 Sauter, D. 4241*
 Savic, C. 4566
 Savic, C. A. 4144
 Sayes, C. 3501
 Sayes, C. M. 3455, 4489
 Scalise, D. M. 3985*
 Scandellaria, C. 4236
 Scanduzzi, L. 3536
 Scanlan, L. D. 3909*
 Scarano, W. R. 3205, 3819, 3831, 3837*
 Schacht, C. M. 4581
 Schaefer, E. 3261
 Schaefer, H. 3314, 4279
 Schaeffer, J. 4602
 Schäfer, A. 3300
 Schaffert, A. 4402*
 Schappe, T. 3943
 Scharf, P. R. S. 3468
 Scharkin, I. 3135
 Scheele, C. 3270
 Schelegle, E. 3591
 Schember, I. 3251, 3252
 Schermbek, J. 4193
 Schewitz-Bowers, L. 4726
 Schexnayder, M. 3486, 3523
 Schichlein, K. D. 4664*
 Schieffer, D. 3719*
 Schjindel, L. v. 3984
 Schildknecht, S. 3565
 Shilt, I. 4120
 Schilz, J. 3629*
 Schladweiler, M. C. 3494
 Schleizinger, J. 3010, 3875
 Schleizinger, J. J. 4232*, 4501
 Schlipf, M. 4515
 Schlosser, A. 3668
 Schlosser, C. 4093
 Schlosser, P. M. 4581
 Schluppman, K. 4128
 Schmandt, B. 3151
 Schmidt, C. 3326
 Schmidt, F. 4034
 Schmidt, K. 3686, 4239
 Schmidt, L. 4307
 Schmieder, F. 4239, 4741
 Schmitt, C. 3420, 4398
 Schmokel, C. 3446
 Schneider, C. D. 3017*
 Schneider, L. 3226, 4464
 Schneider, S. 3147*
 Schneiderjan, E. 4537
 Schneiderman, A. 3901
 Schnellmann, R. G. 3035
 Scholl, E. 3355
 Scholl, E. H. 4358, 4374
 Schoof, R. A. 4183
 Schorn, M. 4465
 Schott, E. 3927, 3932*, 3941, 3943
 Schroeder, J. 3415, 3417
 Schroeter, J. 3477
 Schuck, D. C. 3288
 Schuld, J. 4450
 Schuler, J. 3492
 Schuller, A. 3790, 3791, 4216
 Schuller, A. J. 3599, 4217, 4222*
 Schulte, A. 3746*
 Schulte, A. M. 3736
 Schulte, J. M. 4447
 Schultz, C. 3740*
 Schultz, D. R. 4009
 Schultz, J. E. 3180*
 Schumacher, E. 3615
 Schumacher, F. R. 2041
 Schumacher, K. 3338*
 Schumann, P. 3360, 4010
 Schumann, S. 3590*, 3592
 Schutte, M. 3319
 Schwab, A. 3235
 Schwab, C. H. 2002
 Schwarz, K. 3326, 3518*
 Schweiger, N. 3141, 3638
 Schwengberg, S. 3379, 3383*, 3798, 4134
 Sciaky, D. 4493
 Scinicariello, F. 3304, 3335, 3336
 Sciuscio, D. 3323
 Scottichini, G. 4457
 Scott, E. 3913*, 3923
 Scott, E. T. 3485

Scott, J.	2015, 4138	Sharma, R. S.	3278,	Simoens, D.	4341	Sobolewski, M.	3134,
Scott, J. L.	4298*		3292*, 3559*	Simolin, M.	4312		3144, 3911
Scott, L.	3461	Sharma, S.	3156, 3182, 3562, 3885	Simon, C.	4225*	Sobolewski-Terry, M.	3093
Scott, R.	3171	Sharma, U.	4140	Simon, L.	3872	Soe, M. T. M.	4345*
Scott, S.	3398	Shatz, M.	3420	Simona, S. C.	4560	Soeira, T. V. R.	4454
Seal, S.	4490	Shaub, J.	3897	Simones, T. L.	4479	Soendergaard, C.	3746
Seaton, T.	3832	Shawlot, W.	3095	Simonson, K.	3408	Soerianto, W.	3521*
Seaton, T. A.	3228a	Shea, K.	3452	Simpson, J. S.	4057	Sogbanmu, T. O.	4532*
Sededji, E.	3776*	Shea, K. I.	3453	Simpson, N. E.	4159	Soggia, F.	4187
Sedgwick, A.	2007*, 4035*	Shea, N.	4452	Sims, J. L.	4580*	Solanki, S.	4608*, 4609
Sedykh, A. Y.	4360, 4394, 4395	Shea, R.	4726	Simsek, O.	3200*	Soler, N.	3635
Seemann, F.	3610, 4509, 4645*	Sheabar, F. Z.	3657*	Sinclair, G.	3562	Soliman, R. H.	3230
Segreti, J.	3390	Shehjar, F.	3111	Singanamalli, A.	4130	Solis-Heredia, M. J.	3834
Seguin, R. P.	3378	Shelton, M.	3399	Singh, A.	4140*	Somach, R. T.	3587
Seguret, M.	3051	Shemesh, U.	3524*	Singh, A. D.	3936	Somalraju, S.	2028
Sehgal, N.	4652*	Shen, A.	3191, 4223	Singh, D.	4001, 4515, 4546	Son, B.	4056
Seid, L.	4693	Shen, J.	3639	Singh, K. P.	4408	Song, C.	4019*
Seipel, F.	4129	Shen, S. Q.	4415	Singh, M.	3421	Song, M.	3374
Seiwert, B.	3638	Shen, Y.	3720, 4249, 4356, 4357, 4363*	Singh, M. P.	3453	Song, M.-K.	4676
Sekhon, U. D. S.	3558	Sheng, W.	4749	Singh, N.	3889	Song, X.	4170
Seki, Y.	4045	Shenoy, S. P.	4344	Singh, N. P.	4436*	Song, Z.	4073
Selwyn, J.	4645	Sheppard, A. M.	3653	Singh, R. R.	3574	Soni, D.	4111
Seman, K.	4252	Sherif, J.	3029	Singh, S.	3616	Soni, S. G.	3193a
Seman, K. M.	3212	Sherlock, S. S.	3835	Singh, V.	3322, 4100	Sonntag, F.	4239, 4741
Sen, A.	4679, 4697	Sherpa, R.	4427	Singhal, A.	4671	Soshilov, A.	4133
Senarath Pathirajage, K. S. S. P.	3156*	Shetty, A. K.	4245	Singletary, E.	3594	Sosnowska, A.	3717
Seney, C. S.	3765	Sheu, M.-L.	4418	Singleton, M.	4451	Sostare, E.	3192
Sengupta, A.	3469	Shi, M.	3334	Sinke, L.	3138*	Sousa, C. A.	4344
Sengupta, M.	3455	Shigeaki, I.	3456	Sinke, L. J.	4387	Sousa, T. C.	3205
Sengupta, T.	4422	Shigekawa, Y.	4171	Sinkey, R.	4063	Southall, M.	4005
Seo, B.	3897	Shillito, C.	3578	Sinopoulou, E.	3870	Southall, M. D.	3307, 4687
Sepehr, E.	3647, 3713	Shimada, M.	3333	Sioutas, C.	3924	Southam, A.	3192
Sepúlveda, M. S.	4524	Shimoga Chandrashekar, D.	3957	Sipes, I. G.	3328	Souza, P. V.	3831*, 3837
Serafini, M.	4009	Shimshock, K. D.	3065*	Sipula, I.	2039	Sovero, A.	3966
Sergent, O.	3635	Shin, E.	4056	Siracusa, J.	4481*	Sozzi, E.	4497
Sergeyev, O.	3195	Shin, J.-K.	3698	Siraki, A. G.	3370	Spade, D. J.	2015*
Sermat, S.	4721*	Shin, M.	3374	Siramshetty, V.	3561	Spaeter, T.	3184
Serna III, C.	4160*	Shin, N.	3098, 3099*, 3102, 3103, 3115	Siramshetty, V. B.	4352*, 4355	Sparfel, L.	3635
Serna, S.	3959	Shinde, A.	3950, 3951	Siu, Y.	3473	Sparks, N.	4432
Serna-Pemberthy, S.	4068	Shinde, A. A.	3979*	Sivankutty, I.	3393	Sparks, N. R.	3157
Serra, A.	4402	Shinoda, T.	3389	Sivaprasad, M.	3952	Sparrey, C.	3870
Serradimigni, R.	3168*	Shipkowski, K.	4307	Sivaraman, L.	3177	Specht, A.	3770, 3948
Serrano, R.	4168	Shipman, J.	4497, 4550	Sivaraman, V.	4497, 4550	Specht, A. J.	4588
Serrano-Candelas, E.	4327	Shipp, E.	4097	Sixsmith, G.	3536	Speen, A. M.	4656
Servant, N. B.	3527	Shirely, P.	4218	Siyam, M. A.	3409	Spiegelhoff, A.	3615
Serve, K. M.	3423, 4740*	Shirley, P.	3576*	Skende, O.	3547, 4511, 4523*	Spigelmyer, R.	3132
Setiawan, V. W.	4275	Shmarakov, I.	3928, 3929*, 3938	Skinner, D. E.	3527	Spijkers, X.	3407
Settivari, R.	3293, 4139	Shockley, K.	4306, 4675	Slankster-Schmierer, E.	3411*	Sprague, A.	4193, 4630*
Sewald, K.	3686, 4741	Shockley, K. R.	4625, 4626	Sleiman, A.	3308*, 3351	Sprando, R.	3712
Sewell, F.	3761, 4695	Shoda, L.	4057	Slepchenkova, A.	4254*	Sprando, R. L.	3647
Sexton, J.	4131	Shoda, T.	3004, 4314, 4574	Slitt, A.	2026, 3547, 3895, 3897, 4504	Spratt, T. E.	3457
Sexton, J. Z.	3272	Shoieb, D.	3636, 4745	Slitt, A. L.	4505, 4511, 4523	Spren, A.	4110
Seybold, D. K.	3960, 4188, 4194*	Shoji, M.	4314	Sloboda, R.	3752*	Spriggs, S. M.	3419
Seymore, T. N.	3173*	Shoji, Y.	3777	Slominski, A. T.	4608	Springer, M. S.	3212
Seymour, A.	3377	Shojiv, M.	4489	Sluka, J. P.	3808, 4016, 4031*	Spurbeck, R.	3053
Shabrang, M.	3045*	Shouldis, L.	4084	Smart, G.	3151	Sri Hari, A.	3970*
Shaffer, C.	3740	Showalter, J. G.	4011	Smieszek, S.	2008	Sridhar, V. V.	3137*
Shaffer, R.	3527	Shree, N.	3604, 4245*	Smiraldo, P.	3071	Sriram, S.	3309
Shafiei, S.	3407, 4117	Shrestha, S.	3006, 4612, 4613, 4616*	Smiraldo, P. G.	3059	Srivastava, R. K.	3230*
Shah, A.	3093, 3570*	Shrestha, U.	4674	Smirnova, L.	3097, 3116, 3637	Srivastava, S.	3915, 3918, 4192, 4255,
Shah, E.	4731	Shughart, T.	4556	Smit, L.	3379		4468
Shah, E. K.	4237	Shukla, R.	2018, 3839	Smith, A.	4168	Srivastava, S. K.	4680
Shah, F.	4490, 4622	Shulfer, R.	3062, 3080, 3084	Smith, A. E.	4479	Srivatsan, E.	3055
Shah, K. N.	4488	Shulfer, R. F.	3325, 3329	Smith, B. M.	4461*	St Hilaire, R. C.	4040
Shah, R.	3355, 3864*	Shuttleworth, B.	2012	Smith, C.	3700	Stepnik, M.	4009
Shah, R. R.	4007, 4017, 4358, 4360, 4374,	Sia, A.-Y.	4610*	Smith, D.	4366	St. Fleur, R.	4652
	4394, 4395	Sidaway, J.	4696	Smith, D. R.	3095	St. Mary, L.	3355, 4088*
Shah, S. A.	3318	Siddiqi, A.	3296	Smith, E.	3790	Staats, H.	3426
Shaikh, S. B.	3520*	Siddique, A. E.	4284*	Smith, G.	3830	Stacey, J. E.	3396
Shaikh, S. R.	3927	Siddiqui, H.	3012	Smith, G. J.	4664	Stacy, A.	4346*
Shakti, R.	4688	Sidhu, H.	4065	Smith, J. N.	4570, 4573*	Stadnicki, J.	3255
Shakya, A.	3331*	Siegel, K. R.	3621, 3623*	Smith, J. S.	3105*	Stafford, A.	3802
Shama, L. N.	4548	Siegwald, E.	4720	Smith, L. C.	3936, 3938	Stahl, M.	4137
Shamblin, T.	3179, 4693	Sierzenga, S.	3630	Smith, M. D.	4567	Stallone, J. N.	3215
Shang, P.	3512	Sijm, D. T. H. M.	3671	Smith, M. R.	3750	Stambersky, A.	3229, 3232*, 3238, 4339
Shankar, K.	4274	Silas, S.	4062	Smith, N.	3452, 3607	Stamper, M.	4059
Shankar, U.	4649	Sillé, F.	2004	Smith, N. A.	3453	Stancliffe, E.	4195*
Shannahan, J.	3951, 3979, 4544	Sillé, F. C. M.	3637*	Smith, P.	3585	Stang, M.	3065
Shannahan, J. H.	3465, 3950, 4454		4282*, 4747*	Smith, P. C.	4271	Stanish, P. C.	3317
Shanto, A. H.	3598	Sills, R.	3053, 3594, 4309	Smith, Q.	4111	Stanko, J.	2023
Shao, K.	3946, 3990*, 4379	Sills, R. C.	4308, 4310	Smith, R.	3856, 3857	Stankoff, B.	3142
Shapiro, K. A.	3090*, 3580	Silva Costa, S.	3770	Smith, R. M.	4541	Stanon, M.	4163
Sharkey, H.	4504	Silva, L.	3658	Smith, T.	3383, 3798, 4134*	Stapleton, P.	3173, 3413
Sharkey, H. E.	3897*	Silva, R.	4307	Smith, W. L.	4679	Stapleton, P. A.	3170, 3198, 4247
Sharma, A.	3081, 3250, 3433, 3545, 3676,	Silveyra, P.	3459, 3930, 3931, 3946, 4061	Smith, Z.	3780*, 4383	Stark, L. M.	4128
	3843, 4041	Simberg, D.	3263	Smith-Roe, S.	4623	States, J. C.	3774, 4298, 4596
Sharma, K.	3459	Simeonov, A.	3726, 4151	Smith-Roe, S. L.	4330	Statia, M.	4113
Sharma, M.	3516, 4166*	Simmel, M. L.	4203	Smyth, T.	4029*	Staub, R. H.	4010*
Sharma, N.	3126*, 4708*	Simmons, A. E.	4176	Snyder, K.	4054	Stayer, K.	4425
Sharma, P.	3907*, 3907	Simmons, S. O.	4157	Snyder, R. J.	4649*	Stayer, K. M.	2040*
Sharma, R.	3694*	Simms, L.	3456, 3461	Sobo, A. K.	3606*	Stebbins, K.	4094

Steen, P.	3740	Sullenberger, C.	3363	Tan, Y.	4256	Thomas, S.	3074, 4596
Steffen, J.	3528	Sullivan, D.	3529	Tanaka, L. F.	3145*	Thomas, S. D.	3775
Steichen, H.	4542	Sullivan, K.	3055	Tandon, A.	4007, 4017*	Thompson, C. M.	3324, 4013
Stein, F.	3207	Sullivan, L.	4065	Tang, C.	4226	Thompson, C. T.	3997
Stein, J.	3224, 3827	Sullivan, M.	3920*	Tang, L.	3573	Thompson, J.	3830
Steiner, M. K.	4598*	Sullivan, R.	4352	Tang, S.	4531	Thompson, L.	3022, 4609
Steiner, S.	3776	Sumikawa, K.	3487	Tang, W.-Y.	4644	Thompson, L. L.	4608
Steiniche, T.	4433*	Sumiyoshi, A.	3806	Tang, X.	3365*	Thoranath, V. F.	3729
Steinritz, D.	3235	Sumner, C. P.	4152*	Tanghal, R. B.	4275*	Thorne, P. S.	4284
Stello, K. M.	2041	Sumner, G.	3089	Tangianu, S.	3138	Thornton, C.	4693
Stemmer, P. M.	3028	Sun, L.	3656*, 3959, 4531, 4567	Tangil, B.	3398	Thornton, D.	3639*
Stepanov, I.	3968	Sun, Y.	3432, 4115	Tanguay, R.	3355	Thornton, J. W.	4433
Stephens, A.	3968*	Sundar, V.	3636, 4745	Tanguay, R. L.	3171, 3185, 3353, 3613, 3887, 4017, 4358, 4411, 4435, 4492, 4573	Thorton, M. N.	2040
Stephens, C.	3422	Sundaram, V.	3725	Tanioka, K.	4314	Thota, S.	3963*
Stepnik, M.	3717	Sunderland, E.	3547	Tanir, J. Y.	3327*	Thrall, S.	4215*
Stermer, A.	3150	Sunderland, E. M.	3563, 4505	Tanis, K.	4420, 4622, 4698	Thrall, S. I.	4214
Sterner, T. R.	3564, 4011, 4033*, 4161, 4199	Sunil, V.	3928, 3935	Tanis, K. Q.	4490, 4641	Thresher, A.	3313
Stethem, C. M.	3816	Sunil, V. R.	3239*	Tanwar, R.	3590, 3592*	Thrikawala, S. U.	4722*
Stevens, D.	3501, 4740	Suresh, N.	3927*	Tao, D.	4151	Thudium, D.	2009
Stevens, M.	3497, 4481, 4663, 4671	Surla, A.	3021, 3537, 4257	Tapaswi, A.	3123, 3272, 4131*, 4237, 4678, 4731	Thunga, P.	4492
Stevenson, A.	3578	Suroia, R.	3910, 3957	Tapper, M.	4462, 4463	Thurairasu, V.	4364*
Stevenson, L.	4438	Susiarjo, M.	2014, 2031	Tardano, F.	4187	Thurman, A.	3204
Stevenson, M.	3461*	Suske, D. Z.	4232	Targeted Protein Degradation Safety Committee, C. W.	4696	Tian, F.	4340
Stevenson, V.	3037	Sussman, E.	3746	Targeted Protein Degradation Safety Committee, S. D. W.	4035	Tian, L.	4083
Stewart, A.	4060, 4066	Susukida, T.	3432*, 3438	Tariq, F.	4230	Tian, Y.	4073
Stewart, J.	3359	Sutake, Z.	3294, 3299, 3380	Tariq, S.	3335*	Tibaldi, E.	3053
Stewart, K. D.	3617*	Suter, M.	3823*, 4349	Taroncher, M.	3800	Ticknor, R. C.	3264
Stietz, K. K.	3609	Sutter, C. H.	4608, 4609	Tasaki, J.	3162, 3163*, 3166	Tieghi, R.	3364
Stinchcombe, S.	4022	Sutter, T. R.	4608, 4609	Tasker, M.	3429	Tienda, A. A.	3109
Stingone, J. A.	4363	Suttiwan, P.	4087	Tasnim, S. M.	4608, 4609*	Tighe, R.	3933, 3943
Stinson, K.	4623	Suva, L.	3872	TatahMentan, M.	3412*	Tighe, R. M.	3927, 3932, 3941, 3958
Stinson, S.	4411*, 4435	Suvorov, A. S.	3195	Tatar Ozkan, T.	3809	Tiley, J. B.	3202, 4271
Stobbs, B.	4167	Suzuki, A.	4050	Tate, Y.	4240	Tillier, F.	3526
Stock, A.	4607*	Suzuki, I.	4147*	Tateshita, M.	3254, 4234	Tilman, J.	3277
Stoelze-Feix, S.	4235	Suzuki, K.	3728	Taube, J. H.	4489	Tilton, S. C.	3386, 3500, 4114, 4132, 4412
Stojakovic, P.	3021, 3537, 4257	Suzuki, S.	3258*, 3507, 4297*	Tavares, J.	3827	Timalsina, B.	4674*
Stoker, S.	4542	Suzuki, Y.	3004, 4574	Tawany Caetano Dos Santos, S.	3224	Timme-Laragy, A.	3159, 3603
Stoker, T. E.	3225	Svensson, M.	3535	Taylor, B.	3915*, 4192	Timme-Laragy, A. R.	3606, 3883, 4513, 4527
Stokey, W. R.	4633*	Svetlik, A.	4224, 4290*	Taylor, C. A.	3095	Timmer, N.	3900
Stolberg, R. N.	3406*	Svoboda, D.	4360	Taylor, J.	3035	Ting, A.	3487, 3515
Stone, A. B.	3807, 4344	Svoboda, L. K.	3272, 4237	Taylor, K. E.	3840	Tinwell, H.	3294
Stone, T.	3385, 4126	Svyatova, E. A.	3452*	Taylor, R. M.	4541, 4542*	Tisch, L.	3975
Stornetta, A.	3074	Swamy, S.	3051	Tchounow, P. B.	3973	Tiscione, S.	3321, 3987, 3991, 4099*, 4478
Stout, M.	4398, 4625, 4626, 4675	Swanzy, E.	3273	Tchounwou, P. B.	3851	Tisdall, M. E.	3409
Stowe, S.	4063*, 4080	Sweder Gold, F.	4027*	Teeny, S.	4280, 4689	Tittipkina, N.	4546
Stradtman, S.	4215	Sweeney, L. M.	3337*, 3564, 4161	Teerlink, J.	4477	Tiwari, S. K.	4170
Stradtman, S. C.	4214*	Sweet Duffy, L.	3756	Tejador-Rojas, F.	3794	Tjalkens, R.	3790, 4216
Stratidakis, A.	4009	Sweet, U. H.	3896*	Tejeda-Benitez, L.	3666	Tjalkens, R. B.	3599, 4217, 4222
Stratton, S. A.	4191	Swierczowski, N.	4037, 4038	Telang, A. C.	4649	Tjeerdema, E. R.	4465*
Straut, A. R.	3346*	Swihart, J.	3951, 3979	Tell, L. A.	3649, 4036	Tjerg, J. A.	3772*, 4592
Strazdins, R.	3093, 3566, 3570	Swihart, J. N.	3950*, 4454	Tellez, P.	3506	tlapa, J. A.	3145, 3609
Street, S. M.	2035, 3749, 3751	Swindle-Reilly, K. E.	4334	Templin, O.	4591	Tober, K.	4550
Streeter, S.	3446, 4736*	Syeda, T.	3592, 4094*	Teng, C.-L.	4499	Tobin, E. E.	3876*
Streifer, M.	3993	Szafran, A. T.	4232	Tennant, A. H.	3362*	Toborek, M.	3618
Strenge, M.	3868	Szafran, B.	3304	Tennessee, J. M.	4508	Toda, S.	3441*
Strilchuk, A. W.	3016	Szarka, A.	3347	Teodoro, T.	3588	Tokarz, D.	3594
Strom, M. M.	4203, 4209	Szarnicki, A.	4251	ter Braak, B.	3398	Tokumoto, M.	3784
Strongin, R. M.	3455	Szule, J.	4290	Terc, J.	4137*	Tokunaga, J.	3253, 3257
Stroud, L. R.	4652	Tabana, Y.	3370	Ternes, P.	4404	Tokuno, S.	3553, 4258, 4304
Stucki, A. O.	3516*, 4166	Tabata, H.	3728	Terrill, M.	3380	Tolbert, J.	4293*
Stucki-Koch, A.	4239	Tabatabaei-Dakhili, S. A.	3370	Terron, A.	3565	Toledo Sales, V.	4034
Stucky, A.	3323	Tabora-Sarmiento, S.	4266	Terry, M. B.	4281	Toler, S. C.	3514*
Sturdivant, R. X.	3455	Taborski, S.	2039	Terse, P.	4041	Tolosa, J.	3801, 4327*
Stutz, J.	3400	Tachibana, S.	3255	Terse, P. S.	4703	Tolton, A.	3803
Su, C.	2015, 4414	Tackett, A. P.	4085, 4656	Tesfaigzi, Y.	3496	Tomasevich, A.	4425
Su, H.	3487	Tadros, M.	3946	Tessier, J. M.	3081	Tomassilli, J. C.	4477
Su, K.	4170	Tagmount, M.	4405	Tetko, I.	2002	Tomita, K.	3490, 3954
Su, W.-C.	3501, 4061	Tahir, N.	3983, 3984*	Tewari-Singh, N.	3229, 3232, 3238, 4339	Tomlinson, S.	3048
Subileau, E. A.	3261*	Tahmasbi, A.	4074	Tewksbury, J.	4526	Tomlinson, W.	3530
Subileau, E.-A.	3687	Takagi, S.	3490, 3954	Thackray, C.	3547, 3563	Tompach, M. C.	3606
Subramaniam, A.	4063	Takahashi, K.	4046*	Thada, S.	3439*, 3511, 4287	Ton, S.	4011
Subramaniam, N.	3782	Takahashi, N.	3683*	Thakar, A.	3053*	Ton, T.-V.	3053, 4308, 4309, 4310
Subramanian, N.	3779	Takahashi, T.	3004, 4314, 4574	Thakor, V. P.	3763	Tong, Z.-b.	3586
Suchy, F.	4124, 4141, 4178	Takahashi, Y.	3430	Thakur, C.	4292	Toohay, C.	3371, 3707, 3709
Sue, A.	3111	Takasu, A.	3824	Thakur, M.	4020, 4143*, 4718*	Tool, L.	3031
Suemizu, H.	4584	Takebayashi, S.	4171	Thamert, R. E.	4140	Tora, S.	4457
Suess, J.	3138	Takeda, R.	3672, 3758, 4258*	Thapa, M.	3055*	Torczynski, K.	4190
Suganuma, N.	4725	Takeshita, J.	4378	Then, J.	3404, 3445*	Torczynski, K. A.	3629
Sugaya, S.	3333	Takeshita, T.	3162, 3166*	Thiede, M.	4450	Torenvliet, B.	3708
Suggs, A. P.	3220	Tal, T.	2029, 3141, 3565*, 3638, 4403	Thies, J. L.	4656*	Tornesi, B.	3177
Suh, E.	3425	Talbot, P.	3276, 3458, 4074	Thilakarathne, R.	3320*	Törnqvist, G.	4732
Suh, E. Y.	3953*, 4541	Talecki, L.	3048*	Thirumalaikumar, V. P.	3107	Torrente, M.	4009
Suh, N.	4685	Tamagno, W. A.	3088, 3089*, 3106, 4454	Thomas, C.	3408, 3611*	Torres Maia, M.	4402
Suh, Y. P.	3112*	Tamamis, P.	3612	Thomas, D.	3042, 3548*, 3721*, 4058*	Torres-Berrios, A. J.	2016*, 3608
Suka, M.	3444	Tampakakis, E.	4598	Thomas, J.	3569	Torrobá, M. B.	3810
Sukale, T.	4075	Tan, C.	3339, 4154, 4579*	Thomas, R. S.	3317	Tosi, G.	3651
Sukhawanit, R.	4150					Totti, S.	3285, 3301
Suki, B.	3393					Toyoda, J. H.	3787
Sulentis, C.	4742*					Toyoda, T.	3976

Tracy, J. W.	4307
Trafton, S. K.	4416
Tran, L.	3029, 4083, 4672
Tran, N.	3370
Tran, P.	3905*
Tran, T. D. B.	3816
Tran, V.	3655, 4689
Tran, V. T.	3595
Trapse, A.	3207, 3208*, 3209
Travers, J.	4259, 4419
Travis, J. C.	3316
Traylor, A. M.	3230
Traylor, B.	3558
Treadway, J.	3390*
Trelles-Sticken, E.	3461
Treuting, P. M.	3365
Trezza, M.	4075
Trietsch, S.	4117, 4120
Tripathi, R.	4344*
Trippe Nagaoka, L.	3224
Trivanovic, U.	4546
Trivedi, A.	3793*, 4466, 4471, 4472
Troncoso, T.	3880*
Tropsha, A.	2001
Troughton, L. D.	4341
Troutman, J.	4023*
Trovò, L.	4543*
Trowbridge, J.	3320
Troxel, W.	4024*
Truong, C.	4065
Truong, L.	3185, 3221, 3353, 3355, 3613, 3887, 4017, 4358, 4411, 4435, 4492
Trushina, E.	4371, 4375
Tsai, H.-H. D.	3373
Tsai, Y. H.	3237
Tsai, Y.-H.	3233*, 3247, 3588
Tse, K.	4137
Tseng, K.	3096
Tsilomelekis, G.	4546, 4547
Tsiodra, I.	3961
Tsui, W. N. T.	3197*
Tsuji, J.	4180*
Tsuji, M.	4179*
Tsuji, T.	4044
Tsunoda, M.	3444
Tubbs, C.	2016
Tucci, N.	3089
Tucker, K.	4607
Tucker, N.	2001*
Tukker, A. M.	3107, 3143, 4203, 4209*
Tuladhar, A.	3280*
Turla, T.	4663
Turner, A.	3809
Tuscano, J.	3964
Tuszynski, M.	3870
Twaroski, K.	3692*, 4566
Tweel, A.	4452
Twum, Y. B.	3457*
Tyler, C. R.	3141
Tyler, S.	4057*
Tyrrell, K. J.	4570*
Tzendzalian, T.	3217, 3844

U

Ubhayakar, S.	3561
Ubick, E.	3908, 3972
Uchiyama, A.	4050
Uddin, M. H.	3826*, 4460
Uechi, L.	3239
Uehara, S.	4050, 4584*
Ueyama, Y.	3519
Ufodu, A. U.	3604
Ukbamichael, A.	4362*, 4391*, 4539
Ullrich, A.	4450*
Um, D.	3222*
Umano, T.	3349
Umbaugh, D. S.	4390*
Umphres, S.	4078
Underwood, P.	4481
Ungarala, V. R.	3457
Unkel, C.	3247*, 3588
Unkel, C. M.	3233, 3237
Unniappan, S.	3855
Upadhyay, S.	3197, 3368
Uppu, R. M.	4439, 4627*, 4628
Urban, A. M.	3836, 4686
Ursu, G. M.	4595*
Ussher, J. R.	3370
Usui, R.	3490, 3954
Uyi, O.	3281

V

Vachiraarunwong, A.	3507, 4297
Vaddella, N.	3211*, 3825
Vahora, Z.	2019
Vaidya, K.	4709
Vaidya, V.	3447
Vaidyanathan, R.	4135, 4144*
Valcarcel-Hernandez, V.	3142
Valdelamar-Caballero, C.	3645
Valdez, G.	4525*
Valdez, R.	4412
Valdez, R. M.	4114, 4132*
Valentin, J.-P.	4034, 4701*
Valentine, P. A.	3528
Valentovic, M.	3030*
Valenzuela, A.	3579
Valenzuela, A. E.	3140, 3498, 3917, 3922, 4208
Valko, K.	3353
Vallez, M.	3350
van Berkel, C.	3379
van de Laar, D.	4630, 4637*
van de Steeg, E.	4534
van de Walle, I.	3031
van de Water, B.	3138, 3270, 3271, 3357, 3674, 3688, 3983, 4370*, 4387, 4404*, 4486
van den Broek, L.	4117, 4120
van der Hout, H. A.	3671*
van der Zalm, A.	3361
Van Deventer, D.	4276*
van Duinen, V.	3407
Van Dunk, C.	3992
Van Hee, J.	3853
van Kaam, S.	4535
van Kessel, H.	3138
van Kessel, H. W.	4486
van Kleef, R. G.	3811
van Loenen, P.	3383
van Marle, A.	3810
van Neel, T.	4261
van Neel, T. L.	4260*
van Offenbeek, J.	3810
van Pletzen, C.	4264*
van Puijvelde, G.	3536
van Rossum, P.	3175*
van Schaick, M.	4370
van Schijndel, L.	3983
van Schooten, F. J.	3671
van Schooten, F.-J. J.	4534
van Veen, S.	3271
van Vught, R.	3031*
Van Winkle, L. S.	3418, 3498, 3917, 4669
Vandenberg, L. N.	4688
VandenBussche, A.	4713
Vandermeulen, E.	3188
Vanderpuye, C.-M.	2039, 3482
Vandyousefi, S.	3661
Vang, D.	3964
vanVleet, T.	3280
Varella Gonsioroski, A.	3203
Vargas, A.	4068
Vargas, J.	4515
Vargesson, N.	4696
Varghese, A.	4307
Vargues, T.	3721
Varikuti, S.	3927
Varma, R. K.	4471
Varone, A.	4723
Vasoya, J.	3535
Vasquez, B. S.	3685
Vassaux, G.	4311a
Vasukuttan, V.	4148
Vaughan, E.	3280
Vaughan, K.	4224
Vaught, C.	3647
Vayas, K.	3928, 3935, 3939
Vayas, K. N.	3239, 3241, 3934
Vaze, N.	3961, 4546
Vaziri, M.	3514
Vazquez, M.	3527, 4345
Veerappan, A.	3464
Vega, B.	4723, 4733
Vega, B. A.	4729*
Veh, E. M.	3667*
Veiga-Lopez, A.	2019, 3201
Velayutham, M.	3941, 4467
Veldkamp-Baak, I.	3383
Velie, T.	3853
Veltmaat, L.	4220

Veluru, S.	3238
Venkatesan, S.	3604*
Venkatesan, A. K.	4292
Venkatesh, P.	4669
Venkatraman, A.	4737
Venneman, K.	2037
Venneman, K. K.	4294*, 4641
Venosa, A.	3937
Ventos, V.	4311a
Ventura, F.	4136, 4140
Venugopalan, A.	4727
Venz, A.	4170
Vera-Colón, M.	4432*
Verdugo, D.	3037
Vergis, J.	3113
Verma, N.	3889*, 4100*, 4102
Verma, R. R.	3193a*
Vermersch, E.	4230, 4699
Verras, A.	3535
Versnik, G.	3167
Verstraeten, S.	4166
Verwiel, A. H.	3997, 4604
Verzi, M.	4547
Vessey, J. D.	4027
Veteleanu, A.	3809
Viant, M. R.	3192, 3660
Vidal, E.	3635
Vielee, S.	4682
Vielee, S. T.	3094, 3101, 3104, 3113, 3124, 3769, 3778, 4594*
Vig, E.	4024
Vignaux, P. A.	3723
Vijaykumar, S.	3748*
Vilay, M. M.	4541
Vilcassim, R.	3948, 4063, 4080*
Villalta, P. W.	4326
Villareal, L.	2019, 4293
Villeneuve, R.	3690, 4112
Villeneuve, D.	3360, 4010, 4463
Villeneuve, D. L.	3359*, 4462
Vincent, B.	3045
Vincent, M.	3183, 3982, 4279*
Vincent, M. J.	3314
Vincent-Hall, T.	3901
Vincent-Hall, T. D.	3308, 3316, 3351
Vincent-Hall, T. J.	3309
Vincken, J.-P.	2021
Vinken, M.	4122*
Vinsonhaler, K.	3420
Visone, R.	4230, 4658*, 4699
Viswanadha, R.	3079
Vitale, O.	3827
Vitorino Maia, A. I.	3101
Vitucci, E.	4193*
Vitucci, E. C.	3495
Viviani, B.	3565, 4009*
Vlaming, M.	3810
Vlasakova, K.	4698
Vo, B.	3891
Vo, J.	3877*
Vogel, C.	3964*
Vogt, S.	4553
Vojta, S.	3895, 4505, 4511, 4523
Volety, P.	3839*
Volz, D.	3705, 3873
von Bölscház, G.	3326
Vonmoos, F.	3323
Voorbach, M.	3280
Vose, A.	3941
Voyer, C.	3812*
Voyles, C.	3412
Vrhovac Madunic, I.	3025, 4059*
Vries, R. G.	3708, 4113
Vrolijk, M. F.	3671
Vu, J. P. Q.	3487*
Vue, T. Y.	3480, 3792
Vulpe, C.	3037
Vulpe, C. D.	4405
Vuong, V.	3367
Vuong, V. H.	4344

W

W Herbert, G. W. H.	4551
Wabra, J.	4328
Wagener, T. L.	4085
Wages, P.	2020
Wagh, S.	3332, 4044
Wagner, D.	3429
Wagner, D. J.	3427*, 4730
Wagner, D. L.	3406
Wagner, J.	3127
Wagner, J. J.	3794
Waheed, U.	4654
Wahlang, B.	3010, 3616, 3617, 4501
Waidyanatha, S.	4578, 4583, 4626
Wakayama, M.	3349
Walk, S. T.	4593
Walker, H.	3401
Walker, J.	4537
Walker, L.	2039*
Walker, M.	3560, 4452
Walker, M. A.	3220, 4447*
Walker, P.	3697, 4243*
Walker-Franklin, I.	4550*
Wall, J. T.	3317
Wallace, D.	4087
Wallace, D. P.	3035
Wallace, J.	4167
Wallace, V.	4740
Wallbank, D.	3277
Wallqvist, A.	4374, 4383
Walters, G.	3506
Walz, A.	3865
Wambaugh, J. F.	3562*, 4189
Wang, A.	4168, 4307*
Wang, B.	3311, 3327, 4006
Wang, C.	3279*, 4443*
Wang, D.	3755, 3768, 4414, 4689
Wang, E.	4111*
Wang, G.	3263
Wang, H.	3108*, 3121, 3123, 3615*, 3897, 4073, 4275
Wang, H. J.	4051
Wang, J.	3426, 4319, 4607
Wang, J.-S.	3573
Wang, K.	3378, 3609, 4369*
Wang, L.	3050, 3096*, 3462, 4576
Wang, M.	4749
Wang, P.	4051
Wang, Q.	3091*, 3103, 4052
Wang, R.	3587, 4490
Wang, S. I.	4235
Wang, S.-L.	3436*
Wang, T.	3139, 4367*
Wang, W.	3951
Wang, W.-R.	4182, 4193
Wang, X.	3440*, 3549*, 4247, 4322, 4363
Wang, Y.	3116*, 3388*, 3533*, 3681, 3843, 4062, 4087*, 4284
Wang, Z.	3679, 4292*, 4702*
Wani, S.	3693
Wanibuchi, H.	3507
Wanyama, J.	4104*
Ward, A.	3556*
Ward, B.	4608
Warmka, J.	3740
Warner, G.	3817, 3820, 3904
Warner, G. R.	3170, 4367
Watanabe, E.	3394*
Watanabe, K.	4031
Watanabe, K. H.	4016
Water, B. v.	3984
Water, B. v. d.	3673
Waters, K. M.	3386, 3500, 4114, 4132
Watford, S.	3814
Watkins, P. B.	4607a
Watson, A.	3906
Watson, C. J.	4568*
Watson, L.	4253
Watson, S.	4580
Watson, W.	3631
Watson, W. H.	3010, 3616, 4501
Watt, M.	3217
Watt, M. A. L.	3844*
Watts, J. A.	4649
Weaver, J. R.	3685
Weaver, S.	3465
Webb, E.	4318*
Weber, R.	3192
Webster, T. F.	3627*

- Wedler, M. 4486
 Wei, B. 4749
 Wei, L. 4051
 Wei, N. 4052, 4053
 Wei, Y. 4244*
 Wei, Z. 3016, 3017, 3018
 Weigand, T. M. 3628
 Weinburg, J. 3755
 Weiner, A. M. 3192
 Weinschutz Mendes, H. 3106*
 Weinstock, D. 3245
 Weis, M. 3919
 Weise, D. R. 3506
 Weissner, N. 4709
 Weissmann, A. 4379*
 Weitekamp, C. 3562
 Weitekamp, C. A. 3317
 Weitz, K. K. 4570
 Wellnitz, J. 2001
 Wells, E. 4096
 Wells, E. M. 3776
 Wells, M. J. 3910
 Welmaker, B. 3625
 Wen, L. 3387*
 Wen, X. 3020, 3022*, 3032
 Weng, J. 3062, 3080, 3084
 Weng, M.-w. 3322, 3325, 3329*
 Weng, Z. 3322*
 Wenninger, P. 3667
 West, C. K. 3479*
 West, F. G. 3370
 West, M. 4629
 Westerink, R. 4535
 Westerink, R. H. 3797, 3811*
 Westlund, K. W. 4551
 Westmoreland, S. 3177
 Wetmore, B. A. 3225, 4152, 4153
 Wetzel, B. 3131
 Wevers, N. 3407
 Wexler, A. 3579, 4208
 Wexler, A. S. 3498, 3917, 3922
 Weyer-Nichols, C. 3439
 Whalley, P. 3285
 Wharey, L. 4310
 Wheeler, M. 3982, 4279
 Wheeler, V. 4268
 White, E. 4429
 White, J. 4148, 4708
 White, J. C. 4546, 4547, 4549
 White, K. 3348*, 3853
 White, P. A. 4330
 White, S. 4508
 Whitehead, A. 4437
 Whitehead, H. 4189
 Whiteley, L. O. 4706
 Whitfield, J. 4303*
 Whitmer, C. 3152
 Whittaker, M. H. 3310, 3311, 3327, 3994*
 Wicha, M. S. 4679
 Wichrowski, N. J. 4011*
 Wickliffe, J. 4080
 Wickramasekara, S. 3086*
 Wickramasinghe, N. S. 3918
 Wiczorek, R. 3456, 3461
 Wiczorkiewicz, L. 3849*
 Wieggers, J. A. 4493
 Wieggers, T. C. 4493
 Wiggins, B. 4659
 Wignall, J. 3420
 Wijaya, L. 3271*, 3357, 3674
 Wijaya, L. S. 4387
 Wijesinghe, M. R. 4397
 Wijnakker, J. 3708
 Wik, L. 3673
 Wikoff, D. 3183, 3314, 4005*, 4279
 Wikoff, D. S. 3653, 3982
 Wilflingseder, D. 3381
 Wilker, H. B. 4068*
 Wilkinson, K. J. 3922
 Wille, T. 3235*
 Willett, K. 4693
 Willett, K. L. 3179
 Willett, R. 4129
 Willi, L. 3554
 Williams, A. 4197
 Williams, A. J. 3338
 Williams, B. A. 3835*
 Williams, C. H. 4683
 Williams, D. 4384
 Williams, G. A. 3013*, 3014
 Williams, J. M. 4446*
 Williams, L. 2018, 2032*, 3151*
 Williams, S. N. 4619*
 Williams, T. 3548
 Williams, W. C. 3494
 Willighagen, E. 4370
 Willis, C. 4152
 Willis, C. M. 3362, 4153, 4155*
 Willis, K. 3692
 Willming, M. 3305, 3306
 Willoughby Sr, J. A. 3401*
 Willoughby, J. 3256, 3293
 Willson, C. 4675
 Wilsey, A. 3390
 Wilsey, J. 3080
 Wilson, D. 3136*
 Wilson, J. K. 4220*
 Wilson, L. B. 4492
 Wilson, M. 2017, 3492, 3562, 3625
 Wilson, M. M. 3835
 Wilson, M. R. 3130*
 Wilson, R. J. 3140*
 Wilson, S. 4241
 Wilson-Frank, C. 3052
 Windsor, K. 3100*
 Winer, L. 4164
 Winkelman, L. 3673
 Winkler, M. L. 4328
 Winn, N. 4266
 Winny, G. 3133, 3891
 Winter, S. 4272
 Winz, C. 4685*
 Wise Jr., J. P. 3101, 3104, 3124, 3769, 4682
 Wise Sr., J. P. 3101, 3769, 4682
 Wise, J. L. 3101, 3113, 3124, 3769, 3778, 4682
 Wise, J. P. 4605
 Wise, J. T. 3101
 Wise, J. T. F. 3778*, 3787, 4682
 Wise, Jr., J. 4594
 Wise, Jr., J. P. 3094, 3113*, 3778
 Wise, Q. 3101*
 Wise, R. M. 3787, 4682
 Wise, Sr., J. P. 3787
 Wise, Sr., J. P. 3113, 3778
 Wiss, L. 3300
 Wisser, O. 3469
 Witkowiak, K. 3098
 Witkowiak, K. M. 3102, 3103, 3115*, 4512
 Wittenborn, E. 3893, 3902
 Wittenborn, E. C. 3881*
 Wittman, E. 4447
 Wohlman, I. 3278
 Wojewodzic, M. W. 4009
 Wojtowicz, A. 3696*
 Wolberg, A. S. 3017
 Woldetensae, M. 3426
 Wolf, K. 3385
 Wolf, K. K. 4126
 Wolfe, T. M. 4593*
 Wolton, K. 3175
 Wondra, H. 3425, 4738*
 Wong, A. 3664
 Wong, J. 3063, 3064*, 3065, 3075, 3077
 Wong, K. 3603*, 4048
 Wong, L. 3754*
 Wong, M. 3458
 Woo, D.-H. 4235
 Woo, W. 4083
 Wood, A. 3398
 Wood, C. 4487
 Wood, K. B. 4300
 Wood, S. 4150
 Woodcock, M. 2015
 Woodford, B. 3746
 Woodhead, J. L. 4607a
 Woodlief, T. 3893*, 3902, 4634, 4724
 Woodlief, T. L. 3881
 Woodruff, T. 3320
 Woods, R. 3920
 Woodward, J. M. 4519*
 Woolbright, B. L. 3035
 Wooliever, E. F. 4552
 Woollorton, J. R. 3528*
 Wopken, P. 4535
 Wopken, P. J. 3811
 Word, L. J. 3356
 Workman, T. 4101
 Worth, C. 4489
 Wotring, J. 4131
 Wotring, J. W. 3272
 Wray, C. 3141*
 Wright, C. 3465, 3562, 3885
 Wright, F. 4193, 4492
 Wright, F. A. 3373, 4491
 Wright, G. 3197
 Wright, R. O. 4275
 Wright, S. H. 3541, 4571, 4629
 Wright, Z. 3714
 Wu, C. 4533, 4717*
 Wu, C.-C. 3133, 3188, 3891, 4445
 Wu, C.-T. 3640*, 3858*
 Wu, C.-W. W. 3462
 Wu, H. 2038, 3699*, 4051
 Wu, J. C. 4242
 Wu, K. 4238, 4582
 Wu, L. 4415
 Wu, P. 4650
 Wu, P.-Y. 4396*
 Wu, S. 3062, 3080*, 3325, 3329, 3582, 3890
 Wu, S.-W. 3161, 3652
 Wu, X. 3649, 3881, 4036*, 4299*, 4572
 Wu, Y. 3551*
 Wu, Z. 3023*, 3681
 Wurm, D. 4424*
 Wyatt, B. H. 4493
 Wyrzykowska, E. 3717
 Wyseure, T. 2007
- ### X
- Xenophontos, X. 3612
 Xia, H. 4228
 Xia, M. 3054, 3726, 3727, 3815, 4150, 4151, 4259, 4419, 4495
 Xia, Q. 3334
 Xia, Z. 3084
 Xiang, Q. 3873
 Xiang, S. 3037
 Xiao, S. 3626, 3880
 Xie, C. 4685
 Xie, F. 3512
 Xie, J. 3582, 3890*
 Xie, L. 4453
 Xie, T. 3032*
 Xie, Z. 4192
 Xing, Z. 3002*, 3015
 Xu, J. 3023, 3387, 3388, 3535, 3681*
 Xu, L. 3357, 3378, 3688*
 Xu, M. 4308, 4309*, 4310
 Xu, M. M. 3679, 3680, 4047
 Xu, R. 3726
 Xu, T. 3726*, 3727, 4151, 4259, 4495
 Xu, X. 4366
 Xu, Y. 4453*
 Xu, Z. 3808*, 4383
 Xue, X. 4293, 4299
- ### Y
- Yaeger, M. 3927
 Yaeger, M. J. 3932
 Yajima, T. 4583*
 Yamada, A. 3490, 3954
 Yamada, N. 3004
 Yamada, T. 3199, 3253, 3257, 3349, 3860, 4291*, 4376
 Yamada, Y. 3389, 3438
 Yamagata, R. 3777
 Yamaguchi, E. 4629
 Yamaguchi, N. 3441, 4656
 Yamamoto, D. 4046
 Yamamoto, T. 4043
 Yamanishi, E. 4171
 Yamashita, N. 3777
 Yamazaki, H. 4584
 Yan, C. 4006
 Yan, J. 4093
 Yanagisawa, H. 3444
 Yang, A. 4051
 Yang, I. 3465
 Yang, J. 3020*, 3022
 Yang, M.-J. 3955
 Yang, P. 4131, 4244
 Yang, Q. 4228
 Yang, S. 3815*, 4150
 Yang, S. I. 3165
 Yang, T. 2003*
 Yang, W. 4572
 Yang, X. 3009, 4632
 Yang, Y. 3037, 4453, 4599
 Yang, Z. 3898
 Yano, K. 3490, 3954
 Yanouri, O. 3790, 4216
 Yanouri, O. A. 3599*
 Yanouri, O. R. 4217
 Yard, B. 4312
 Yaroshuk, T. 3904
 Yasmin, F. 4533*
 Yates, N. E. 3599
 Yates, R. 3873
 Yauk, C. L. 4330
 Yaw, A. 3201
 Yazbeck, C. 3916*
 Yazzie, S. 3480*, 3792, 3921, 4600
 Yazzie, S. J. 4592
 Ye, J. 4163
 Ye, L. 4151
 Ye, X. 4500
 Yelleti, A. 3160*
 Yen, T. H. 3677*
 Yeung, C. 3034
 Yeung, P. 3376
 Yeung-Luk, B. 3637
 Yi, J. 4635
 Yianni, V. 3711
 Yin, L. 3392*, 3830
 Yin, M. 4095
 Yip, A. 3744*
 Yiting, L. 3430
 Yoder, J. A. 4728
 Yokohira, M. 3174
 Yokota, S. 3199*
 Yon, T. 3261
 Yoneda, N. 4584
 Yoo, B. 3514
 Yoo, B. R. 3494*
 Yoon, C. 3955
 Yoon, S.-H. 4056
 York, A. 4444
 Yoshida, K. 3349
 Yoshigae, A. Y. 3205
 Yoshii, M. 4700
 Yoshikawa, T. 4050
 Yoshimoto, T. 3441
 Yoshinari, K. 4378
 Yoshino, K. 3456
 Yoshioka, Y. 3860
 You, D. 3908*, 3972
 Younan, J. 3517, 4610
 Younce, K. M. 3446*
 Young, J. 3443, 4639
 Young, S. L. 3225
 Youngberg, A. 4556
 Youngblood, V. L. 3966*
 Youngs, L. 3285, 3301
 Yourick, J. J. 3647
 Yourick, M. R. 3647
 Yu, C. 3843, 4062
 Yu, F.-Y. 3652*
 Yu, I. 3391
 Yu, J. 4453
 Yu, S. 3582
 Yu, T. 3955, 4749
 Yu, X. 3874*
 Yu, X. J. 3392, 3830
 Yuan, C. 3582, 3890
 Yuan, K. 3006, 4612
 Yuan, S. 3012, 3634
 Yueh, Y. L. 4580
 Yuk, J. E. 4676
 Yumiketa, Y. 3487
 Yun, J.-W. 3007, 3187
 Yun, K. 3165*
 Yu-Rice, Y. 2034*, 4002
 Yurkow, E. 4247
- ### Z
- Zablah, M. 3779*
 Zaccaria, K. 3304*, 3335, 3336
 Zach, K. 4339
 Zacharewski, T. 3000, 3780
 Zacharewski, T. R. 3696
 Zafar, F. 4654
 Zafar, I. 3248
 Zafiratos, M. 3390
 Zagha, E. 3549
 Zagorski, J. W. 4720
 Zahn, D. 3638
 Zahoor, M. Y. 4612, 4613*, 4616
 Zahoor, Y. 3000, 3006

Zaiat, M.	4454	Zhu, H.	3626, 4259, 4334, 4367, 4377, 4547
Zakharov, A.	4042, 4704	Zhu, J.	3195*, 3533
Zalaznick, J.	4044	Zhu, L.	3759, 4521, 4743
Zalc, B.	3142*	Zhu, S.	3332*, 3856, 4130
Zalma, B. A.	4429	Zhu, T.	3971
Zaman, A.	3691	Zhu, W.	4407
Zamprogno, P.	3027*, 3469	Zhu, Y.	3547, 3563*, 3720
Zane, D.	4034*	Zhu, Z.	4405
Zanetti, F.	3323	Zhuang, Y.	3020, 3022
Zanotti Russo, M.	4187	Zhurgenova, A. A.	4449
Zanotti, S.	4706	Zickgraf, F. M.	4404
Zarei, P.	3194	Ziemann, C.	4328
Zauber, E.	4096	Zierden, H. C.	3822
Zavidil, J.	4326	Ziesenitz, M.	3638
Zdybel, S.	3717	Zimmer, C.	3260*
Zegura, B.	4059	Zimmerman, S.	4281
Zelikoff, J.	3992	Zimmerman, Z.	3012, 3634*
Zelikoff, J. T.	3464, 3619, 3914, 4278	Zingales, V.	3659*
Zelinski, M.	4016, 4031	Ziv-Gal, A.	3839
Zelko, I.	3918, 4468	Zmuda, J. M.	4268
Zelko, I. N.	3915	Zolkowski, L.	3048
Zeng, J.	4511*, 4523	Zolkowski, L. A. K.	3396, 3397*
Zengin, A. N.	3782, 3785	Zong, Y.	4749
Zenitsky, G.	3122, 3572, 4636	Zooq, S. J.	3041
Zequiri, E.	4661	Zou, J.	4707*
Zevallos-Morales, A.	3201	Zühr, E.	4128
Zhai, J.	3125	Zulkowski, N.	4508
Zhan, J. J.	3595	zur Nieden, N.	3184
Zhan, T.	3626	Zurlinden, T. J.	4581
Zhang, A.	4191	Zuverza-Mena, N.	4546, 4547, 4549
Zhang, C.	3321, 3679, 3856, 4478*	Zweifel, L. S.	3095
Zhang, G.	3788, 4587	Zychowski, K.	3480, 4600
Zhang, J.	3332, 3424*, 3456, 3533, 3753, 3880, 4030, 4123, 4368, 4591, 4597	Zychowski, K. E.	3792*, 3921*
Zhang, K.	4345, 4719*		
Zhang, L.	3172, 3197, 3727, 3846, 4055*, 4334*, 4690, 4707, 4717		
Zhang, M.	4317*		
Zhang, M. x.	3759		
Zhang, P.	3169*, 3189, 4632		
Zhang, Q.	3451, 3465, 3562, 3625*, 4016, 4019, 4031		
Zhang, Q.-Y.	4572		
Zhang, R.	4241, 4271, 4317, 4319, 4702, 4711		
Zhang, S.	3084, 3390, 3538, 3680, 4055, 4313*, 4690		
Zhang, W.	3544, 3950, 4292		
Zhang, X.	2021, 3125, 3139, 3561, 3942, 4123*, 4238, 4330*, 4338*, 4545		
Zhang, Y.	2036, 3002, 3015, 3172, 3720, 3846, 4055, 4289*, 4337, 4453, 4523, 4707, 4711*		
Zhang, Z.	2000*, 4244, 4396		
Zhao, D.	3651		
Zhao, H.	3097, 3582*, 3890, 4322		
Zhao, J.	3054, 3512, 3726, 4151, 4259		
Zhao, L.	3096, 4324, 4329*, 4525		
Zhao, P.	4603		
Zhao, S.	3424		
Zhao, S. R.	4242		
Zhao, T.	4151, 4238, 4419		
Zhao, X.	3582, 4052*, 4340*		
Zhao, X. x.	3759		
Zhao, Y.	3334, 3531*, 3647, 3713		
Zhao, Z.	2015		
Zhel'tov, E. J.	4447		
Zheng, S.	3064, 3076*, 4238		
Zheng, X.	4082*		
Zheng, Y.	4651		
Zhitkovich, A.	4595		
Zhong, Y.	3561, 4047*, 4287		
Zhou, C.	3821*, 4531, 4545		
Zhou, F.	3759*		
Zhou, G.	4330		
Zhou, J.	2006*, 3037*, 3213		
Zhou, L.	4690*		
Zhou, P.	3239, 3242, 3245, 3934, 3935, 3939, 3940		
Zhou, R.	4319		
Zhou, T.	4545		
Zhou, T. s.	3759		
Zhou, X.	3123, 3424, 4131, 4200, 4476, 4603		
Zhou, Y.	3748, 4337		
Zhou, Z.	4632*		
Zhu, C.	4749		

- (S)-nicotine analogs 3455
- 1,2,4-Trimethylbenzene 4626
- 1,2,4-trimethylbenzene 4625
- 1,3-Butadiene 3342
- 1,4-Dichlorobenzene 3029
- 1,4-Dioxane 4292
- 1,9-Dimethyl nonanedioate 4715
- 2,1,3 4042
- 2,2'-Dimorpholinodiethyl ether (DMDEE) 4623
- 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) 4609
- 2,3,7,8-tetrachlorodibenzo-p-dioxin 3780, 4437
- 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-t 4636
- 2-amino-9H-pyrido[2,3-b]indole 4677
- 2-ethyltoluene 4675
- 3,3'-Dichlorobiphenyl (PCB-11) 4572
- 3,4-Dichloroaniline 3192
- 3,5-diethoxycarbonyl-1,4-dihydrocollidine 2040
- 3D cell system 4248
- 3D printing 3625
- 3-nitrobenzanthrone 4302
- 4G/5G Electromagnetic Fields 3174
- 4-vinylcyclohexene diepoxide 4068
- 6PPD 4676
- A. calamus*, Beta Asarone 4116
- A549-NRU assay 3507
- AAV 4717
- Accelerated Ovarian Failure 4068
- Acephate 3319
- Acetaminophen 2037, 3001, 3004, 3011, 3016, 3019, 3183, 4005, 4279, 4687
- Acetaminophen, Capmatinib 3014
- acetaminophen, ERA 3686
- Acetaminophen, Osimertinib, Afatinib 3013
- Acetic acid 3382, 4148
- acetylcholinesterase 3236
- Acrolein 3457, 4176, 4655
- Acupen 4488
- acute toxicity 3997
- ADC 3538
- ADC, LC-MS/MS, ... 4047
- ADCs 4661
- Adipogenesis 4301
- Adipose 3617
- Adipose tissue 3284
- Adverse Outcome Pathways 3395
- Adverse Outcome Pathway; FAIR 4009
- afidopyropen 4579
- Aflatoxins 3994
- Agents 3507, 3716, 4043, 4262, 4324, 4352, 4691
- Aging 3089, 3101, 3123, 3180, 3196, 3228a, 3570, 3577, 3578, 3655, 3666, 3786, 3789, 3818, 3854, 4204, 4207, 4213, 4219, 4220, 4222, 4418, 4466, 4619, 4657
- Agomelatine 4057
- AHR 3964
- AHR ligands 4727
- AI Fluency; Workforce Development 3414
- AICAR 3316
- AI-Driven Analysis 3037
- Air pollution 3498, 3792, 3792, 3909, 3921, 3968
- Albuminuria 3897
- alcohol 4619, 4694
- Alcohol; Prescription medication; Self-poisoning 4262
- alcohol-liver injury 3862
- ALD, TASLD, liver injury 3482
- Aldehydes 4070, 4662
- Alkyl substituted naphthalenes 3171
- Alkylated furans 3554
- Alkylating Agents 3025, 3239, 3242, 3245, 3262, 4334, 4344
- all-trans retinoic acid 4618
- alpha synuclein, Exosomes, Red blood cells, Exodus 4636
- Alternative Splicing 4308
- Alternative substances 4522
- Alternatives Assessment 2025, 3327, 3348, 3354, 3369, 3377, 3717, 3756, 3989, 4150, 4173, 4283, 4369, 4620
- Alternatives to Animal Testing 2007, 2020, 3027, 3031, 3042, 3072, 3116, 3138, 3162, 3163, 3166, 3175, 3191, 3253, 3254, 3257, 3265, 3266, 3267, 3281, 3289, 3337, 3343, 3347, 3349, 3353, 3355, 3356, 3361, 3363, 3364, 3372, 3373, 3379, 3380, 3381, 3385, 3388, 3399, 3404, 3461, 3538, 3560, 3587, 3675, 3681, 3686, 3697, 3706, 3707, 3708, 3711, 3723, 3726, 3754, 3764, 3809, 3810, 4008, 4012, 4013, 4029, 4033, 4093, 4112, 4116, 4121, 4124, 4127, 4130, 4136, 4141, 4144, 4151, 4160, 4169, 4170, 4171, 4175, 4175, 4178, 4223, 4239, 4242, 4311a, 4354, 4474, 4485, 4620, 4648, 4699, 4732
- Alveolar macrophage, Emphysema and COPD 3910
- Alzheimer's disease, APP/PS1 mice, mitochondria, d 4371
- Alzheimer's 3046
- Alzheimer's disease 3582, 3703, 3965, 4195, 4203, 4204
- Aminoglycosides 3038
- Aminopyridines 3807
- Amiselimod 4043
- Ammonia 3960
- Amorphous Silicon Dioxide 3513
- AMP-activated protein kinase 3180
- Amyloid 3596
- Amyloid Beta, Alzheimer's Disease, LRP1, BACE-1 3704
- anakinra, etanercept 4261
- Analogue 4014
- Analysis 3286
- Aneuploidy 3825
- Angiotensin-II, Phenylephrine 4228
- aniline 4090
- animal model alternatives 4138
- Animal Welfare 4046
- antibody and payload 4047
- Antibody drug conjugates 3365
- Antigen-Presenting 3056, 3425, 3536
- Antimicrobial 4102
- Antimicrobial peptides; ulcerative colitis 4287
- Antioxidants 3036, 3127, 3158, 3200, 3645, 3646, 3666, 3797, 3832, 3924, 4415, 4423
- Anti-PD-1 Antibody 3431
- Antiretroviral toxicity 4364
- Anti-sense oligonucleotide, Gene therapy 4048
- antisense oligonucleotides 4706
- Antivirals 3353
- AOP 4230
- APOE4 3597
- Apoptosis 3262, 3513, 3567, 3581, 3639, 3788, 3801, 3899, 4212, 4407, 4587, 4590, 4707
- Aquaculture, pollutants, infection, phytochemicals 4018
- Aquatic Toxicology 2016, 3151, 3167, 3192, 3359, 3610, 3767, 3826, 3851, 3852, 3891, 4018, 4138, 4439, 4443, 4450, 4458, 4459, 4460, 4509, 4645, 4668
- Archived Tissue, Retrospective Exposure 3875
- Arsenic 3436, 3767, 3773, 3779, 4267, 4268, 4268, 4270, 4298, 4388, 4593, 4599
- Arsenic, cadmium, lead 4731
- Arsenic, Lead, Cadmium, Hexavalent Chromium 3637
- Arsenic, Uranium 3782
- Arsenic, Zinc 3775
- arsenic, zinc, zinc finger protein 3775
- Arthritic 4488
- Artificial Intelligence 3548, 3721
- Artificial Intelligence, Off-Targets 3718
- Artificial intelligence; Machine learning 3725
- Aryl Hydrocarbon Receptor 4492, 4742
- Asbestos 4004, 4671, 4740
- Asbestos, Thresholds, Biologically Safe Dose 4663
- Aspartame, Lead 3661
- Asthma 4132
- Astrocytes 3046, 3093, 3790, 3862, 3890, 4216, 4222
- Atrazine 4202
- autism 3145
- Autism Spectrum Disorder 4748
- Autoimmune 3423, 3425, 3431, 4286, 4600, 4727, 4738, 4739
- azoxystrobin 3141
- B Cell 3606, 3964, 4067, 4724, 4727, 4742
- Bandrowski's base 4723
- basal forebrain cholinergic neurons 3581, 4201
- Beginning of extrapolation 3304
- Behavior 3088, 3089, 3096, 3118, 3144, 3146, 3153, 3167, 3169, 3182, 3188, 3219, 3592, 3596, 3638, 3767, 3783, 3804, 3900, 3949, 4060, 4226, 4403, 4460, 4466, 4518, 4520, 4653
- Behavioral 3096, 3147, 3155, 3174, 3183, 3218, 3477, 3714, 3793, 3802, 3858, 3864, 4130, 4654
- Benzalkonium Chlorides 3378
- benzene 3315, 3915, 3918
- benzo(a)pyrene 4434
- benzo[a]pyrene 3132, 3825
- Benzo[a]pyrene and 8-methyl-benzo[a]pyrene 4411
- Benzo[b]fluoranthene 4312
- berberine 3180, 4296
- Beryllium 4458
- Biliary Excretion 3679
- Bioaccumulation 4465
- Bioanalysis 4632, 4749
- Bioavailability 3066, 3401, 4023, 4119, 4212, 4365, 4465, 4561, 4564
- Biocompatibility 2033, 3745
- Biocompatibility; ISO 10993; Extraction 2032
- Bioinformatics 2004, 2005, 3021, 3053, 3151, 3161, 3210, 3215, 3295, 3317, 3350, 3360, 3473, 3782, 3938, 4010, 4029, 4195, 4230, 4245, 4308, 4310, 4366, 4370, 4373, 4379, 4386, 4390, 4399, 4414, 4466, 4486, 4487, 4490, 4492, 4493, 4494, 4495, 4606, 4613, 4622
- Biological Modeling 3256, 3262, 3353, 3548, 3549, 3551, 3560, 3562, 3627, 3649, 3861, 3887, 3990, 4019, 4022, 4023, 4036, 4198, 4241, 4371, 4391, 4581, 4604, 4607a, 4627, 4628, 4713
- Biologics 3379
- Biomarkers 2004, 3020, 3022, 3028, 3098, 3102, 3103, 3249, 3280, 3447, 3473, 3476, 3597, 3629, 3714, 3770, 3805, 3860, 3867, 3868, 3901, 3943, 3952, 3973, 4063, 4080, 4101, 4190, 4195, 4211, 4257, 4272, 4280, 4290, 4308, 4309, 4321, 4322, 4323, 4343, 4454, 4627, 4628, 4629, 4632, 4635, 4636, 4637, 4638, 4639, 4640, 4641, 4688, 4701, 4733
- Biomaterial 4449
- Biomonitoring 3454, 3554, 3636, 3914, 4182, 4183, 4184, 4185, 4194, 4198, 4270, 4455, 4505
- Biotech Products 3350, 3719, 4047, 4051, 4163, 4397, 4709, 4714
- Biotherapeutics 3042
- Biotransformation 3303, 3553, 3554, 3684, 3779, 4463, 4547, 4559, 4628
- Biotransformation and Toxicokinetics 3352, 3544, 4565
- Bispecific Antibodies 3536
- Bisphenol A 3221, 3584, 4560, 4643, 4735
- Bisphenol analogues 3624
- Bisphenols 3155
- Blood Brain Barrier 3808, 4144
- Blood lead 4183
- Blood-Brain Barrier 3094
- Blood—milk barrier 3363
- Bone 3280, 3872, 4548, 4645
- botanicals 2020, 2023, 3019, 3275, 3605, 3640, 3644, 3645, 3647, 3648, 3713, 4260, 4488, 4554
- Breast Cancer 3639
- Breast Cancer, Lymphohematopoietic Cancer 3988
- Breath Volatiles 4639
- Brevetoxin 3802
- Brevetoxin, red tide 4446
- Brominata™ 4325
- Bronze Casting 4640
- BTEX 3514
- Buprenorphine 4691
- Burn Pit 3508
- Butyl Oligomer (C21H40) 4003

- Butylparaben 3581
- C. elegans* 3573
- C. elegans* 3191, 4223, 4594
- Cadmium . 3108, 3607, 3695, 3784, 4256, 4269, 4301, 4585
- Cadmium (Cd), Colorectal cancer (CRC), metastasis 4293
- Cadmium and Lead 3630
- Cadmium exposure, Secondhand smoke, Cotinine level 3454
- Cadmium, Arsenic, Zinc 4414
- Caenorhabditis elegans 3882
- Calibration of toxicity reference values ... 3317
- Calicheamicin 4210
- Cancer organoids 4140
- Cannabichromene 3556
- cannabidiol 3358, 3641, 4071
- cannabidiol and trazodone 4212
- cannabinoid 3146, 3179, 3650
- Cannabinoids 3556, 3647, 4693, 4721
- Cannabis 3409, 3472, 4265, 4746
- Cannabis, Cocaine 4692
- Cannabis, Metals, Organic contaminants 3755
- Carbon Black 3957
- Carbon Tetrachloride 3002, 3015, 3018, 3336
- carbon tetrachloride, toluene 3335
- Carcinogenesis 3003, 3035, 3308, 3312, 3314, 3322, 3329, 3492, 3537, 3668, 3683, 3736, 3774, 3775, 3964, 4005, 4043, 4131, 4280, 4291, 4292, 4294, 4295, 4296, 4297, 4298, 4299, 4300, 4301, 4302, 4303, 4304, 4306, 4307, 4308, 4309, 4310, 4311, 4311a, 4321, 4326, 4327, 4392, 4413, 4590, 4592, 4603, 4605, 4631, 4641, 4678, 4679, 4682, 4683, 4684, 4685, 4686, 4687, 4688, 4690
- Carcinogens 3062
- Cardiopulmonary 3470, 3515, 3772, 4254, 4467
- cardiotoxicity 3151, 3383
- Cardiovascular System 3277, 3387, 3390, 3463, 3488, 3514, 3634, 3702, 3785, 3848, 3881, 3915, 3918, 3966, 3981, 4045, 4068, 4078, 4118, 4120, 4134, 4135, 4159, 4168, 4225, 4226, 4228, 4230, 4231, 4232, 4233, 4234, 4235, 4236, 4237, 4238, 4239, 4240, 4241, 4242, 4243, 4244, 4245, 4247, 4248, 4249, 4251, 4253, 4254, 4257, 4259, 4260, 4261, 4268, 4468, 4531, 4541, 4598, 4635, 4700, 4701
- CAS 60144-85-6 4628
- CBD, THC 3275
- Cefazolin 3541, 4571
- Cell Communication 4324
- Cell Culture 3032, 3274, 3387, 3399, 3407, 3457, 3458, 3505, 3585, 3645, 3673, 3681, 3684, 3685, 3719, 3772, 3823, 3878, 3886, 3910, 4082, 4106, 4109, 4142, 4163, 4258, 4290, 4408, 4409, 4417, 4418, 4503, 4558, 4668, 4728
- cell culture, ROS 3503
- Cell Lines, Transfected 4131
- Cell Painting 3810
- Cell Proliferation 2037, 2038, 3039, 3273, 3378, 3654, 4139, 4303
- cell therapy 4053, 4726
- Cellular Plasticity 4131
- Cellular Reactive Nitrogen and Chlorine Species 4628
- cereblon 3150
- Cetylpyridinium chloride 3427, 4416, 4730
- Chemical allergens 3260
- Chemical Allergy 3252, 3253, 3510, 4484
- Chemical and Biological Weapons 3230, 3231, 3234, 3235, 3236, 3238, 3239, 3240, 3241, 3242, 3243, 3244, 3245, 3247, 3249, 3375, 4339
- Chemical Characterization 3057, 3063, 3070, 3071, 3077, 3082, 3086, 3355, 3491, 3492, 3510, 3644, 3729, 3730, 3731, 3737, 3740, 3749, 3926, 4085, 4151, 4185, 4417, 4439, 4639, 4746
- Chemical Entity 3013, 3014, 3248, 4197, 4662
- Chemical exposures 4195
- Chemical Hazard Assessment 3178, 3264, 3288, 3314, 3327, 3338, 3357, 3358, 3361, 3442, 3622, 3756, 3762, 3888, 4011, 4033, 4088, 4091, 4128, 4149, 4158, 4173, 4199, 4267, 4269, 4279, 4325, 4373, 4387, 4449, 4476, 4486, 4626, 4671, 4712
- Chemical imaging 4589
- Chemical of Concern 3053, 3322, 3412, 3427, 3446, 3625, 3749, 3898, 3926, 4249, 4393, 4416, 4526, 4550, 4655, 4730
- Chemical Nomenclature 4197
- Chemokine, Signalling 3439
- Chemokines 3691, 4592
- Chemoproteomics 2028
- Chemotherapy 3557
- Children's Health 3223, 3412, 3422, 3444, 3942, 3985, 4200
- Chlorambucil 4324
- Chlorinated acetaminophen 4627
- Chlorinated compounds 3574, 3762
- chloropicrin 3234, 4669
- Chlorpromazine 4415
- chlorpromazine, bosentan, fialuridine ... 3677
- Chlorpyrifos 3590, 3591, 3594
- Cholestasis 4122, 4563
- Chromatography prediction models 4639
- chromium 3778
- Chrysotile 3497
- Cigarette Smoking 3045
- Circadian rhythm 3139, 3154
- Cisplatin 3020, 3025, 3642
- Clastogen 3667
- Climate change 3281, 3540, 4325, 4657
- Climate Change; Neuroinflammation 4466
- Clinical Toxicology 3042, 3043, 3045, 3047, 3052, 3054, 3127, 3421, 3452, 3619, 3721, 3757, 4058, 4264, 4634, 4649
- Clinical Trials/Human Studies 2031, 3022, 4272
- Clozapine 3370
- CLP classification 4458
- CNS 3809, 4714
- Coagulopathy 4332
- Cocaine 3092
- combustion 3492
- Comparative 3052
- Computational Data Integration 3350
- Computational Modeling 3547
- Computational Toxicology 2000, 2001, 2002, 2004, 2005, 2020, 2021, 3074, 3254, 3288, 3313, 3317, 3350, 3356, 3525, 3526, 3564, 3622, 3626, 3648, 3649, 3660, 3716, 3717, 3723, 3814, 3840, 3887, 4010, 4011, 4012, 4014, 4016, 4018, 4021, 4022, 4024, 4025, 4026, 4030, 4031, 4032, 4033, 4057, 4083, 4151, 4155, 4196, 4277, 4306, 4352, 4353, 4354, 4355, 4356, 4357, 4364, 4365, 4366, 4367, 4369, 4374, 4375, 4377, 4378, 4379, 4381, 4383, 4384, 4385, 4386, 4387, 4388, 4389, 4393, 4395, 4396, 4399, 4400, 4403, 4485, 4490, 4491, 4581, 4622, 4627
- Consumer products 3755
- Copper 4637
- Copper oxide nanoparticles 3973
- Cortical Organoids 4498
- cosmetic ingredient 4562
- Cosmetic Products 3754
- Cosmetics, Tox21 4495
- CRISPR 3302
- CRISPR screen 4405
- Cross-species translation 3048
- Crystalline silica 4288
- CSTF2 4044
- Cumulative 3987
- cuproptosis 3008
- Cutaneous or Skin Toxicity 2020, 3059, 3193a, 3235, 3238, 3245, 3250, 3261, 4169, 4449, 4530, 4608, 4609
- cyanazine, imidacloprid, atrazine 3359
- cyanobacteria 3176
- Cyclicity 3220
- cyclodextrins 4697
- Cynomolgus Macaque 3024
- cynomolgus monkey 3868
- Cytochrome C Oxidase 3805
- Cytochrome P450 2038, 2040, 3002, 3026, 3131, 3132, 3156, 3540, 3559, 3616, 3711, 4165, 4413, 4425, 4503, 4513, 4572, 4584, 4670, 4694
- Cytokine, Signalling 4525
- Cytokines 3096, 3405, 3471, 3702, 3772, 3806, 3943, 3947, 4051, 4220, 4261, 4471, 4679, 4731, 4734, 4735, 4741, 4749
- Cytoplasmic Vacuolization 4623
- Cytotoxicity 2021, 3008, 3035, 3054, 3063, 3078, 3097, 3178, 3250, 3267, 3369, 3376, 3398, 3405, 3457, 3538, 3654, 3698, 3788, 4059, 4071, 4072, 4073, 4341, 4342, 4347, 4415, 4530, 4587, 4593, 4611, 4623, 4674, 4741
- Dapagliflozin 4678
- DART 3846
- Data Integration 4384
- DDX, TCPMs 2016
- Decidualization 2018
- Deep Learning 4395
- degraders 3150
- DEHP 4461
- Deiodinase Inhibition 3292
- Dendritic Cells, Mast Cells 3234
- Dermal Absorption 4023
- Dermal Absorption Assay, Skin Integrity Testing 3763
- DESI-MSI 4516
- Developmental Neurotoxicity Compounds 3355
- Developmental Toxicity; Post-Natal 3120, 3187, 3217, 3303, 3442, 3607, 3608, 3611, 3831, 4244, 4745, 4747
- Developmental Toxicity; Prenatal 2015, 2027, 3152, 3164, 3172, 3176, 3177, 3178, 3190, 3193a, 3201, 3204, 3211, 3212, 3276, 3426, 3499, 3594, 3607, 3829, 3846, 3866, 3877, 3962, 4066, 4069, 4128, 4141, 4178, 4274, 4349, 4414, 4525, 4598, 4652, 4747
- Developmental/Teratotoxicity 2016, 3150, 3151, 3156, 3161, 3162, 3163, 3166, 3175, 3179, 3180, 3185, 3192, 3193a, 3402, 3864, 3873, 4129, 4267, 4269, 4331, 4332, 4422, 4448, 4618, 4693
- Dexamethasone 3549, 4615
- Diabetes 4601
- diazinon 3337
- Dichloroacetic Acid 3152
- Dichlorodiphenyldichloroethylene (DDE) 3597
- Dichlorodiphenyltrichloroethane (DDT) ... 3596
- Diclofenac, Ketoconazole, Nitrofurantoin 4404
- Diesel exhaust particulate matter 3944, 3966
- Dietary Supplements 3664
- diethylene glycol 3421
- Diethylstilbestrol 4294
- Dihydroxyacetone 3463
- Diisononyl Phthalate 3194, 3206, 4409
- Di-isononyl phthalate (DINP) 3314
- Diisopropyl fluorophosphate (DFP) 3589
- Diisopropyl fluorophosphate 3233, 3247
- Dimethyl sulfoxide 4314
- Dimethyl Terephthalate 3325
- Dioxin 3439, 3616, 3617, 3780, 4019, 4437, 4492, 4608, 4609, 4742
- Dioxin, PAH 3964
- Dioxins 3550
- diphenhydramine 3290
- Disposable pods, E-cigarettes, Oxidative Stress, ... 3458
- DNA Adducts 4316, 4329
- DNA Damage 4312
- DNA Damage and Repair 4682
- DNA damage, American alligator, Comet assay, Metal 3787
- DNA methylation, wildfire smoke 4642
- DNA Repair 2030, 3092, 3351, 3774, 3876, 4210, 4316, 4329
- DNT; ToxRefDB; Hazard 3814
- Dose-Response 3040, 3099, 3105, 3250, 3304, 3312, 3331, 3334, 3353, 3455, 3628, 4001, 4435, 4446, 4454, 4461
- Dosimetry 3049, 3456, 3477, 3501, 3564, 4047, 4180, 4565
- Doxorubicin 3557, 4233, 4631, 4635
- Doxorubicin, Dexrazoxane 4251
- Drilling fluid 4449
- Drinking water 3892
- Drinking water, BMD, TFC adenomas 3308
- Drosophila melanogaster* 4508
- Drug delivery 3549
- Drug development 3537

Drug Safety and Efficacy	4142	Environmental Toxicology ...	2012, 3000, 3010, 3033, 3109, 3116, 3130, 3134, 3156, 3176, 3193, 3205, 3211, 3248, 3283, 3296, 3298, 3302, 3374, 3437, 3483, 3485, 3487, 3491, 3493, 3496, 3575, 3593, 3612, 3623, 3626, 3631, 3634, 3695, 3769, 3791, 3830, 3838, 3843, 3851, 3875, 3889, 3891, 3897, 3915, 3918, 3922, 3933, 3942, 3943, 3945, 3953, 3956, 3958, 3959, 3961, 3967, 4092, 4131, 4143, 4150, 4207, 4221, 4256, 4277, 4305, 4339, 4342, 4384, 4397, 4432, 4447, 4450, 4456, 4467, 4484, 4497, 4517, 4531, 4532, 4538, 4546, 4547, 4549, 4554, 4572, 4603, 4611, 4636, 4738, 4743	Fatty acid metabolism, Mitochondria, HepG2, HUH7	3695	germline transmission	4052
Drug-Drug Interaction (DDI), FDA/EMA guidelines	3559	Enzymes, Exposure Limits	3306	FDA Animal Rule	3249	GHS Mixtures Equation, acute oral toxicity	4100
Echo examination	4240	EPFRs	3488	Female Reproductive Toxicity	3626	Glass Fiber	3352
E-cigarette aerosol	4069	Epidemiology ...	3298, 3438, 3627, 3776, 4096, 4221, 4267, 4268, 4269, 4273, 4274, 4275, 4276, 4279, 4282, 4357, 4363, 4634, 4686	Fenbucanazole	3291	Glia	3122, 3139, 3790, 3912
E-cigarette, Benzaldehyde, Veratrylaldehyde	3457	Epigenetics	3117, 3169, 3195, 3210, 3221, 3593, 3971, 4286, 4305, 4309, 4310, 4388, 4436, 4492, 4643, 4644, 4646, 4648, 4649, 4650, 4652, 4653, 4739	Fenofibrate	4374	GLP-1RA	4345
E-Cigarettes	3460, 4067	Epitranscriptomics	3122	Ferroptosis	3346, 4417	Glucocorticoids	4722
Ecotoxicology	3360, 3612, 3826, 4138, 4356, 4442, 4443, 4446, 4448, 4453, 4454, 4457, 4459, 4460, 4461, 4588	Essential Metal Dyshomeostasis	3769	Ferroptosis Reactive oxygen species	4680	Glucosamine	3166
EDSP	3289	Ethanol	3307, 3872, 4653	Ferroptosis; ROS; Lipoperoxidation	3368	Glucose Homeostasis	3670
Education 3043, 3408, 3409, 3410, 3411, 3413, 3414, 3415, 3416, 3417, 3418, 3657		Ethanol, PPARα	4501	Fertility	3291, 3816, 3834	Glucuronidation	3293
Education Outreach	3410, 3412, 3415, 3416, 3418, 3419, 3420	ethylene dibromide	3311	Fiber toxicology	4659	Glutathione	3049, 4423, 4631, 4680
Efavirenz	4617	Ethylene Oxide	4322	Fibrinolysis	3016	Glyoxalase-1	4425
EGFR, JNK, Autophagy, Regeneration	3013	Eucalyptus Oil	4264	Fibrosis	4343	Glyoxylic acid, Oxalic acid	3326
Egr1, Drug Induce Liver Injury	2037	Eucalyptus Smoke	3494	fine particulate matter	4432	GlyphAgo	4057
ELCR	3478	EV	3860	Fire Response	3762	Glyphosate	4318
Electronic cigarette, Oxidative Stress, Dry Eye	3471	Everolimus	3539	flame retardant	3151, 3985	goat; NLME; risk assessment	4036
Electronic cigarettes	4083, 4255, 4662, 4666	Evidence map	4307	flame-retardant	3717	goat; sheep; risk assessment	3649
Electronic Cigarettes, Disease-in-a-dish Modeling	3276	Ex Vivo	3448	Flash Flood	4473	GPCR	4259
Elesclomol-copper	3040	Exatecan	4707	flavor	4666	granulosa cells	3200
Embryo	3172, 3606, 3834, 4434, 4618	Excess Lifetime Cancer Risk	3479	Florfenicol	3649, 4036	graphene oxide	3851
Embryonic Stem Cells	4141	excipients	4697	Flow Cytometry	3440	green tea extract and kava root extract	3713
Emerging Contaminants	4448	Excitotoxicity	4236	Fluorescence	4316	Guinea pigs	3113
Endocrine Actives	4157	Experimental animals	4449	Fluorescent Reporters	3357	gut microbiome	4593
Endocrine Disruptors	2016, 2017, 2019, 3067, 3144, 3149, 3157, 3205, 3214, 3220, 3227, 3272, 3279, 3281, 3284, 3285, 3287, 3299, 3303, 3502, 3581, 3623, 3626, 3835, 3839, 4126, 4200, 4273, 4461, 4482, 4601, 4643	Exposome	3419, 4282, 4493	Fluoride	4423	Halogenated Hydrocarbon	3029
Endocrine Toxicology	3224, 3281, 3283, 3291, 3292, 3295, 3296, 3300, 3302, 3608, 3792, 3851, 3966, 4201, 4408, 4499, 4615	ExpoSS; Computational Toxicology; Food Toxicology	3298	Food	3658	Hazard	3328
Endocrine; Androgens	3283, 3289, 3296	Exposure Assessment	3062, 3077, 3084, 3091, 3099, 3181, 3307, 3326, 3328, 3331, 3339, 3469, 3522, 3524, 3550, 3562, 3625, 3658, 3663, 3766, 3770, 3888, 3945, 3960, 3982, 3985, 3987, 3988, 3991, 3992, 3993, 4001, 4004, 4013, 4084, 4099, 4180, 4181, 4182, 4183, 4188, 4193, 4194, 4196, 4197, 4198, 4199, 4200, 4258, 4275, 4282, 4392, 4477, 4515, 4539, 4542, 4556, 4640, 4671	Food Additives	3653	Hazard Identification/Reduction ...	3059, 3061, 3181, 3856, 4199, 4283, 4523, 4556, 4667
Endocrine; Estrogens	3270, 3284, 3297, 3349, 3839, 4146, 4157, 4685, 4688	Exposure, Environmental	3120, 3169, 3173, 3209, 3226, 3298, 3423, 3465, 3504, 3506, 3515, 3579, 3617, 3635, 3755, 3776, 3892, 3898, 3910, 3914, 3927, 3928, 3934, 3935, 3939, 3957, 3960, 3968, 3970, 3972, 4096, 4101, 4183, 4191, 4192, 4195, 4231, 4270, 4322, 4363, 4473, 4496, 4529, 4555, 4588, 4599, 4611, 4646, 4675, 4678	Food Safety	3161, 3295, 3339, 3554, 3624, 3627, 3643, 3649, 3650, 3651, 3652, 3653, 3654, 3657, 3658, 3659, 3660, 3661, 3662, 3663, 3665, 3668, 3801, 3976, 3982, 3994, 4012, 4036, 4110, 4325, 4532, 4554	Heart Failure, HFpEF, mitochondria, ...	4228
Endocrine; Other	3180, 3290, 4483	Extracellular vesicle	3635	Food Toxicity	2022, 3330, 3634, 3651, 3652, 3659, 3665, 3667, 3671	Heat Stress	4468
Endocrine; Receptor ...	3149, 3287, 3288, 4601	extracellular vesicles	4056	Formaldehyde	3996, 4481	Hematopoietic .	3150, 3443, 3773, 3867, 4706
Endocrine; Thyroid	3135, 3213, 3285, 3286, 3292, 3293, 3294, 3297, 3299, 3301, 3302, 3385, 4022, 4126, 4158, 4373	Extracellular vesicles (EVs)	3860	Formaldehyde, Histone, Chromatin Assembly, ChIP-Seq	4650	Hematopoietic Stem Cells	3538
Endometriosis; NR4As; EMT	3197	Extractables	3071, 3074, 3075, 3082, 3085, 3086, 3640, 3734, 3741, 3749, 4564, 4715	Formulation	3267	Hematotoxicity	3531, 3538, 3773, 3906, 4137, 4250, 4624, 4697
Endometrium	4469	Extractables and Leachables	3729, 4027	Fragrances	3382	Hemochromatosis, SLC30A10	4597
Endothelium	3918	Eye irritation	4346	Functional Observational Battery	3219	Hemocompatibility, Transfusion equipment	3083
endotoxemia	3005	Fasudil	3791	Fungi	3643	HEOR	4272
ENDS	3456, 3478, 4086			Furan	3554	HepaRG	3687
Energetics	4624			GARDskin Medical Device	3072	Hepatic	2026, 3001, 3002, 3015, 3684, 3692, 3694, 3844, 3880, 4057, 4429, 4496, 4519, 4538, 4607a
Energy expenditure	3855			Gastrointestinal	3121, 3371, 3376, 3533, 3671, 3714, 3778, 4110, 4113, 4117, 4137, 4187, 4409, 4549, 4566, 4597	Hepatocyte proliferation	4291
Environmental Fate	2013, 3900			Gene Expression/Regulation	3003, 3188, 3223, 3436, 3609, 3880, 3929, 3939, 4048, 4052, 4298, 4302, 4378, 4404, 4411, 4423, 4596, 4642	Hepatocytes	2038, 3000, 3300, 3400, 3532, 3545, 3647, 3676, 3677, 3678, 3679, 3680, 3681, 3682, 3685, 3691, 3693, 3698, 3703, 3711, 3713, 3719, 3874, 4109, 4136, 4139, 4165, 4368, 4503, 4559, 4584, 4637
Environmental Justice	3422, 3570, 3756, 3992, 4282, 4357			Gene Regulation	3702	Hexanitrohexaazaisowurtzitane (CL-20)	4624
Environmental tox	3943			gene therapy	4710	Hexavalent Chromium 3094, 3101, 3113, 3769, 3778, 4453, 4605, 4682	

HIV, Alcohol, Hepatocytes, Apoptotic bodies, Progr	4430	<i>In Vitro</i> and Alternatives	2007, 2008, 2022, 2023, 2025, 2028, 2036, 3021, 3027, 3034, 3061, 3078, 3097, 3135, 3148, 3150, 3199, 3202, 3216, 3252, 3256, 3266, 3270, 3274, 3275, 3279, 3294, 3299, 3300, 3343, 3344, 3358, 3362, 3363, 3364, 3365, 3366, 3373, 3374, 3376, 3377, 3378, 3379, 3380, 3382, 3383, 3384, 3385, 3386, 3388, 3390, 3391, 3392, 3393, 3396, 3397, 3400, 3401, 3402, 3404, 3406, 3407, 3411, 3435, 3456, 3468, 3493, 3502, 3512, 3516, 3528, 3533, 3545, 3559, 3584, 3611, 3620, 3622, 3674, 3675, 3677, 3678, 3679, 3682, 3683, 3687, 3690, 3693, 3694, 3695, 3696, 3700, 3706, 3707, 3708, 3709, 3710, 3712, 3713, 3738, 3745, 3754, 3764, 3803, 3809, 3811, 3876, 3945, 3984, 4059, 4072, 4075, 4076, 4091, 4102, 4106, 4107, 4111, 4112, 4113, 4114, 4115, 4118, 4119, 4120, 4121, 4122, 4123, 4125, 4126, 4127, 4129, 4132, 4133, 4135, 4136, 4137, 4138, 4140, 4143, 4144, 4145, 4147, 4148, 4149, 4150, 4152, 4153, 4154, 4155, 4156, 4157, 4161, 4162, 4163, 4164, 4166, 4167, 4168, 4173, 4174, 4175, 4176, 4178, 4187, 4236, 4237, 4239, 4249, 4251, 4258, 4259, 4315, 4340, 4346, 4387, 4390, 4399, 4403, 4405, 4427, 4450, 4480, 4489, 4498, 4529, 4535, 4550, 4553, 4558, 4562, 4568, 4580, 4583, 4664, 4697, 4732
Human health	3174	<i>in vitro</i> cell models	3387
Huntington's Disease	3584	<i>In Vitro</i> Skin Model	4175
Hyaluronic Acid	3082	<i>In vitro</i> toxicology	4661
Hydrogen sulfide	3805	<i>In Vitro</i> , Human-on-a-Chip Cortical Networks	4142
Hyperoxia	4673	<i>In Vivo</i> Models	2018, 3013, 3014, 3023, 3024, 3056, 3217, 3375, 3389, 3415, 3446, 3452, 3487, 3642, 3835, 3838, 3848, 3850, 3853, 3855, 3858, 3859, 3860, 3861, 3862, 3865, 3872, 3916, 3957, 3970, 3973, 4006, 4053, 4068, 4121, 4142, 4320, 4435, 4468, 4509, 4527, 4593, 4626, 4635, 4680, 4716
Hypersensitivity	4723	Indium	4587
Hypertension	4227, 4256	Indium tin oxide	4725
Hypothalamus	3224	Indole-3-Carbinol; dextran sulfate sodium	4287
IL-11; Fibrosis	4289	Indole-3-carbinol; SEB	3511
Image Analysis	4017	Induced Pluripotent Stem Cells	3175, 3270, 3271, 3273, 3277, 3357, 3383, 3394, 3396, 3397, 3512, 3688, 3692, 3798, 4134, 4147, 4159, 4168, 4203, 4234, 4251, 4566
Imaging	4561	Infection	3852, 3932, 4472, 4664, 4722, 4743
Imaging agent	4042	Inflammation	3031, 3090, 3106, 3238, 3260, 3263, 3372, 3431, 3438, 3439, 3449, 3470, 3480, 3503, 3523, 3580, 3589, 3670, 3686, 3705, 3815, 3818, 3856, 3902, 3908, 3915, 3921, 3924, 3927, 3933, 3936, 3937, 3941, 3947, 3950, 3958, 3971, 3974, 3979, 4106, 4110, 4118, 4220, 4284, 4286, 4287, 4288, 4289, 4290, 4531, 4540, 4544, 4566, 4619, 4674, 4721, 4722, 4734, 4737, 4743
Imatinib	4024	Inflammation, Microglia, Astrocyte, Ultrafine PM	4208
Imidacloprid	2018, 3991, 4099	Inflammatory Exacerbations	3937
Immune systems	3271	Inhalants	4079
immune-related adverse effects	3056	Inhalation	4148
immunochemical analysis	3652		
immunogenicity	4729		
Immunotoxicity	3009, 3426, 3429, 3430, 3431, 3432, 3433, 3436, 3437, 3438, 3440, 3441, 3442, 3443, 3447, 3448, 3449, 3452, 3461, 3462, 3490, 3700, 3881, 3932, 3954, 3955, 3976, 4018, 4067, 4070, 4111, 4497, 4509, 4666, 4717, 4722, 4723, 4724, 4729, 4732, 4733, 4735, 4737, 4740, 4741, 4746, 4747, 4749		
Immunotoxicology	3045, 3234, 3241, 3381, 3427, 3429, 3433, 3435, 3446, 3460, 3852, 3921, 3936, 3940, 3975, 3979, 4107, 4143, 4216, 4217, 4255, 4459, 4467, 4529, 4664, 4710, 4720, 4721, 4724, 4725, 4726, 4727, 4728, 4729, 4730, 4734, 4736, 4739, 4742, 4748		
immunotoxicology, inhalation	4660		
<i>in silico</i>	2020		
<i>In Silico</i> Predictions	3510		
<i>in vitro</i>	3255		
		Inhalation Toxicology	3064, 3070, 3093, 3134, 3248, 3249, 3344, 3375, 3382, 3391, 3425, 3464, 3468, 3470, 3475, 3478, 3480, 3481, 3488, 3489, 3490, 3492, 3493, 3494, 3496, 3497, 3498, 3504, 3505, 3506, 3508, 3509, 3514, 3515, 3516, 3521, 3522, 3523, 3524, 3544, 3566, 3761, 3908, 3911, 3919, 3920, 3921, 3930, 3935, 3937, 3940, 3944, 3950, 3951, 3954, 3958, 3959, 3961, 3962, 3965, 3968, 3970, 3972, 3979, 3997, 4004, 4030, 4062, 4070, 4071, 4074, 4075, 4108, 4114, 4125, 4145, 4162, 4166, 4167, 4208, 4231, 4400, 4481, 4526, 4600, 4610, 4625, 4626, 4642, 4655, 4656, 4659, 4661, 4662, 4671, 4675, 4746
		Inorganic Arsenic	3990, 4305
		Interindividual variability	3983
		International Harmonization	3747, 3989, 4363
		<i>In-Vitro</i>	3878
		<i>In-vitro</i> inhalation	3518
		Iodoacetic Acid	3203
		Ionizing Radiation	4631
		Iron	3346, 3607, 3788
		ISO 10993	2033, 3079
		ISO 10993, ASTM F1980, IEC 60601-1, ISO 18562	3064
		ISO 10993, Extractable, Skin Irritation	3063
		ISO 18562, ISO 10993, gas pathway	3076
		ISO 18562-1:2024	3744
		ISO 18562-4, Condensate, Solvent Selection, Mucus	3075
		Isotope Metallomics	4630
		IVIVE	4133
		IVIVE, long-term culture, metabolism	3389
		Japanese medaka	3851
		Juvenile Toxicity	3155, 3187, 3333, 3421, 3949, 3969
		KEAP1-NRF2	4302
		Keratinocytes	3260
		ketohehexokinase inhibitor; liver; PPAR	2006
		Key characteristics	4005
		Kidney	3011, 3020, 3021, 3022, 3023, 3024, 3025, 3026, 3027, 3028, 3029, 3030, 3031, 3032, 3033, 3034, 3035, 3036, 3037, 3038, 3039, 3230, 3325, 3464, 3541, 3640, 3897, 3922, 3944, 4044, 4240, 4374, 4472, 4571, 4574, 4602, 4649
		kidney, Liver, smal intestine, Incucyte	4121
		Kinase	3334
		Knockouts	3857, 4044
		Knowledgebase	4363
		Large Language Models	4352
		Large language models (LLMs)	4396
		LC-MS	4192
		Leachable Selection; Container Systems	2034
		Leachables	3063, 3075, 3082, 3085, 3086, 4003, 4086, 4564, 4712, 4715
		Lead	3105, 3106, 3120, 3123, 3133, 4237, 4357, 4588, 4604, 4607, 4678
		Lead, Nickel, Antimony, Copper, Zinc	3474
		Learning and memory	3147
		Lipid	3680
		Lipid Nanoparticle	4054
		Lipids	3450, 3605, 3677, 3699, 3928, 3929, 3950, 3951, 4374, 4491, 4510, 4541, 4617
		Lipids, Hepatocytes	4519
		Liquid Crystal Monomers	4232
		Liver	2008, 2011, 2036, 2037, 2039, 2040, 3000, 3001, 3002, 3003, 3004, 3005, 3006, 3008, 3009, 3010, 3011, 3013, 3014, 3016, 3017, 3018, 3019, 3026, 3291, 3294, 3324, 3374, 3380, 3389, 3395, 3466, 3482, 3532, 3534, 3545, 3556, 3616, 3617, 3631, 3632, 3648, 3673, 3674, 3675, 3676, 3680, 3682, 3683, 3685, 3686, 3687, 3690, 3691, 3692, 3693, 3696, 3697, 3698, 3699, 3700, 3702, 3704, 3705, 3706, 3709, 3710, 3718, 3779, 3780, 3782, 3860, 3874, 3893, 3969, 3973, 4044, 4122, 4136, 4139, 4161, 4164, 4279, 4289, 4291, 4294, 4297, 4312, 4320, 4374, 4429, 4453, 4471, 4511, 4517, 4538, 4563, 4612, 4613, 4616, 4619, 4621, 4630, 4637, 4641, 4670, 4736, 4737
		Liver organoid	3710
		Liver, Cyp2e1, Hepatotoxicity, Metabolism, Obesity	3007
		Liver-derived organoids	3708
		LNP	4055
		Lung stem cells	3942
		Lung; Pulmonary or Respiratory System	3393, 3441, 3450, 3455, 3472, 3483, 3489, 3494, 3495, 3496, 3508, 3512, 3591, 3705, 3859, 3914, 3916, 3928, 3929, 3930, 3934, 3938, 3939, 3942, 3959, 3962, 3971, 3980, 4029, 4070, 4074, 4079, 4132, 4156, 4311a, 4427, 4552, 4555, 4585, 4655, 4660, 4661, 4670, 4673, 4676, 4682, 4740
		Lupus Nephritis	3944
		Luteolin, Atrazine	3296
		Lysosome	4288
		machine learning	3257, 3555, 3722, 3723, 4349, 4356, 4357
		machine learning, RNA-seq	4368
		MACHINE LEARNING; Neural-ODEs	2000
		Machine-learning-based image analysis	3191, 4223
		Macrophage	3241, 3433, 3437, 3441, 3450, 3785, 3912, 3938, 3939, 4288, 4289, 4529, 4540, 4660, 4676, 4731
		Mammary Glands	3198
		Manganese	3091, 3098, 3100, 3103, 3109, 4203
		Manganese, Dopamine, Addiction	3092
		Manganese, Iron	3099, 4597
		Manganese, Manganism, Histone Deacetylases	3117
		Manganese; Iron	3102
		Manganese; Iron; Welding; MRI	3102
		Manganism, Histone Deacetylases	3114
		Maresins (Specialized Pro-resolving Mediator)	3504
		MASH, High Fat Diet, Calpain	2039
		MASLD	4612
		Mass spectrometry imaging	3953
		Mast Cells	4730
		Materials Science	3058
		Maternal exposure delayed effects	3436
		mathematical modelling	3509
		Mating Behavior	3224
		Matrigel Replacement	3399
		Mechanism	2027
		Mechanism of action	3647

- Mechanisms 2014, 3016, 3026, 3030, 3040, 3121, 3231, 3346, 3398, 3427, 3429, 3638, 3639, 3934, 4043, 4111, 4225, 4228, 4288, 4292, 4294, 4295, 4307, 4316, 4384, 4432, 4472, 4502, 4523, 4540, 4551, 4586, 4595, 4723, 4730
- Medical Device 2032, 2033, 2035, 3057, 3058, 3059, 3060, 3061, 3062, 3064, 3066, 3067, 3068, 3069, 3071, 3072, 3074, 3076, 3077, 3078, 3079, 3080, 3081, 3083, 3084, 3085, 3087, 3325, 3728, 3729, 3730, 3731, 3732, 3733, 3734, 3736, 3737, 3738, 3740, 3741, 3742, 3743, 3744, 3745, 3746, 3747, 3748, 3751, 3752, 3753, 4169
- Medicinal plants 3645
- medico-legal toxicology 3421
- Meiosis 3221
- Melanoma 4296, 4300
- Membrane Biology 3025, 3216
- menthol 3030
- Mercury 3107, 3127, 4589
- Mercury; Carbon Disulfide 3304
- MET, HGF, Regeneration 3014
- metabolism 4244
- Metabolic Activation ...3703, 4219, 4315, 4323
- Metabolic signaling 3966
- Metabolic syndrome 3951
- Metabolism 3139, 3194, 3287, 3370, 3432, 3555, 3556, 3604, 3605, 3611, 3699, 3805, 3807, 3833, 3893, 4204, 4371, 4383, 4389, 4416, 4425, 4499, 4519, 4545, 4567, 4583, 4617, 4653, 4694, 4738
- Metabolomics 3192, 3198, 3260, 3473, 3574, 3655, 3879, 3953, 3969, 4186, 4192, 4228, 4295, 4404, 4463, 4494, 4631
- Metabonomics 3028
- Metal Mixture Modeling 3636
- Metals 3068, 3091, 3094, 3097, 3100, 3103, 3104, 3111, 3114, 3115, 3119, 3127, 3158, 3309, 3444, 3474, 3475, 3580, 3583, 3607, 3623, 3629, 3630, 3636, 3637, 3646, 3695, 3736, 3766, 3767, 3769, 3770, 3772, 3773, 3774, 3775, 3776, 3777, 3778, 3779, 3780, 3781, 3783, 3784, 3785, 3786, 3788, 3826, 3965, 3974, 3992, 4070, 4184, 4186, 4187, 4190, 4191, 4211, 4256, 4268, 4298, 4301, 4413, 4414, 4442, 4447, 4457, 4458, 4536, 4544, 4586, 4587, 4588, 4589, 4590, 4592, 4593, 4594, 4595, 4596, 4597, 4598, 4599, 4600, 4601, 4602, 4603, 4605, 4606, 4640, 4644, 4682, 4725, 4731
- methanol 4225
- Methods/Mechanism 2012, 3001, 3306, 3362, 3370, 3400, 3712, 3861, 3875, 4028, 4047, 4109, 4123, 4149, 4174, 4548, 4574, 4632, 4638
- methyl chloride 3310
- Methyl isothiocyanate 4684
- Methylation 4310
- methylene diphenyl diisocyanate 3740
- Methylglyoxal, Acetaldehyde, Flavors, Coolants 3458
- Methylmercury 3777
- MHC Class I pathway; Persistent organic pollutants 4744
- Micro and nano plastics 2012
- Micro and nano plastics 2012
- Micro- and nanoplastics 4535
- Micro or nanoplastic 4536
- Microarray 4385, 4436
- Microbial derived indoles 4608
- Microbiome 3108, 3141, 3465, 3573, 3604, 3655, 3670, 3671, 3778, 3837, 3935, 4434, 4471, 4500, 4522, 4599
- Microcystin-LR 3000
- microelectrode array 3811
- Microfibers 3955
- Microfluidics 3366
- Microglia 3815, 4220, 4525
- Microglia, Inhibitory synapses 3793
- Micro-nanoplastics (MNPs) 4551
- Microphysiological system ...3031, 3407, 4117, 4118, 4120, 4135
- Microphysiological systems 3401, 3690, 3706, 4171, 4583
- microplastic 4540, 4542
- microplastic fibers 4555
- Microplastic, Transfer Learning 4362
- Microplastic; Small non-coding RNAs 4545
- Microplastics 2011, 2013, 3413, 3437, 3830, 4145, 4459, 4529, 4538, 4550
- Microplastics and Nanoplastics 4553
- Microplastics, Inhalation 4539
- Microplastics, UV-aged, intestinal organoids 4534
- Microplastics; Developmental Toxicology; Zebrafish 3165
- Microscopy 3594
- miRNA 3239
- Mitochondria 3575, 3877, 3981, 4164, 4204, 4329, 4375, 4649
- Mitochondria, bioenergetics, *C. elegans* 3630
- mitochondrial dysfunction 3801
- mitochondrial toxicants 3400
- Mixed Plant Protection Products 4091
- Mixtures 2029, 3386, 3622, 3627, 3629, 3637, 3638, 3781, 3783, 3883
- Mixtures Toxicology 3623
- Mixtures, Transporters, Liver, PFAS 4523
- ML/AI 3714
- MNP 4541
- Model animal 3852
- Mode-of-Action 2009, 3152, 3171, 3285, 3293, 3301, 3308, 3312, 3324, 3333, 3342, 3392, 3398, 3668, 3718, 3810, 4095, 4291, 4311, 4370, 4680, 4684
- mold 4743
- Molecular Models 2001, 3171, 3345, 4024, 4028, 4360, 4407, 4437
- monoclonal antibody 4709
- Monosodium Glutamate 3653
- Mood 3115
- Morris 3218
- Mouthwash 3307
- MPS, NAM 3384
- MRI 3806
- MRI, Welding 3091
- MRI, Welding, Cerebellum, Glutamate 3098
- MRI; Welding 3103
- MTHFR 3127
- Multi-component nanomaterials 3544
- Multi-omics 3206
- Muscle Toxicity 3786, 4418, 4532, 4698
- Mustard agents 3262
- Mutagen 3667, 4545
- Mutagenesis 4304, 4389, 4586
- Mycotoxin 3668, 4273
- mycotoxins 3654, 4001
- myelin 3142
- Myeloid cells, imaging 3447
- Myocarditis 3431
- nabilone 3146
- N-acetyltransferase-2 4677
- NAM 3293, 4241
- NAMkind Intestine 3371
- NAMkind liver 3707, 3709
- NAMs 3351, 3660, 3711, 4134, 4230, 4315
- NAMs, hiPSC-CMs, Electrophysiology 4159
- NAMs.network 3717
- Nanoparticle 3391
- Nanoparticles ...2000, 2010, 2013, 3033, 3153, 3170, 3173, 3198, 3212, 3441, 3513, 3555, 3557, 3722, 3725, 3831, 3837, 3911, 3919, 3961, 3974, 3975, 3976, 3979, 3980, 3981, 4055, 4214, 4226, 4250, 4252, 4284, 4353, 4362, 4443, 4544, 4552, 4553, 4558, 4725
- nanoplastics 4145, 4214, 4215
- Nanotechnology 4530
- Nanotoxicology 3919
- Nanotubes 3980
- nanotubes 3980
- Natural Products 2021, 3032, 3318, 3448, 3534, 3573, 3639, 3641, 3642, 3643, 3646, 3650, 3666, 3811, 4071, 4116, 4242, 4303, 4465, 4488, 4615
- natural toxins 3176
- NCE 3023, 3387, 3533, 3681
- Neonicotinoids 2019
- Neonicotinoids 3987
- Nervous System 3394, 3419, 3568, 3614, 3806, 3858, 4006, 4142, 4206, 4333, 4371, 4407, 4520, 4524, 4561
- netarsudil 4341
- Network 4494
- Network analysis 4349
- Neural Organoid 4129
- Neurodegeneration ...3567, 3579, 4211, 4219, 4507
- Neurodegenerative Disease: Parkinson's Disease 3569
- neurogenic inflammation 3591
- Neuroinflammation 3233, 3237
- Neurological impact 3970
- neuropharmacology 4124
- Neurotoxicity 3415
- Neurotoxicity; Developmental 2023, 3088, 3089, 3106, 3107, 3125, 3130, 3131, 3132, 3133, 3134, 3135, 3136, 3137, 3138, 3140, 3142, 3143, 3144, 3146, 3147, 3148, 3149, 3154, 3155, 3158, 3218, 3219, 3320, 3356, 3499, 3587, 3590, 3637, 3797, 3877, 3896, 4032, 4060, 4066, 4088, 4128, 4129, 4202, 4211, 4223, 4498, 4507, 4645
- Neurotoxicity; Metals 3088, 3089, 3090, 3091, 3093, 3094, 3095, 3098, 3099, 3101, 3102, 3103, 3104, 3105, 3106, 3107, 3108, 3109, 3111, 3112, 3113, 3115, 3117, 3118, 3119, 3120, 3121, 3122, 3123, 3124, 3125, 3130, 3133, 3143, 3571, 3580, 3583, 3586, 3636, 3777, 3781, 3783, 3804, 3813, 3911, 3919, 4203, 4209, 4211, 4217, 4646, 4745
- Neurotoxicity; Pesticides 3137, 3237, 3247, 3551, 3565, 3566, 3588, 3591, 3592, 3593, 3594, 3598, 3600, 3601, 3602, 4089, 4202, 4215, 4224, 4417
- Neurotoxicology 2012, 2029, 3046, 3048, 3090, 3100, 3101, 3104, 3107, 3108, 3111, 3112, 3114, 3118, 3123, 3124, 3136, 3139, 3141, 3145, 3193, 3233, 3236, 3237, 3240, 3247, 3277, 3365, 3485, 3498, 3519, 3567, 3568, 3570, 3574, 3575, 3577, 3578, 3579, 3582, 3584, 3585, 3586, 3588, 3590, 3592, 3596, 3600, 3610, 3614, 3618, 3638, 3642, 3660, 3742, 3789, 3790, 3791, 3792, 3793, 3794, 3797, 3798, 3799, 3800, 3801, 3802, 3803, 3804, 3805, 3807, 3808, 3809, 3810, 3811, 3812, 3814, 3815, 3882, 3890, 3896, 3899, 3903, 3908, 3912, 3953, 4123, 4124, 4130, 4147, 4160, 4201, 4202, 4204, 4206, 4207, 4208, 4210, 4212, 4213, 4214, 4215, 4216, 4217, 4219, 4221, 4222, 4224, 4248, 4390, 4516, 4518, 4535, 4551, 4560, 4572, 4589, 4625, 4636, 4657, 4691, 4693, 4721
- Neurotransmitter 3098, 3102, 3581, 3598, 3862, 4223, 4507, 4567
- new approach methodologies 3034, 3374, 4125
- New Approach Methodologies, Machine Learning, qPCR 4367
- New Approach Methodology 3527, 4108
- New Approach Methods 3344, 3403, 4010, 4033
- New Approach Methods (NAMs) 4032
- NGRA, NAMs, population variability 3373
- Nickel 3736, 3997
- Nickel alloy 3736
- Nicotine 3461, 4224
- Nicotine Pouches 3461
- Nitrate 3052
- Nitrate/Nitrite 3982
- Nitric Oxide 4028
- Nitroalkoxyacetanilides 4439
- Nitrosamines 3982, 4321
- Nonanimal 3255, 3352, 3399, 3406, 3712, 3999, 4140, 4170, 4346, 4480
- non-animal model 2008
- noncoding RNA 4309
- Nonenzymatic biotransformation 4627
- Non-Genotoxic 3688
- Non-human primate 4487
- Non-Mammalian Species 3164, 3221, 3346, 3359, 3417, 3630, 3802, 4508, 4694
- Nose 4181, 4548
- novel pyridinium oxime 3243
- N-pyrrolidino protonitazene 3047
- Nrf2, Extracellular vesicles 4292
- NTP compound library 3392
- Nuclear Opacities 4331
- Nutrition 3650, 3704, 3928, 4275, 4630
- Nutritional Toxicity 3444, 3448, 3664, 3670
- Occupational Exposure 4188

occupational exposure limit	3315	Parkinson's Disease	3566	PFAS 2027, 2028, 2029, 2031, 3046, 3077,	Polyamide (PA)	4534	
Occupational Health	3914, 4180, 4221	Particle	3391	3217, 3225, 3503, 3582, 3622, 3753, 3875,	Polybrominated diphenyl ethers	3619, 4362	
Occupational Toxicology	4250, 4667	Particulate Matter	3493, 3502, 3914,	3888, 3892, 3900, 4122, 4182, 4405, 4498,	Polychlorinated biphenyl	3293	
Occupational Toxicology; Permitted Daily		4343, 4657		4507, 4509, 4513, 4523, 4524, 4583, 4724,	Polychlorinated Biphenyls	3140, 3145,	
Exposure;	4002	Particulate Matter, Oxidative Stress,		4736	3609, 3612, 3614, 3615, 3618, 3618		
Ochratoxin A	3668	Organoids	3963	PFAS mixtures	4527	Polycyclic Aromatic Hydrocarbons	3131,
Ocular Toxicity .. 3242, 3246, 3471, 3726, 3850,		Particulates	2011, 3021, 3081,	PFAS; VOCs	3632	3132, 3171, 3386, 3472, 3499, 3500, 3610,	
3902, 4331, 4332, 4333, 4334, 4336, 4338,		3173, 3352, 3485, 3488, 3489, 3491, 3508,		PFBS, PFHxA, PFHxS, 6:2 FTOH	3886	3635, 3762, 3823, 4021, 4114, 4132, 4330,	
4339, 4340, 4341, 4342, 4343, 4344, 4345,		3544, 3842, 3917, 3922, 3924, 3944, 3947,		PFHpA	4133	4349, 4412, 4412, 4573, 4645	
4346, 4347, 4705		3949, 3951, 3952, 3955, 3960, 3965, 3966,		PFHxS	3154	polyester	4555
OECD 451 , OECD 453, 10993-3, US EPA TG		3969, 4247, 4343, 4515, 4557, 4660, 4667		PFOA	4469	Polyethoxylate	3287
870.4300, ...	3747	Passive Headspace Concentration	2035	PFOA and PFOS	3178	Polyethoxylate	3287
off-target safety assessment	3526	PBPK-PD model; Tilmicosin; Pasteurella		PFOA, PFOS, GenX, PFBS	3838	Polyethylene	4536
Oleic Acid	3704	multicida;	3656	PFOA, PFOS, PFAS mixture	3891	polyethylene terephthalate	3852
Olfaction, Sensory irritation,		PCLS	4741	PFOA; PFHxA; PFPeA; PFBS	2030	polyfluoroalkyl substances	3813
Fragrance	4181	Peat & Eucalyptus Wood Smoke Condensate		PFOS 3889, 3899, 4503, 4524, 4613, 4748		Polymeric PFAS	3888, 3900
Oligonucleotides	3530, 4368	 3945		PFOS (perfluorooctane sulfonate)	4020	Polystyrene	3153, 3170
Omics	3465	Pentachlorophenol	4744	PFOS, PFAS	4516	polystyrene nanoplastics	4558
Oncogene	4426	Per and Poly Fluorinated Substances 3880		PFOS, Ribosomal Proteins (RPS6, RPS7),		Polytetrafluoroethylene	4515
Oncolytic virus	4051	Per- and Poly Fluoro Alkyl substances (PFAS)		Uncoupling	4020	polyurethane	3734
One Health	3418	 3136		PFOS, TCDD	3006	polyvinyl chloride	4611
Optical Imaging	2011	per- and polyfluoroalkyl substances 2026,		Pharmaceuticals	3049, 3146, 3183,	Preclinical Assessments	3024, 3218,
Oral Toxicity	3405	3903, 4519		3193, 3204, 3290, 3305, 3306, 3531, 3535,		3219, 3246, 3263, 3371, 3387, 3405, 3407,	
Organofluorine	4025	Per- and polyfluoroalkyl substances (PFAS)		3561, 3577, 3824, 4025, 4035, 4041, 4042,		3440, 3519, 3522, 3525, 3533, 3535, 3558,	
Organoid, Organ-on-a-chip	3388	 3547, 3881, 4396		4056, 4261, 4377, 4426, 4488, 4576, 4696,		3561, 3761, 3853, 3857, 3865, 4035, 4048,	
Organoids	3694, 4163	Per-and Polyfluoroalkyl Substances 3882		4709, 4714		4050, 4055, 4078, 4335, 4658, 4696, 4704,	
Organoids, Risk assessment	3376	Percutaneous Absorption	3993	Pharmacokinetics	3204,	4717, 4749	
Organoids; spheroids; single-cell RNAseq		Perfluoroalkyl Substances (PFAS)	3895	3309, 3401, 3540, 3541, 3550, 3555, 3558,		Predictive chemistry	4199
..... 4390		Perfluorobutane sulfonic acid (PFBS) 3209		3564, 3972, 4036, 4042, 4561, 4567, 4570,		Predictive Toxicology .. 2007, 3034, 3042, 3148,	
Organ-on-chip	4170	Perfluorodecanoic acid (PFDA)	3840	4571, 4576, 4579, 4581, 4629, 4713		3343, 3384, 3404, 3550, 3628, 3712, 3718,	
Organ-on-Chip, Microphysiological System,		Perfluorinated Agents 2014, 2026, 2027,		Phase II Metabolism	3556, 4192	3726, 3803, 4008, 4016, 4025, 4027, 4057,	
Liver	3678	2028, 2030, 2031, 3046, 3136, 3142, 3154,		Phenobarbital	2038	4119, 4133, 4140, 4158, 4176, 4235, 4354,	
organophosphate	3247, 3588	3167, 3209, 3426, 3503, 3547, 3603, 3632,		Phenobarbital, Membrane Transporter ... 2038		4355, 4364, 4376, 4378, 4385, 4392, 4397,	
Organophosphates	3039,	3751, 3752, 3753, 3840, 3844, 3873, 3874,		Phenotype	3681	4662	
3233, 3235, 3236, 3237, 3240, 3243, 3319,		3875, 3876, 3877, 3877, 3878, 3878, 3879,		Phosgene	3249	Pregnancy	2014, 3823, 4061
3589, 3591, 3732, 3834, 4092, 4154, 4161		3880, 3882, 3883, 3886, 3887, 3889, 3890,		Photoallergy	3999	Prenylated phenolics	2021
organotin compound	4173	3891, 3896, 3897, 3898, 3901, 3903, 3904,		phototoxicity	3266	Proarrhythmia	4234
Organs-on-Chip	4658, 4699	3906, 4133, 4152, 4182, 4201, 4206, 4244,		Phthalate	3227, 3985, 4016, 4408	Probablistic, Dietary	4099
Osteoblast	3175	4274, 4297, 4447, 4463, 4469, 4470, 4491,		Phthalates	2015, 2017, 3194,	Proficiency testing	3292
Otseconazole	4039, 4331, 4332	4496, 4498, 4499, 4500, 4502, 4504, 4505,		3196, 3206, 3214, 3227, 3284, 3314, 3324,		Proposition 65	3310, 3311, 3994
Other (open text box) .. 3052, 3066, 3094, 3212,		4507, 4508, 4510, 4511, 4515, 4516, 4518,		3604, 3818, 3831, 3832, 3837, 4213, 4232,		propylene oxide	4162
3304, 3335, 3336, 3346, 3371, 3395, 3584,		4519, 4520, 4522, 4525, 4526, 4527, 4549,		4311, 4367, 4409, 4482, 4495		Proquinazid	4559, 4565
3653, 3721, 3769, 3930, 4058, 4061, 4146,		4575, 4583, 4616, 4634, 4683, 4728, 4748		phthalates (mixture)	2015	Prostate Cancer	3879
4146, 4170, 4188, 4250, 4256, 4306, 4408,		perfluorooctane sulfonate	4514	Physiologically Based Pharmacokinetics		Protein Allergy	3430
4424, 4474, 4487, 4508, 4650		Perfluorooctane sulfonate (PFOS)	4501	 3319, 3337, 3349, 3549, 3557, 3562,		Protein Structure	3345, 3704, 4024, 4028
Ototoxicity, Exposure Limits	3305	Perfluorooctane sulfonic acid	4201	3725, 4019, 4021, 4023, 4391, 4504, 4539,		Protein Translation	4424
Ovarium	3831	perfluorooctanesulfonamide	3873	4569, 4570, 4573, 4579		Proteins	4632
Ovary 2017, 3196, 3203, 3214, 3222, 3297,		Perfluorooctanesulfonic	3606	Physioxia	3274	Proteome 2028, 4469, 4470, 4568, 4595, 4621	
3792, 3825, 3832, 3838, 3842, 4016, 4031		Perfluorooctanoic Acid	3144, 3446,	Pioglitazone	3703	Proteomics	2031, 3028, 3113, 3370,
Oxidative Injury .. 3036, 3152, 3231, 3274, 3603,		3902, 4508		PIPERINE	3642	3466, 3722, 3903, 4174, 4302, 4349, 4375,	
3813, 3832, 3902, 3941, 4331, 4332, 4415,		Perfluorooctanoic acid (PFOA)	4470	Placenta	2019, 2031, 3173, 3177, 3201,	4424, 4426, 4446, 4497, 4511, 4594, 4621	
4424, 4670		perfluoropentanoic acid	3906	3202, 3212, 3223		proteotoxicity	4595
Oxidative Stress	3646, 3799	Perfluoroundecanoic Acid	3844	Plant-based meat alternatives	3658	Pubertal	3303
Oxidative Stress, <i>Drosophila melanogaster</i>		peroxisome—mitochondria	3893	plastic	4532	Pyraclorobin	3443
..... 4089		Persistent Organic Chemicals	3006, 3142,	Plasticizers	3369	Pyranine Conc (G204)	4562
Ozone 3928, 3929, 3930, 3932, 3936, 3938,		3222, 3619, 3752, 3881, 3882, 3893, 3899,		Plastics, Toxicology, ICU	4482	Pyrethrins, Piperonyl Butoxide, N-Octyl	
3939, 3941, 3941		3904, 4122, 4439, 4517, 4524, 4612		Platelet	3530	bicyclolep	4108
Ozone and Other Advanced		Pesticide (propargite)	4417	Plethysmography	4654	Pyridostigmine bromide, Permethrin	3793
Oxidations	4439	Pesticides 2018, 2019, 3190, 3191, 3200, 3201,		Plutonium, asbestos, aromatic amines, lead		QSAR	3351, 3986, 3989, 4011,
Paclitaxel	3806	3272, 3286, 3321, 3330, 3565, 3567, 3575,		 4180		4025, 4083, 4259, 4327, 4355, 4360, 4368,	
PAHs	4307	3587, 3589, 3590, 3596, 3597, 3598, 3601,		PM2.5	3422	4376, 4381, 4394, 4395, 4521	
Palmitoyltransferase	4586	3602, 3864, 3987, 4009, 4015, 4088, 4089,		PM2.5, Air Sensor Monitors, Policy, Exposome		QSAR, Read-Across, Expert Rules	4564
Pancreas 3290, 3603, 3606, 3608, 3883, 4510,		4090, 4091, 4092, 4093, 4094, 4096, 4097,		 3422		Quaternary Ammonium Compounds- QAC s or	
4683		4098, 4099, 4100, 4101, 4102, 4232, 4262,		PMR	4690	quats	4197
Parabens	3839, 4200	4276, 4318, 4394, 4478, 4483, 4559, 4565		Pneumocyte	3388	Quercetin, Atrazine	3283
Paraquat	3566, 3601, 3602	petroleum constituents	4021	Pollution	4442	R-1132(E): trans-1,2-Difluoroethylene 4258	
		Peyer's patches	3976				
		PF-06835919	2006				

- Rabbit 3866
 rat 4565
 rat and human 4559
 Read-across 4003, 4014, 4015, 4027, 4474, 4715
 Read-across, TTC, No-Effect Levels 4012
 Receptor 3182, 3528, 3703
 Receptor; Aryl Hydrocarbon 3855, 3964, 4411, 4435, 4513, 4608, 4609
 Receptor; G-Protein Coupled 4427
 Receptor; Nuclear Hormone 3609, 4157, 4296, 4300
 recycled plastic 3258
 Refinement 3761
 Regeneration 3011, 3025
 Regulatory Science/Regulatory Toxicology 2001, 2006, 3041, 3064, 3080, 3081, 3084, 3292, 3307, 3313, 3319, 3321, 3325, 3338, 3364, 3458, 3478, 3479, 3559, 3566, 3643, 3661, 3664, 3716, 3730, 3737, 3824, 3845, 3991, 3994, 4005, 4027, 4039, 4042, 4046, 4056, 4084, 4093, 4095, 4160, 4171, 4246, 4318, 4386, 4474, 4476, 4477, 4481, 4483, 4557, 4569, 4686, 4687, 4690, 4695
 Regulatory/Policy 3060, 3069, 3320, 3384, 3409, 3497, 3738, 3746, 3920, 3990, 4364, 4458, 4476, 4478, 4480, 4560, 4703
 Renal 3029, 3271, 4542
 Repeated exposure 3688
 Reproductive and Developmental Toxicity 3067
 Reproductive and Developmental Toxicology 2013, 2014, 2019, 2027, 3147, 3153, 3157, 3162, 3163, 3164, 3166, 3170, 3173, 3174, 3177, 3181, 3191, 3195, 3198, 3199, 3200, 3201, 3202, 3203, 3206, 3207, 3209, 3210, 3211, 3215, 3216, 3217, 3220, 3221, 3222, 3225, 3228a, 3276, 3310, 3311, 3392, 3523, 3619, 3814, 3816, 3817, 3820, 3823, 3824, 3825, 3829, 3833, 3835, 3836, 3839, 3840, 3843, 3844, 3845, 3846, 3866, 3981, 4052, 4062, 4063, 4069, 4097, 4253, 4273, 4336, 4367, 4388, 4432, 4448, 4469, 4470, 4625, 4644, 4736, 4748
 Reproductive System 2018, 3199, 3202, 3213, 3225, 3817, 3833, 3967
 Reproductive Tract; Female 3177, 3213, 3225, 3227, 3839, 4031, 4127, 4502
 Reproductive Tract; Male 3205, 3226, 3830
 Respiratory 3076, 3377, 3462, 3469, 3509, 3859, 3952, 4181, 4188, 4193, 4536, 4610, 4654, 4657, 4675
 Respiratory Sensitization 3435, 3510
 Respiratory Toxicology 3070, 3234, 3244, 3386, 3393, 3457, 3474, 3480, 3481, 3483, 3495, 3498, 3502, 3503, 3504, 3505, 3507, 3513, 3516, 3517, 3518, 3519, 3520, 3521, 3524, 3620, 3917, 3929, 3936, 3941, 3955, 3957, 3975, 4004, 4073, 4074, 4077, 4081, 4082, 4108, 4145, 4148, 4156, 4162, 4166, 4167, 4176, 4194, 4284, 4481, 4489, 4546, 4550, 4555, 4655, 4656, 4658, 4659, 4664, 4666, 4667, 4668, 4669, 4673, 4704
 Retrospective Study 4711
 Risk Assessment 2005, 2025, 2029, 2033, 2035, 3048, 3057, 3060, 3062, 3067, 3068, 3079, 3080, 3084, 3141, 3172, 3251, 3252, 3253, 3256, 3257, 3261, 3265, 3279, 3289, 3304, 3305, 3306, 3309, 3310, 3311, 3313, 3317, 3318, 3320, 3321, 3322, 3323, 3326, 3328, 3329, 3330, 3331, 3332, 3333, 3335, 3336, 3337, 3338, 3339, 3342, 3344, 3351, 3361, 3373, 3395, 3463, 3478, 3479, 3497, 3506, 3518, 3527, 3537, 3620, 3628, 3651, 3658, 3659, 3663, 3664, 3721, 3731, 3733, 3734, 3740, 3742, 3743, 3747, 3751, 3757, 3762, 3764, 3789, 3808, 3888, 3889, 3909, 3920, 3983, 3985, 3987, 3988, 3990, 3991, 3992, 3993, 3994, 3996, 3998, 3999, 4001, 4003, 4004, 4005, 4006, 4014, 4015, 4019, 4027, 4039, 4073, 4077, 4081, 4086, 4097, 4099, 4107, 4108, 4116, 4124, 4154, 4180, 4196, 4272, 4277, 4312, 4362, 4365, 4379, 4391, 4393, 4396, 4400, 4420, 4473, 4477, 4478, 4486, 4505, 4521, 4539, 4556, 4557, 4560, 4562, 4570, 4579, 4641, 4659, 4684, 4687, 4695, 4710
 Risk communication 3412
 RNA velocity; PROGENy; endothelial cells; immune r 3511
 Rotenone 3567
 RPTC, 3D spheroids 3023
 RSV 4585
 RT-PCR 3255, 3799, 4115, 4165
 S5(R3) Industry Guidance-long half-life drugs 4039
 Safer Design 3345, 3518, 3717, 4197
 Safer Design/Green Chemistry 4365
 Safer Design/Green Toxicology 4402
 Safety Assessment; Drug Development, Translational 3452
 Safety Evaluation 2006, 2009, 3023, 3041, 3174, 3243, 3251, 3255, 3261, 3280, 3307, 3318, 3326, 3328, 3332, 3334, 3366, 3379, 3382, 3389, 3409, 3430, 3525, 3526, 3527, 3532, 3535, 3537, 3548, 3553, 3558, 3644, 3653, 3661, 3697, 3737, 3741, 3745, 3754, 3755, 3757, 3759, 3798, 3846, 3853, 3857, 3866, 3868, 3980, 3984, 3999, 4014, 4039, 4041, 4045, 4046, 4052, 4053, 4055, 4056, 4117, 4120, 4134, 4148, 4238, 4257, 4264, 4317, 4335, 4352, 4381, 4484, 4546, 4632, 4690, 4700, 4702, 4705, 4706, 4707, 4708, 4711, 4715, 4717
 safety margin 4058
 Safety Pharmacology 3040, 3267, 3383, 3390, 3394, 3396, 3397, 3526, 3527, 3528, 3530, 3707, 3709, 3848, 4035, 4048, 4058, 4159, 4234, 4241, 4243, 4248, 4368, 4420, 4654, 4696, 4699, 4701, 4707, 4713
 Salivary Gland 3049
 Sarcopenia 4615
 Secondary Pharmacology 4355
 Seizure 3394
 Semaglutide 4345
 S-equal 3112
 Sevoflurane 4526
 Sex differences 3616, 3930, 4247, 4673
 Sex-differences, Alzheimer, Transcriptomic 4617
 Short Chain Fatty Acids 3935
 SH-SY5Y cells 3800
 Signal Transduction 3402, 3520
 Silica 3033, 3331, 3950
 Simulation Studies 4306
 Single cell analysis 4609
 Single-Cell RNA-Seq, PFAS 3838
 siRNA 3381, 3481, 4711
 skeletal malformations 3175
 Skin 3262
 Skin Explant 3261
 Skin Sensitization 3265
 Skin Sensitization, Skin Irritation, NAMs 4116
 sleep, LED, blue light 3854
 Sodium Chromate 4594
 Software 4581
 Spatial Omics 4603
 Spatial transcriptomics 3789, 4411
 Spearmint Essential Oil 3318
 Sperm 3195
 Spinosad 3200
 Spontaneous Convulsions 4702
 Stability 3849
 Steatosis, Asparaginase, amino acid insufficiency 4429
 Stem Cells 3105, 3125, 3157, 3271, 3272, 3275, 3276, 3378, 3710, 4115, 4117, 4209, 4237, 4679, 4685
 STEMI (ST elevated myocardial infarction) 3848
 Steroidogenesis 3283, 3296
 Stress 3417
 Stress response 4071
 Sulforaphane 3448
 Sulfur Mustard 3242, 3245
 sunscreen 3764
 Super-Resolution Microscopy 3427
 Surfactants 4346
 Sustainability 3327, 3422, 4283, 4482
 Sustainable Development 4402
 Synthetic Vitreous Fiber 4659
 Systematic Evidence Map 3335, 3336
 Systematic Reviews 4007
 Systems and Integrative Toxicology 3355, 3366, 4366, 4402, 4405, 4493, 4613, 4615, 4616
 Systems Biology 3873, 4186, 4375, 4422, 4494, 4607a, 4610, 4612, 4617, 4618, 4673, 4698
 T Cell 3009, 3056, 3363, 3406, 3432, 3436, 3443, 3460, 3791, 4053, 4436, 4487, 4723, 4726, 4735
 T cell, mitochondria, cytokine 4725
 Tapinarof 4608
 Tau pathology, Parkinson's Disease 3601
 TBBPA, Ahr 2 receptor, cartilage, hemoglobin, ... 3156
 t-Butyl alcohol 3308
 TCDD 3439, 3696
 TCDD, PFOS 4612
 TCDD, Resveratrol, I3C, FICZ 4436
 TD50, Acceptable Intake, ICH M7 3313
 Temphos 3834
 Testis 2015, 3829, 3842, 3967
 Testis Organ Culture 3199
 Tetrabromobisphenol A (TBBPA) 3156
 Tetrahydrocannabinol 3449
 Thallium (Tl); Gallium (Ga) 4448
 THC, CBD 4721
 Theophylline 4654
 thirdhand exposure 4078
 Three-dimensional *in vitro* model 3392
 Threshold of Toxicological Concern 3066
 Thresholds of Toxicological Concern 4013
 Thrombosis 3531
 Thymus, Immunotoxicity, microRNAs 4436
 Thyroid 3294, 3300
 Thyroid hormone disruption 4158
 Thyroid Model 4126
 Tin 3731
 tire-derived airborne contaminant 4676
 Tissue Engineering 4251
 Titanium dioxide 3976, 3981
 Titanium Dioxide and Polyamide-12 3198
 Tobacco Products 3045, 3405, 3456, 3458, 3461, 3462, 3463, 3464, 3465, 3466, 3467, 3468, 3469, 3472, 3474, 3475, 3476, 3477, 3479, 3825, 3843, 4060, 4061, 4062, 4063, 4065, 4068, 4075, 4076, 4077, 4078, 4080, 4081, 4082, 4083, 4085, 4086, 4125, 4253, 4254, 4255, 4536, 4656, 4658, 4666
 Topoisomerase 3054
 Tox21 4151
 ToxCast; database 4399
 Toxicity reference value 4604
 Toxicity; Acute 2008, 3001, 3008, 3011, 3016, 3017, 3018, 3019, 3020, 3030, 3038, 3176, 3187, 3230, 3248, 3421, 3507, 3534, 3553, 3574, 3614, 3641, 3678, 3694, 3708, 3761, 3806, 3916, 3937, 4041, 4102, 4233, 4264, 4344, 4428, 4453, 4547, 4624, 4691, 4699, 4703, 4704, 4710
 Toxicity; Chronic 3018, 3037, 3041, 3194, 3226, 3358, 3601, 3680, 3775, 3781, 3782, 3906, 3910, 3917, 4085, 4089, 4113, 4203, 4235, 4292, 4344, 4545, 4563, 4705, 4709, 4745
 Toxicity-induced fibrosis 3673
 Toxicodynamics 3984
 Toxicogenomics 3072, 3138, 3190, 3301, 3348, 3354, 3357, 3359, 3360, 3647, 3673, 3674, 3688, 3983, 3984, 4095, 4098, 4230, 4243, 4305, 4370, 4383, 4405, 4411, 4420, 4485, 4490, 4620, 4622, 4642, 4643, 4676
 Toxicokinetics 2000, 2022, 3342, 3413, 3547, 3548, 3560, 3561, 3690, 3725, 3807, 3886, 3895, 3982, 4016, 4155, 4198, 4396, 4504, 4523, 4524, 4527, 4573, 4575, 4580
 Toxicology 3666
 Toxicovigilance, Machine learning, ... 4364
 ToxProfiler 3398
 tradipitant 2008
 Traffic-related air pollution 3917
 Training/Professional Development 3411, 3413, 3416
 trans-1,2-difluoroethene 4304
 Transcription Factors 2037, 3100, 3112, 3114, 3117, 3228a, 3467, 4404, 4437, 4522
 Transgenerational Effects 4643
 Transgenic Models 3140, 3332, 3568, 3578, 4597
 Translational safety 2005
 Translational Toxicology 3045
 Transporter Protein 3699

TRANSPORTERS	4629
Trichloroethylene	3010, 3574, 3631, 4407
Tricyclic pyrone	4371
Trimethylbenzenes	3920
triphenyl phosphate	3273, 3374
Tropane alkaloids	3660
Tungsten	3772, 4592
Tyrosine kinase inhibitors	4257
Uncertainty assessment	3723
Undergraduate Student	3052, 3369, 3417, 3418, 3455, 3460, 3583, 3878, 3879, 3927, 4067, 4210, 4224, 4296, 4300, 4301, 4425, 4630
Undernutrition	3845
Uranium	4600, 4603
uranium and arsenic	3629
Ursolic acid	4300
Uterus	3194
UV filter	3764
Vancomycin	3036
vaping	3030, 3465
Vasculature	4112
Vehicle	3759
Vinyl Acetate Monomer	3312
Vinyl Chloride	3053, 3482, 3836, 4630, 4686
Viral Infection	3521
virtual controls	3816
Virus	4051
Vitamin D	4664
Volatile Organic Compound(s)	3010, 3076, 3188, 3391, 3490, 3494, 3495, 3514, 3625, 3632, 3743, 3744, 3836, 3918, 3926, 3954, 4080, 4084, 4193, 4255, 4623, 4639
Wastewater Treatment	4454
Weight of evidence	3998, 4008
Weight of Evidence (WOE) approach	3824
Weight of Evidence Approach	4102
Weight-of-Evidence	4687
Wildfire	3791
Wildfire Smoke	3908
wildfire smoke, wood smoke	4660
Wildfire, Burn Pit, WUI	4231
Wildfires	3921
Wood Smoke; Sex Differences; Wildfire	3945
Woodsmoke	3489
Xenobiotic Transporters	3540, 3541, 4428, 4465, 4568, 4571
X-ray Fluorescence; Bone; Soft Tissues; Optics	4588
Xylene	3442
Zalunfiban	3848
Zearalenone	4273
zebrafish	2030, 3106, 3153, 3158, 3161, 3162, 3163, 3179, 3603, 3606, 3887, 3891, 4358, 4403, 4527
Zebrafish embryos	3192
Zebrafish, New Alternative Methods	4146
Zinc	3444
Zinc, Nickel	4601
Zonation	3006, 4613