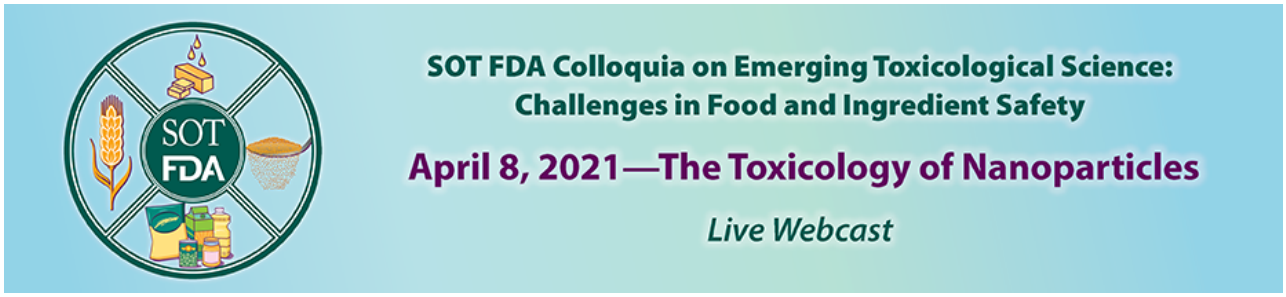

Real-Time Captioning

Note: This is not a transcript.

April 8, 2021

The Toxicology of Nanoparticles

Schedule (All times are Eastern US, UTC -4)

9:00 AM–9:05 AM	Welcome George Daston, SOT President, Procter & Gamble Company, Mason, OH
9:05 AM–9:10 AM	Overview and Speaker Introductions Rick Canady, NeutralScience LC3, Camano Island, WA
9:10 AM–9:45 AM	Lessons Learned from Nanomaterial Characterization: Critical Quality Attributes That Influence Biological Properties Anil Patri, NCTR, Jefferson, AK
9:45 AM–10:20 AM	Standard Dose Measurement for Nanomaterials: What to Include in Exposure and Toxicity So That We Can Bound Dose Estimates for Safety? Christie M. Sayes, Baylor University, Waco, TX
10:20 AM–10:30 AM	Break
10:30 AM–11:05 AM	Dosing-Related Challenges in Toxicity Studies and Risk Assessment of Titanium Dioxide in Food Walter Brand, RIVM, Bilthoven, The Netherlands
11:05 AM–11:40 AM	Practical Application to Regulatory Toxicology: Issues Faced in Consideration of Developing Health Guideline Values Lynne Haber, University of Cincinnati Risk Science Center, Cincinnati, OH
11:40 AM–12:40 PM	Roundtable Discussion Moderator: Rick Canady, NeutralScience LC3, Camano Island, WA

All speakers
Timothy Duncan, US FDA, Bedford, IL
Agnes Oomen, RIVM, Bilthoven, The Netherlands

Welcome
George Daston, SOT President, Procter & Gamble Company, Mason, OH

If we could have the next slide? Thank you. I am George Datson, and I'm the president of the Society of Toxicology. Welcome to the SOT FDA Colloquium on nanoparticles.

The colloquium series has been a very successful partnership between FDA CFSAN and SOT. This is our sixth year of operating this joint colloquium series and this is the 25th event. It's been tremendously successful. The real purpose is to make sure that we can share with both our FDA colleagues and with others the latest information on the science of toxicology that would be useful in terms of understanding the safety or lack of safety of food and food ingredients.

These colloquia are intended for FDA personnel and also open to the public, and they're open at no cost. And they're also recorded and available along with the slides that are presented on our website, SOT's website, which is toxicology.org.

The reason why we make these open and free, and accessible, is because of our mission. I won't read this to you, but I think it's clear that we have 8,000 members in the Society of Toxicology and every single one of them became a toxicologist because they care about the public's health and the environment's health. We really want to make this a safer and healthier world and having this colloquium series open and accessible is working towards that mission.

Just so you know, this is what the website looks like. Go to toxicology.org and you'll find a tab with the SOT FDA colloquium information. You can go through all these materials and find previous colloquia. This one will be uploaded as well. You can also, if it turns out you're not a member of the Society of Toxicology but feel like this is something that you want to be involved in, there's membership information, so we would love to have you join as a member.

I want to acknowledge and thank the organizing committee for the SOT FDA colloquia. These people are volunteers and have taken their time to think through lists of topics and really help in getting together top-notch colloquia that are state-of-the-art and informative as possible. I thank them and hope we can all acknowledge their efforts. With that, I want to introduce Rick Canady, who will be the colloquium chair. And he will go through the agenda and be the master of ceremonies. Thank you.

Overview and Speaker Introductions
Rick Canady, NeutralScience LC3, Camano Island, WA

Hi, everyone, and good morning, or good afternoon, depending on where you are. Let me go through a brief overview of why we are doing this colloquium. And the goal. And also go through the introductions of the folks who will be doing the speaking and participating in the panel.

I spent a lot of my career developing health-based guidance values, and it's generally a challenge to translate what we find in the open literature, study findings, into the numbers and confidence estimates that we used to make decisions for any substance. As you're aware, for the current literature of nanomaterials, the task is even harder. We put together this colloquium to explore the challenges for data development and data integration to encourage the development of best practices for linking the understanding of exposures to the understanding of effects. We have four speakers joining two panelists and each speaker will have 30 minutes so that we can leave time for brief clarification questions. I'll go through a quick introduction.

The first speaker is Dr. Anil Patri, who directs the US FDA's nanotechnology core facility and is also chair of the Nanotechnology Task Force in the FDA's Office of the Commissioner. He was formally deputy director and principal scientist of the National Cancer Institute's Nanotechnology Characterization Laboratory. He has had quite a long history in directing research, functional assays and interpreting results for real-world products for nano. We asked Dr. Patri to provide a view to the physical and functional characterization challenges presented by nanomaterials. As he has experienced them in programs he directs at FDA and had directed at NCL.

Following Dr. Patri is Dr. Christie Sayes of Baylor University, we've asked Dr. Sayes to explore further challenges and potential solutions. Christie understands the characterization challenges for nano from an instrumentation level through to the interpretation of the level of nanomaterials in various practical settings. As I hope we see through each of these talks, integration across contexts as Christie excels at. It's critical for nanomaterial evaluations. Carrying all of that information through to toxicology study is the next order challenged. She has some ideas about that.

Following Dr. Sayes is Dr. Walter Brand of the National Institute for Public Health and the Environment, RIVM, in the Netherlands. RIVM connects independent research and public health serving as a trusted advisor to the Netherlands and the EU. As part of providing that trusted advice he has been involved in and published evaluations of the toxicology of nanomaterials used in consumer products. He will do a deep dive into one nanomaterial currently used and currently in focus in Europe, and elsewhere, as an example of some of the challenges faced.

Dr. Lynne Haber of the University of Cincinnati Risk Science Center. We invited Lynne to present both an integration viewpoint as well as a view to the practical challenges that are amplified in the exposure of toxicology comparisons for nanotechnology. She's someone who reads study after study across all endpoints for toxicology and epidemiology and distills out the very relevant dose responses and mechanistic information to develop health-based guidance values. I have shared stacks of studies with her and look forward to getting through some of the recent evaluations.

We have a full hour for panel discussion with all speakers and I hope you will join us. We increased the firepower of the panel with two senior scientists in regulatory environments. Each of them has deep experience in research for uses and products. In addition to contributing to the discussion as each added panelist sees fit I asked both to review the slides and offer questions. For the entire panel to consider as a way of introducing or starting the discussion. All participants should have received these questions earlier this week and we will keep an eye on the chat for questions from participants.

The first added panelist is Dr. Agnes Oomen of RIVM in the Netherlands. Dr. Oomen is a senior scientist at the Center for Safety of Substances and Products and served on numerous EU projects and expert committees from an integrated perspective on the relation from data development and risk management for nanomaterial uses.

The second added panelist is Dr. Timothy Duncan of the US Food and Drug Administration. Dr. Duncan is a research chemist and principal investigator at a part of FDA at Chicago that was set up for regulated products. He's published on nanomaterial release for packaging and food and on nanosensors for measuring food safety. He has a particular interest in the transmission of data between exposure scientist and toxicologists ensuring that safety standards are met for nanotechnology.

These next few slides give the questions that Dr. Oomen and Dr. Duncan have prepared. I ask you to refer to them and think about them during the presentations and again at the end we will go into a panel discussion. These are the questions for Dr. Duncan that he prepared after reading through the slides.

With that let's turn to the first presentation, which is Dr. Anil Patri. Thanks very much.

Lessons Learned from Nanomaterial Characterization: Critical Quality Attributes That Influence Biological Properties
Anil Patri, NCTR, Jefferson, AK

Good morning, it's a pleasure to participate in the colloquium and I would like to thank the Society of Toxicology colleagues for organizing this, and also to Rick Canady for the invitation and my colleagues including Jason for helping this session for you today. Risk asked me to present on lessons learned from nanomaterial characterization and how changes to material properties influence biological effects from my past and current experiences from the FDA. There are many nuances as you all know because nanomaterials are different, standard disclaimer: the views expressed are my own and should not be considered as an official position or policy of FDA. I don't have any conflict to declare.

The objective of my presentation today is to showcase the importance of nanomaterial categorization, monitoring various physico-chemical attributes to understand the interactions of biological impacts in intro and in vivo. Each has its nuances, and some of my colleagues will discuss some of the detail, for example with titanium dioxide. But I will provide an overview with my past experience. The attention to detail in this characterization is vital for generating good reproducible data to make any kind of decisions, whether scientific or regulatory decisions,

especially due to the additional complexity nano materials bring compared to small molecules.

Next slide. I would like to provide an overview of the FDA focus on nanomaterials so that we are all on the same page. The mission of the FDA is to protect public health by ensuring safety and efficacy and security of products it regulates and to advance public health by helping to speed innovation. Nano materials are used in many FDA-regulated products. The majority of the submissions that we've seen are in the area of drug products with more than 700 submissions followed by devices. Food additives and food contact material constitutes a smaller portion of this portfolio. Understanding the scientific advances and challenges is critically important. As a submission of these products are becoming increasingly more complex.

The FDA does not have a regulatory definition of nanotechnology even though there are other definitions outside with the National Nanotechnology Initiative in Europe and elsewhere. We felt the existing regulatory work and review process adequately identified and managed potential risks associated with nano materials and regulated products. Meaning we did not have any new regulation but published several guidances and documents related to nanotechnology to help industry. So, the overarching guidance include the following points for the main guidance where we consider whether a material or end product is engineered to exhibit properties or phenomena that are attributable to its dimensions all the way up to a micron. Usually there are many considerations for the nanometers elsewhere, but we extended that to a micron size as long as dimension-related properties are novel and are used in these products.

That nano material can have various different compositions and properties and they are not one type of material so that makes it challenging. They can be organic and carbonaceous materials and inorganic particles used in gold and silver. Metal oxide, there's a whole host of different kind of particles. The measurement tool to elucidate the vast range of properties can be simple in some cases or get significantly complex. One has to be aware of the differences to elevate for structure-activity relationships to ascertain biocompatibility and safety and efficacy of these products.

This is the data I’ve been presenting for quite some time. We have seen an increase in drug product submissions containing nanomaterial, my colleagues for the Center of Frugs surveyed the submissions and the trend continues increasing the product submissions containing drugs. Over 700 products containing nanomaterial are submitted. They've evolved from the simple systems to highly complex multifunctional structures. Liposomes constitute one third so there's a lot of research in the FDA with liposomes with cancer indications constituting one third of the submissions. And intravenous administration is utilized in 60% of the submissions. You can get additional details from the report we published last year, and you can find the link below. The FDA is very flexible to support innovation by working with the sponsors for nanotechnology product development.

We have issued final guidances about the current thinking of nanotechnology products to help industry. This can be downloaded on the FDA website. The guidance pertinent to food is the third guidance in the previous slide. My wifi is slow and now we are on the following slide. Without spending too much time on the

details of these guidance. It's helpful and pertinent to food or CFSAN, the third guidance is applicable where we have emerging technologies but also nanotechnology for the safety and regulatory status for food ingredients and food contact substances.

The FDA has invested heavily in research facilities due to the emerging technologies at White Oak campus in Maryland and Jefferson campus in Arkansas and both are equipped with state-of-the-art instrumentation, so it provides great opportunity for collaboration within the FDA and to also engage in interagency collaborations to conduct projects of mutual interest and benefits. From our colleagues if there are any questions would like to collaborate and get in touch here at one of us at White Oak or in Jefferson campus.

These core facilities have a lot of instrumentation because of the new challenges with nanomaterials and helps us with understanding and developing the critical quality attributes. Going through some of these properties such as size, surfaces, impurities, and how to measure them and utilizing appropriate methods. Often multiple methods are needed to ascertain these attributes and the instruments that are measured are not typically used in small molecule characterization and regulatory submissions. Except spectroscopy that are typically used in those submissions. The rest of them listed here are more advanced, electron microscopy, nanomaterial characterization as well as scaffolding and refraction techniques and so on.

The unique properties of nanomaterial can interfere with broad absorption, scattering, fluorescence, can interfere with many biological assays. Most of the in vitro assays include the 96-well plate assays and typically use of sorbents so prior knowledge of these attributes of the nanomaterial being studied can assist in designing studies with appropriate controls. And we have seen even starting from endotoxins, that’s the firs thing when we conduct the studies, endotoxin chemistry and some of the in vitro immunotoxicology assays.

Unlike many of the drug products that utilize nanomaterial as drug delivery vehicles where they are engineered to be suspendable for a considerable amount of time, unlike those, many metals that can only stay suspended if they don't have any coatings. Once we introduce [inaudible], then they start agglomerating, aggregating and completely become insoluble. The interaction of these conglomerate cells might be different from individual particles and protein coatings may provide stability but also [inaudible]. Sorry, somebody’s talking. Can you mute your phones, please? Thank you.

Protein coatings may provide temporary stability, but they may alter the nanomaterial behavior because what you're seeing is the interaction of the proteins with the cells and biological systems. So, adequate characterization of these materials is suspension, if necessary, for experimental conditions that you'll encounter during in vivo and in vitro studies.

Here I want to showcase a very good example which is liposomes. One third of the drug product submissions containing nanomaterials include liposomes. It's a very simple methodology. The main factor is to be consistent for any product that comes

to the FDA. When you publish, reproducibility is key to have the data set. So, liposomes as the structure or the cartoon illustrates are composed of a lipid bilayer. In some cases, is multiple layers are possible with an aqueous inner compartment that encapsulate drug molecules, and these are mostly aqueous soluble but there are some drug molecules that can be encapsulated into the lipid bilayer. This seems to be a very simple product but there are many attributes that one should monitor as each may change the biological activity. An example here. Size is measured by scaffolding, gives you hydrodynamic size and they look very homogeneous many nuances. You can see the structure, whether they are unilamellar or multilamellar methods such as nanoparticles tracking analysis or laser diffraction provide additional details of size measurement. Fractionation followed by analysis, this is the image on the right, they provide more analysis that sometimes you don't see with the dynamic light scattering.

The additional methods that include zeta potential is an indicator of charge, mobility even if you coat the product and glycol, you'll still see the difference in the surface charges. Ion content for ammonia sulfate ions that are used, and lipid analysis, and then the free encapsulated drug analysis and drug release profiles.

A slight change in these properties such as size and compilation, size of the liposome, the composition of the lipids, free or encapsulated drug may have different biodistribution. The safety and efficacy profile containing the liposomes. Appropriate monitoring as a function of time is a reproducible product.

The slide illustrates the complexity of each of the hydrodynamic size measurements. Some of them are deceptively simple, such as the dynamic light scattering, because it is used in many of the drug product submissions. More than 50 percent use DLS. It is simple and effective in cases where you have more homogeneous size of material. This measurement becomes complicated when you have more poly dispersed material. Often multiple methods can provide a picture about the size and the glomeration state. If there are more than micron size, DLS might have limitations. And so, one may have to use laser diffraction and other fractionation methods. One challenge that we have in the size measurement is they are applicable buffer such as water or saline but can become more complex if you want to measure the size in cell culture media, the content proteins because the density changes in the protein attraction changes the size and they may become poly dispersed and difficult to analyze. Especially soft material. So, depending on the type of particle that's being measured. Because one could use those or metallic and metal oxide nanoparticles.

Fractionation method is very useful but, in some cases, you cannot put a nanomaterial on a column. This is where asymmetric and centrifugal field fractionation typically used for protein analysis but also for more than a decade or two that they are being used for nanomaterial analysis. This does not utilize stationary plates, so there is no column packing. They can provide a more detailed picture of the poly dispersity. As with any other method it has its limitations with membrane and concentration and other factors. It also depends on the detectors that you use. It provides a better picture of the poly dispersity of the sample.

The slide illustrates the reason why such detailed size measurements in the example of three different size liposomes they are not commercial but research grade.

Hundred 150 and 200 nanometers with the drug loaded in there. The characteristics of these three different liposomes are identical as you can see on the slide but there are some differences depending on the size heterogeneity. The uptake seems to be similar where the tumor accumulation seems to be different. This is an animal model, the tumor. The 150-nanometer size in this case it's predominantly accumulating in the tumor. The uptake of larger-sized liposomes is greater than the 100-nanometer liposome, that's on the bottom right draft. So, the overall conclusion to take away from this slide in this study is that size plays a role in distribution even though for pharmacokinetics may be similar, local toxicities may be different depending on the slide or size.

Jumping into the complex challenges with metal oxide nanoparticles, as I mentioned these polymers and liposomes maybe one thing but once you get into graphene or metal oxide it can complicated. So, here's an example of titanium dioxide nanoparticles using the protocol to suspend this material. It provides a relatively narrow size distribution. If you take a real-world sample, it can be less useful, and one has to utilize laser diffraction and has the dynamic range. The bottom right shows the poly dispersed sample with many sizes and the size distribution in suspension. Proper methods need to be used especially if you know it can form agglomerate.

Another challenging example is carbonaceous material. Added to the fact that they are completely insoluble. Graphene, it becomes partially suspended but for most part. While graphene can stay suspended from longer periods. Breaking the larger agglomerates to become smaller graphene plates that get suspended. So, the question is whether this the same material, but the sample preparation is different. So, they may have different biological effects. It is most likely that [inaudible]. There is an echo, sorry.

Surface coatings can also cause differential protein binding. The top portion is an uncoated particle. Here the slide illustrates the differences that the surface coatings can cause. This is an example of why surface coatings can influence behavior, the top portion is in uncoated particle versus the bottom portion which is coated and has less protein binding. As illustrated on the right the uncoated particle gets deposited into the liver and spleen. While the coated particles have not gotten into the liver and spleen quickly, but they may eventually get the liver and spleen because the coating may come off. Coatings can have dramatically different in vivo and in vitro outcomes.

And the surface charge this illustrates as an example of dendrimer, which is a polymer with a different branch molecule. In this case we looked at different surface coated dendrimers. On the left panel is the cell viability assay that show that the cytotoxin releasing LDH the cell viability. On the right it demonstrates the hemolytic potential of a mean surface dendrimers whereas the neutral dendrimers are non-hemolytic. The good thing about the nanomaterial is that you can engineer the surfaces to have a different charge so as you gradually coat these amines, it becomes more and more neutral. From 100% amine to more neutral amine dendrimer, it shows there is a decrease in platelet aggregation but also decrease in hemolysis and decrease in cytotoxicity. So, these surface charges can have influence in vivo and in vitro.

The last lessons learned is the purity. In many cases it's made with some kind of surfactant to stabilize them. Sometimes you buy them commercially and they do not tell you the composition that's present, so this is the case study with gold nano rods which are synthesized with trimethyl ammonium bromide as a surfactant and followed by polyethylene glycol protein to make it more suspendable and neutral. These are the characterizations that go in. You can see one has to monitor if you purify. On the bottom right panel, you can see the residual CTAB that's present and only after the sixth wash it disappears.

The reason it becomes critical is the cytotoxicity of these gold nano rods. By themselves they are not cytotoxic, but it shows the toxicity that is a publication in the bottom right. And what we realized is when we took these samples, we filtered the samples, and the cytotoxicity came from the filtrate which does not have the particles. That is attributed to the CTAB which is a surfactant impurity that is present in the sample. Even after the initial purification by this group. The gold nano rod didn't show any cytotoxicity. Sometimes these characterizations include small electron microscopy and diffraction techniques, but one has to get into the detail.

Here's a summary, significant increase as I illustrated in drug product submission that we have seen at FDA. These nanomaterial understandings become critical and go from simple to more complex systems and because of the research both internal and external research, knowledge about nanomaterial evolved for the past two decades. Many nanomaterial properties experience biological interactions and alter safety. Advanced understanding of these properties and nuanced differences would enable better products to market. I would like to acknowledge my colleagues from the FDA, many current and past colleagues and also the Nanotechnology Task Force colleagues that we work with on a regular and daily basis in some cases that have contributed to this talk. Thank you.

Rick Canady: Thanks very much, Dr. Patri. We have a few minutes for some brief clarifying questions, if anybody has one, please send your question through chat in the WebEx program. And meanwhile let's queue up the next set of slides.

Our next speaker is Dr. christie Sayes from Baylor University. She's going to dive into more of the analytical and characterization challenges of nanomaterials as mentioned earlier. And without further ado let's jump in. Thank you very much, Dr. Sayes.

Standard Dose Measurement for Nanomaterials: What to Include in Exposure and Toxicity So That We Can Bound Dose Estimates for Safety?
Christie M. Sayes, Baylor University, Waco, TX

Thank you, Rick. It is a pleasure to be here. First can you remind me how to open the chat window to be able to put questions in.

Canady: If you highlight the coordinator, you should be able to send a chat through.

Sayes: I see, so it’s at the very bottom to the right-hand side of the WebEx screen.

Thank you all for the invitation to speak with you today. My email address is on the slide in case you would like to contact me after the talk.

I was trained as a chemist, but I practice toxicology. I mostly use cells in culture but commonly used zebrafish model and rodents. I was charged with this question: Standard Dose Measurement for Nanomaterials: What to Include in Exposure and Toxicity So That We Can Bound Dose Estimates for Safety? I hope I highlight some points here that will help us to get through some of these questions. I don't have any conflicts. I'm in academia.

My objectives for today's talk are to introduce analytical methods to assess exposures and presenting challenges relating with nanomaterial dose in toxicology to nanomaterial concentration in the actual product. I'd like to introduce an area that's getting some attention recently. Which is to justify speaking about using a mixtures toxicology approach in nanotoxicological research.

There are many analytical methods that a researcher can use to characterize focal nanomaterials used in their study, be it in food or drugs in devices or even contaminants in the environment. The types of study range from human and ecotox to performance or failure analysis of the final product. And clinical applications in biological sciences. The analytical tools can be categorized based on the question you want to ask and answer. Such as, are there nanoparticles present? There are different analytical methods that are available in more labs simply to answer that question. You then might want to ask more complicated questions such as how many particles or what type of particles. And for this we have to get increasingly specialized and start thinking about chromatography, mass spec, and infrared detection, imaging for which I can give an example and everybody's favorite, electron microscopy.

The question I think that's on a lot of our minds these days is how these nanomaterials or -particles changed from before you did the exposure to after. So, we can characterize these particles very well and should be proud of ourselves for being able to characterize the nanomaterials, but we have to do a better job of asking of have they transformed and maybe why, and for that we need increased specialization such as X ray and more hyphenated techniques like LCMS-MS. So, this pyramid structure is laid out from top to bottom in increasing instrument availability. And increasing specialization when we look at this pyramid from the bottom to the top, that's both good and bad. It's good because it enables us to move forward with collaboration and finding teamwork settings, but it’s bad if you need a quick answer in the middle of your product development. So now I'm going to transition to show some examples showing how we can apply this pyramid using analytical techniques. For model exposure in different media and accessing dose, followed by identifying hazards.

This is text, you can read it yourself. In life cycle considerations, one of the most focal questions is how the nanoparticles transformed throughout the product life cycle. It's one of the most challenging questions to address but the literature has a plethora of information to lean on to be able to look at how people have tried it and then improve upon it. Specialized and multiple instruments need to be used. As Dr.

Patri previously said, sample preparation is key in a three-step process that we use to first detect and identify and quantify.

Here I will run through an example that won't be so difficult to digest this. Giving an example of a stepwise approach using electron microscopy to analyze TiO2 particle morphology before versus after incorporation into a product formulation. In this case it's paint, a little outside the scope of food. But this principle still remains the same. Here's the stepwise process for paint formulation process. We used a regular mixer in the laboratory on a more industrial scale to prepare and mix the base paint with the nanomaterials. We poured the formulation onto the drywall and allowed the nanoparticle paint to dry. We used a Taber Abraser to wear and tear this formulated applied nanoparticle product and collected the worn and torn particles from the matrix and collected the powder for analysis. Electron micrographs were used to be able to image the worn and torn particles.

I don't have time to go through the energy disperse but if you don't mind believing me for now. First of all, we had to use scanning electron microscopy for this analysis because TEM proved too difficult to analyze the particles with this formulation. But the EDX pattern shows that some of these circles or spheroidal particles, these separated individual particles in the top and bottom panel of the slide, these were indeed TiO2 particles or agglomerates. Because the scale is so large, it's agglomerated drywall with the carbonaceous material with latex that was in the paint as well as the TiO2 particles intact in this matrix. So, what we could be exposed to in the beginning of the process is if there was an occupational exposure or if this was a nanoparticle embedded food product. What we're exposed to as the pristine phase is different to what we are exposed to after formulation.

So, when we think about modeling the exposure is in these various media and matrices. The studies are designed to ask how many and what type of particles might be in the sample. And like I said I leaned on the literature a lot for these methods. The methods for which people are analytically and bioanalytically measuring and characterizing and detecting and quantifying the particles in the different complex matrices. The data collected is most relevant for extrapolating exposure concentration to the biological dose which is something we need more funding for and more trainees working on this type of extrapolation.

One way we often look at modeling the exposure in what the biological dose might be is hyperspectral imaging. It can be used to qualitatively and semi-quantify the nanoparticles in this case gold nanoparticles inoculated in the culture. If you read this panel to the left over to the other panel C. A is a typical fluorescent image of the cells stained in blue, mitochondria in red, and the cytoskeleton in green, we can easily see the different components of the cell culture itself. When you have this particular image in a hyperspectral imaging microscope, you would identify a potential region of interest in the cell to be able to further analyze, we look for a scan across the entire image. We can identify the ROI as indicated in the dotted boxes. In some of the cells we see an agglomerate or aggregate of the gold nanoparticles, whereas in blue, this is a smaller agglomerate in the cells, and we know that they are gold. Because in addition to being able to look at this hyperspectral image, the dark field image. We are also able to get the spectra of that region of interest. In panel C, we

see this particular peak is indicative of a gold nanoparticle. This is a way for us to semi-quantify what type of particles are maybe inside the cell versus outside the cell.

We've all seen images of the nanoparticle corona. This is an image of the 25 nm gold nanoparticles before versus after incubation with albumin. Obviously, the top is before and then B is after. Electron microscopy is used to qualitatively assess what is in the sample, and it gives us good information of that nanoparticle itself. But this is little information about the nanoparticle crown.

We can use mass-spec to give us more details regarding the composition of the crown. And for this study we wanted to not only see what proteins were present in this crown but also if the nanoparticle properties itself changed and what the composition of the crown might be. Here we used carboxyl group to confer a negative charge and we used a polymer peg to confer neutral charge and for the third sample we used an amine group to confer a positive charge on the nanoparticle surface. We also varied the incubation time with the albumin and interestingly the same sets of proteins were identified in all the samples. Subtle differences can be seen across the positive and neutral and negatively charged particles as well as over the different incubation times. If you're looking back at this presentation the ST 1 and ST 2 represent the shorter and longer incubation times with positive or negative or gold nanoparticles. More interpretation of this data can be found in the paper by Madison Stewart. She was an undergraduate researcher in my laboratory, worked three years as a researcher to help put together this paper and we are very proud of that.

Examples of assessing dose-response. How can we ascertain the dose? The million-dollar question we ask is did we really deliver the dose we intended to through these serial dilutions? I mentioned that because in the literature the most common study design that people use on a dose-response is over a serial dilution. The answer is useful for dose range finding studies, for weight of evidence evaluations, and for accurately recording dose relations. I find that more discussion and teaching and learning is needed in our community for designing the appropriate dose response relationship for ascertaining which dose did the biological systems see where we got a particular response? We know it requires unknown concentration at the beginning of the study and often, I’m not saying good or bad, what happens is we use that serial dilution as the dose on our X axis on our graphs and we wonder if that's the best way to represent that.

Here is an example of a systematic study identifying trends in nanoparticle exposure to a biological system. In this case we used silver and copper nanoparticles. These were done similarly to the previous study where we conferred a positive, negative, and neutral charge onto the surface of the silver or copper nanoparticles. Across the top row is the silver exposure for the physiologically environmental relevant bacterial. At the bottom is the copper exposures. For all of these graphs the Y axes is measuring bacteria inhibition. Unfortunately, I don't have time to get into the exact disc diffusion method that's used in this particular study, but bacteria inhibition is shown on the Y axes where the nanoparticles have more antibacterial properties when you go up on the Y axes. The X axes is the exposure concentration, and that scale is one to four and eight micromolar when referring to the nanoparticle molarity.

What we see here across the six different graphs is that each nanoparticle, the copper or silver particles, induce some different results but the trends are similar.

The blue line in all six graphs indicates that there is an increase in bacteria inhibition as the concentration of the nanoparticles in the system also increases. That is a trend that we see for the blue negatively charged nanoparticles and is also similar for most of the black and the red. Where the red are the neutrally charged nanoparticles and black are the positively charged nanoparticles. So, looking at the charge it's definitely an indicator for us to be able to understand what these effects might be overdose. And how do we report the dose that these particles saw? If you use a systematic study and compare them against each other then there is value in a dataset such as this to be able to read across.

We will change topics to challenges associated with relating nanomaterial doses in toxicology and toxicology to the nanomaterial concentration in products. The first main concept that we are talking about weight of evidence, we need to be able to translate the data that we collect in the laboratory. I'm talking about the analytical and bioanalytical data, not just toxicological, that we do. And read-across studies in an effort to decrease uncertainty in an individual study. Doing the studies in parallel at same time so we eliminate as many variables as possible. And understanding the concentrations and how the exposure method induces different results in concentration or dose.

The first concept to talk about is to give a shoutout to Cuddy et al. in 2016 where proposed a particular approach of weight of evidence for nanoparticle characterization using these multiple lines of evidence to determine size and composition. Analytical and bioanalytical data can fit into it. It is something I've personally used when designing studies. The idea is that the orthogonal methods that you use to answer a single analytical question, the more robust the weight of evidence is. So, don't just measure particle size using electron microscopy; also use it to measure size with centrifugation followed up with dynamic light scattering. If you have two or three different methods to measure particle size, then the weight of evidence increases, the size of the sample it is actually the accurate size of the sample. We can do that for particle composition as well as product composition.

Not only can we use multiple lines of evidence across differing analytical techniques, we can use multiple lines up evidence within a single data set. Here to the right, I encourage my team members to use electron microscopy. We’re always in that center. To assess the qualitative information about the system, we’re looking at cross-sections of individual cells exposed to silica or aluminum. These are exposed to aluminum nanoparticles and the white arrows are pointing to mitochondrial as well as the vesicular types of formations. It will normally only give you this qualitative information, but it's my proposition that we can increase the weight of evidence by not only looking at this first data set but by following up with qualitative assessment and statistical analysis to be able to compare the exposures of the given nanoparticle to that particular cell system. With the deeper dig within one particular methodology, we are able to increase our confidence and our weight of evidence of that characterization that feeds into risk science for decision-making.

Read across studies are used to decrease uncertainty within the study design. Here I was fortunate enough to be involved in a series of studies where the nanomaterial was systematically compared for the bulk of the counterparts. We used in parallel a rodent seed study where we were able to demonstrate a strategy for the assessment of the materials side-by-side simultaneously. With a simulated digestion method for the in vitro study that mimics the conditions that occur in the gastrointestinal tract on the in vivo side of the study. So, we believe this type of study design is useful to evaluate the impact of physical or chemical changes to something like cellulose nano crystals or even something more complicated like functionalized nano celluloses after oral exposure and for future commercial forms that are developed and tailored for other applications in food products or in primacy to go products.

And switching it up similarly to subjecting like in the previous study. Subjecting them to simulated digestion before exposure and characterizing the digested form of those particles. Similar to that we can think about a long exposure as well. There's a couple of different ways we can think about that inhalation: either subjecting particles to the fluid in the trachea then look at the effects for the transformation of the particles after they were incubated in that bio fluid. Or we can say the method of exposure in this case in the delivery of the aerosol as a gravitational settling or with a gentle impaction that's pulsing air into the LI device to measure the differences. In the rate of deposition and the total deposition after a given time of 15 minutes. We show that the gravitational settling only deposits 2000 nanograms to the top of the cell. In gentle impaction we deposited 150,000 nanograms so these two methods, give us a different exposure dose to the cells and culture.

I think I've just got this one particular slide with the example to go through, the last thing I wanted to put up for this audience and discussion is that I think we should continue to think about nanotoxicological resource as a mixture of toxicology or an exposure to a mixture. When we think about this challenge, at the onset, studies can be designed as either equimolar or extra-potent facials. When we think about the ratios of the mixture. For instance, a nano particle. It’s inherently an exposure of a mixture so maybe particle one is represented and could be one particle size, but particle number two is a different particle size, represented here in the green. It could be the nanoparticle itself versus the corona. Or maybe as a formulation of a nanoparticle in a buffer. Either way. Whatever was being exposed to is a mixture of sorts. So similar to how we do dose response relationships.

Another way we can think of this when we bring research in, is by looking at the ratios of the chemicals in your mixture and measuring the responses at will molar ratios, equimolar versus aqua potent values that are a way a toxicologist might think about it. In my laboratory most of the times we stick with these constituent mixtures and we've got two different things we co-expose ourselves to in these known mixtures of 50/50, 30/70, all these different ratios between zero and 100. Here at the bottom, we can think about the same type of idea where you've got maybe two different particle systems but with a third constituent like a buffer or latex paint. For these we can think about the 50% formulation. You're still a 50-50 ratio of your nanoparticles that you're interested in. But that is only 50% of the entire volume, which the other 50% would be the buffer or the formulation for which you’re measuring.

Even within a mixture of the approach we can overlay the dose response relationship when we start thinking about this. You can still overlap some of the ratios, we can collect a lot of data for those relationships. If it gives us an antagonistic response, the way you go about doing that is after you collect the dose response curves for the individual sample that you have. We have led versus the differential LC 50 values across these three different materials and these constituents. We can then start doing our 50-50 ratio for these combinations. We did a 50-50 mixtures of the lead particles connecting the values of the individual if it's above the line it's antagonistic on the line added or below the black solid line to give you for the models and references that are shown over here on the right-hand side of the screen. There's a lot of error of prediction that goes in to, which we put some error bars around these samples.

In summary, there is a lot of variety of techniques available to help assess the exposure. Dosimetry It is and always will be a very important consideration in nanotoxicological research. Every study ought to assess dosing concentration and the target dose into the model system. The challenges associated with this, yes, we are limited by methods and tools and techniques but as we work together as a community to look at hyphenated techniques, then we should be able to move forward with some SOP and standardizations. We look to the literature. Because that's a ton of information in the literature on sample preparation. And mixtures toxicology approaches in nanotoxicological research is needed. We can begin to tease apart what the effects might be in one particular size particle versus another or if there are differences in other contaminants in that matrix.

With that I will leave you with the references on the slide. These are acknowledgments of my research team. And we have funding from Air Force, USDA, from Vireo Advisors, and internally the Gus Glasscock Endowed Fund for Excellence in Environmental Sciences at Baylor University. Thank you very much.

Canady: Thank you very much, Dr. Sayes. I would like to spend a full 10 minutes on that last slide, I hope we will have time to do that. We do have time for one brief question of clarification. And I've asked Anil Patri to deliver that question.

Anil Patri: Just a clarification question from my presentation. There was a slide for a bacterial inhibition assay. In one of those I think the CTAB is used as a surfactant or is it another surfactant. The question is, is inhibition coming from the surfactant itself and nothing to do with the particle?

Sayes: Absolutely. The CTAB was used as a coating agent and is not bound to the surface of either one of the particles, either silver or copper. It was used to confer a positive charge onto the surface of the particles. We have to use a very small amount of CTAB I the synthesis process and go through three different ways of purification to get access surfactant that's not absorbed onto the surface out of the system. You're ultimately left with a .4% CTAB in your final particle suspension. We then in parallel measure the bacteria inhibition of the .4% CTAB in parallel to the nanoparticle system. While CTAB does inhibit bacteria growth, it does not do so to the same extent as the entire particle system of CTAB plus the nanoparticle itself.

Patri: Thank you.

Canady: Thanks very much. So, we have time for a 10-minute break. We will come back at 10:30 East Coast time US. We will restart the presentation with Dr. Brand from RIVM. If you have questions for the panel afterwards, please put them into the chat function to send it to us. We will speak to you again in about 9 minutes.

Break

Canady: So, as we get ready for the next presentation, I wanted to bring folks' attention to the chat function, where some of the questions for clarification have been addressed. If you have questions for the panel, please put them in the chat. To the captioner, your microphone is rather loud, so could you please mute?

I would like to introduce the next speaker. Dr. Walter Brand of the National Institute for Public Health and the Environment in the Netherlands, RIVM. Dr. Brand will take a deep dive into one of the nanomaterials that is currently in research and also in use in products and without further ado, I'd like to turn the mic over to Dr. Brand.

Dosing-Related Challenges in Toxicity Studies and Risk Assessment of Titanium Dioxide in Food
Walter Brand, RIVM, Bilthoven, The Netherlands

Thank you, Rick. Good morning to your all, or good afternoon. My name is Walter Brand, and the topic of my presentation is Dosing-Related Challenges in Toxicity Studies and Risk Assessment of Titanium Dioxide in Food.

I have no conflict of interest to declare. I work as a scientific officer at the National Institute for Public Health and the Environment in the Netherlands, abbreviated in Dutch as RIVM. RIVM conducts research, advises on policy, and helps to implement that policy with the purpose of promoting public health and maintaining a safe and clean environment. RIVM is the Netherlands' main public sector knowledge institute in the field of public health, nutrition, safety, and environmental management, and it's owned by the Netherlands Ministry of Health, Welfare, and Sport. At RIVM, I work at the Center for Safety of Substances and Products. Out main clients are [inaudible].

Canady: Walter, if I could jump in: your microphone seems to be cutting out. So, maybe approach the microphone closer?

Brand: OK, maybe this is better. And also, international clients are the EU, the EU Commission and related bodies, and we also work for the WHO, OECD, and international agencies. Next slide.

The objective of this presentation is find potential causes for differences between divergent outcomes of toxicity studies with titanium dioxide, which are related to dosing. Titanium dioxide in this case is used as an example of nanomaterial. And investigating differences based on the literature review and discuss causes for differences. Finally, I will make some recommendations regarding the challenges related to the dosing in animal studies with nanomaterials.

First, an introduction on titanium dioxide, it is a well-known white pigment, and uses leading to exposure are key, as a food additive to make it white or shiny, and it is called E171 in the EU. And then used in toothpaste, which is being swallowed by young children, and in addition, the use of medicinal products. And lifelong daily intake from food, supplements, and toothpaste has been estimated to be 0.19 mg.kg body weight per day. The white color is caused by particles of the size range of 200-300 nanometers, during the production, also smaller particles are being formed. Different studies determined that about 10-49% of the number of particles in food additives is less than 100 nanometers. However, based on the percentage, less than 50% E171 is not considered a nanomaterial according to the EU definition, that is important to know.

Next slide, titanium dioxide, as well as E171, can have different characteristics, the particle size distribution can be different, it can consist of different crystal shapes, such as anatase or rutile. And in addition to particles can be coated. This can be of influence, as we saw in the presentation by Dr. Patri. There's a lot of debate about the safety and there are many contradictory toxicology studies. And there are no long-term animal studies demonstrating that titanium dioxide is safe; however, during recent years, also many studies appeared that showed in can cause adverse liver effects and induction of intestinal tumor formation. Next slide please.

In 2019, we performed a review of all of the animal studies with titanium dioxide, focusing on liver and intestinal as critical target organs, to structure the information, we used adverse outcome pathways as a kind of structure information. Adverse outcome pathways describe a cascade of key events leading to a final adverse outcome such as tumors. The adverse outcome pathway allowed us to differentiate between early effects and pathological outcomes. We also took into account information on titanium levels detected in liver and intestine. And compared to the level of human organs which we analyzed recently. Then we regard the differences between the studies and particles characteristics, and the study design. Next slide.

This picture illustrates the adverse outcome pathway for the liver. From the left, it moves via a number of key events, such as the generation of reactive oxygen species, mitochondrial dysfunction etc., to adverse outcomes, including liver steatosis, edema, and fibrosis, as well as associated events increased liver weight. Next slide please.

We made an overview table in which we placed the results of all the different in vivo studies that looked at steps in the adverse outcome pathway for livers. In the table, positive studies that found a key event, or adverse outcome were indicated by the color red and negative studies were indicated by the color green, and in some cases, both positive as well as negative results were found by different studies. This was indicated by the color yellow. Next slide please.

And here you can see the table, which is a simplified version of the table, and the publication, leaving out some details. It includes the results of 13 different studies, and the studies have different focuses, and they use different types of titanium dioxide. And the setups which are different. The columns from the left onward, key events are presented, up to the adverse the outcome and the associated event on the right, in accordance with the picture of the AOP that I just showed you. In the

rows you can see increasing doses used by the different studies. As one can see, there are studies using relatively low doses, looking at the early effects, that are often positive, but they don't look at the final adverse outcomes, on the other hand there are studies looking at the final adverse outcomes, using usually high doses that are mostly negative. And these are not looking at key events, however. Like I said, different titanium dioxide was used by many studies, and the positive adverse outcomes, reported here, are from studies that used nanoparticles of titanium dioxide specifically. Next slide.

From the slide, it can be concluded that oral exposure to titanium oxide is early key events in the liver AOP, including generation of reactive oxygen species and inflammatory mediators. That happens at relatively low doses of 5 to 10 milligram per kilogram body weight per day. Early key events could be reversible or unable to trigger the next event, the real at first outcomes are only reported in some studies using titanium dioxide nanoparticles specifically.

Let's have a look at the intestine effect, this illustrates the adverse outcome pathway for the intestine, a cascade of different key events, the adverse outcome can be reached, in this case intestinal tumors. Again, we make an overview table in which we placed the results of the different in vivo studies that look at the steps in the adverse outcome pathway for the intestines, in this case we separate the studies in rat from the stunning in mice, and I will show the rat data here. More or less the same outcome comes for the data for mice. Next slide, please.

These are data of about 10 different studies. And the initiating events, the uptake, in color red, and key events are presented up to the adverse outcome, rows increasing doses used in the different studies. Some studies report positive key effects at relatively low doses; however, the studies don't focus with the adverse outcome, and most of the studies that look at adverse outcomes used relatively high doses and didn't take early effects into account. Next slide, please.

This shows that all exposure to titanium dioxide seems to trigger key events in the adverse outcome pathway in the intestines in some cases, happening at relatively low doses. The final adverse outcome is not reached. Next slide.

General conclusions on the toxicity studies can be drawn. In general, high dose studies just focusing on the adverse outcomes which are negative, while relatively low dose studies report early effects but do not regard the final adverse outcomes. With regard to the liver AOP there are studies with positive and studies with negative outcomes. And we wonder about the causes of the differences and we also took into account the internal concentrations in organs reported by the studies. Next slide please.

With titanium dioxide in tissues, usually determined as elemental titanium. This table presents the significantly increased titanium levels in the liver and different toxicity studies after different doses, indicating the toxicological effect in the liver was seen, the results however don't provide a clear picture. One would expect with increasing sternal dose, higher internal organ concentration, but this is not the case, the results do show increased liver concentrations of about one mg per kg liver, with a three-week exposure to five milligrams per kilogram body weight per day, associated with

liver inflammation in the study, by Talamini. And after a single dose of 5,000 mg per kg, an increased liver concentration of four milligrams per kilogram liver was reported together with increased liver weight, but other studies report increased organ concentrations without toxicological effects, or toxicological effects while there is no significant increased concentration. Next slide, please.

And levels in intestinal tissues, they are only determined in a very few studies. One studies, again the one by Talamini, reports an increased titanium level of one milligram per kilogram, and after three weeks, only five milligrams per kilogram body weight per day. And this is E171, and no increase in small intestinal tissue. At this dose, generation of reactive oxygen species and inflammation were triggered. Next slide.

At present, we have data on the level of titanium in several human organs, including liver and intestine, and these can be compared to the ones in animals. The human organs are actually postmortem tissues from individuals who donated their bodies to science. And mostly elderly people, with an average age of 86. No histopathology was performed on the tissues and we have no medical history and of course no data on the exposure to titanium dioxide as well. The exposure was almost exclusively originates from oral exposure. Next slide, please.

This table presents the titanium levels measured in the different human organs. In some cases, the levels were below the limits of detection. From 0.01 up to 0.16, milligrams per kilogram in liver, and compared with the levels in animal studies and just a second. The levels in intestine were measured in different sections, and the ilium section, and they were much higher and ranged from about 0.02 to 2 mg per kg intestinal tissue. Next slide.

Back to the internal liver concentrations in animal studies for a short comparison, the level reported by Talamini of 1 mg per kg liver is only six to 30 times higher than the median and max concentration in human liver. There are also other animal studies with increased organ concentrations without effects.

When we compare the level reported by Talamini of about one mg per kg in tissue, which generated reactive oxygen species and triggered inflammation, you will notice it’s similar to the levels found in human small intestinal tissues, a medium value of a factor of 4-8 times lower.

So, what can we conclude in this? In animal studies there are increased internal organ concentrations, with or without effects, and even effects without increased concentration, therefore the relationship between the external dose and internal organ concentration is not clear at all. It is worrying that sometimes first effects are seen in animal toxicity studies with apparent external exposure not much higher than detected in human organs. What can be an explanation for the differences between the outcomes of these various animal studies?

We discussed about the differences between the studies causing the different outcomes. Of course the properties of titanium dioxide such as particle size and crystal structure and exposure duration of the study played a role, and possibly offers some general issues; however, and other tissues related to administration

methodology could be of different importance to the toxicological kinetics of titanium dioxide, and possible other nanoparticles as well. And some things are to be said about dose formulation and the method of administration. Next slide.

We saw differences in outcomes in high and low dose animal studies. It is possible that at high doses, when titanium dioxide agglomerate or aggregate, there are reasons to believe that high doses could negatively affect the intestinal uptake and therefore the subsequent effects. Aggregation and agglomeration are also playing a role in the dispersion and the formulation given to animals. The way titanium dioxide is dispersed, could also lead to aggregation and agglomeration, or different protein [inaudible] the difference of the protein information. Also altering the uptake and subsequent effects.

The method of administration of titanium dioxide, the difference between studies, using an oral gavage, leading to a certain high concentration, you can also administer via drinking water or drinking a suspension of water into the mouth, which was done in the Talamini study. We noticed that studies with dietary exposure showed no induction of effects, which could be explained by matrix effects with reduced content with [inaudible], for instance. And from the perspective of risk assessment, it also raises questions about how well these different methods represent the realistic human exposure via different foodstuffs.

There is only one study, a remarkable study performed by a Mexican group, that compare different dosing regimes with titanium dioxide, it studied effects on testes in mice, and compared one dose level by oral gavage, the suspension in water, with three dietary dose levels mixed through animal feed, the effects of the dose given by oral gavage, was similar to up to 260 times higher dose given through feed, which really illustrates the importance of the dozing regime in toxicological studies.

What I would like to summarize, is that the administration methodology including dose, formulation and method of administration likely effects of the absorption, the kinetics and subsequent toxicological effects of titanium dioxide after oral exposure, and likely of other nanomaterials as well. Some recommendations that link to the questions, that we saw, are one, that physical chemical properties of the material exposed must be characterized, both the material itself as well as the material in the delivery medium. And it is also recommended to further research the effects of dose and method of administration on absorption. And regarding those levels, we recommend that information from high dose studies should be treated with care. And regarding formulations, those are to be well dispersed, and there are protocols available. And finally, we recommend to determine internal levels in key tissues in toxicological studies in order, in order to be able to relate the external dose to the internal concentrations and effects.

In this slide, you can find the literature referred to during my presentation. And on the next slide, this is my last slide, last but not least, I want to think my colleagues, and my coworkers on all of this work, and of course the funding, the funders, and I would like to thank you for your attention. And of course, I would be happy to take any questions.

Canady: Thank you very much Dr. Brand. We have some time left for brief clarification questions, and remember, we are having a full panel discussion following the next presentation, so if you have general questions or discussion questions, please put them into the chat. In the questions for clarification? Could you please put them into the chat screen?

Jason Aungst: Yes, there was one question here. In the study by Talamini, there were only livers from 2 control mice and 4 exposed mice subjected to histological examination. Inflammatory changes described by the authors are not uncommon in untreated mice. Background pathology findings of mice. More of a statement, not a question.

Canady: Dr. Brand, do you have a response, to the statement about the low number of animals in the Talamini study?

Walter Brand: This is true, I guess. I don't know the study at this moment by heart, but it is not an OECD guidelines study, but yes, this is something which should be considered in interpreting the results.

Canady: Thank you very much. I greatly appreciate your attention to looking across different study platforms and also species and the adverse outcome pathways, that kind of integration is something that we really need with nanomaterials, again, because as we saw on the first two talks, the context, the environment, the dosing regimen, all affect how cells see these particles, and so we need to pay attention to the varying contexts.

I would like to queue up the next presentation, if we could. The next presentation is from Dr. Lynne Haber of the University of Cincinnati Risk Science Center. We asked her to give us both a prospective as a toxicologist looking across a wide range of studies, but also as someone who's trying to integrate the information in developing health-based guideline values, it is quite a challenge and I look forward to her practical insights as well as review of information that she has come across. Thank you very much. Dr. Haber?

Practical Application to Regulatory Toxicology: Issues Faced in Consideration of Developing Health Guideline Values
Lynne Haber, University of Cincinnati Risk Science Center, Cincinnati, OH

Lynne Haber: Thank you, Rick. Audio check, you can hear me fine?

Canady: Coming across fine, thanks.

Lynne Haber: Great, thank you. Thank you, Rick, for the introduction, and thank you to the organizing committee for the chance to share my thoughts with you. Next slide. So I have no conflicts, I realized that this was not explicit on the slide, but I do have a disclaimer that was provided by the Consumer Product Safety Commission, CPSC. I am an employee of the University of Cincinnati and not with the CPSC but the work I will be talking about is informed by the work that I have done as a

contractor for CPSC, on evaluating several nanomaterials. I am not actually presenting the results of any assessment, but I will be presenting the sorts of challenges that we face in doing those assessments. Next slide.

So, the objectives of my talk are to address real-world challenges associated with doing the nano assessments, to highlight some key questions, and present would looks like the proposal for thinking differently about nano research, and research in general, this is a bit different from my usual talks in that going beyond the science to make a broader appeal to this community. Next slide, please.

I'm not conflicted as you saw on the earlier slide, but I do have a specific perspective, and that perspective is informed my specific areas of specialization. That I am a risk assessor, and my values, click to show the rest of the slide. This is all a matter of finding a balance. So, we need the fundamental basics of science, and we also went to the research results to lead to understanding of the effects of nanomaterials in a way that can be used to protect public health, and I want to recognize the earlier talks in the session for illustrating how some of the basic research can be done to really illuminate any questions. Unfortunately, that's not always the case in this field. And as I will be discussing in the rest of the talk, I find it particularly frustrating to do risk assessment work for nanomaterials, the reason is for the well-studied nanomaterials, it's not that we lack data, thousands of publications investigating the toxicity of the nanomaterials, but we do like the data in many cases addressing the key risk assessment questions.

And I want to acknowledge that I'm not experimentalists, there are experimentalists in the audience that have done amazing work with nanomaterials and you know much more than I do about the practical difficulties of obtaining the data, and we've seen some of those practical issues in earlier talks and working with nanomaterials is hard. I want to acknowledge the many years of effort from those who have been in the nano field for longer than I have. I'm a relative newcomer in the field but I've been working this area for several years and I have been a risk assessor for many more years, and I hope that bringing a risk assessor perspective to the experimentalist can mean that together, we can help to move the field forward. Next slide.

So, there does need seem to be a need for developing health-based guidance values for nanomaterials. There are a number of regulatory programs that regulate nanomaterials in commerce, requiring labels for nanomaterial use, and some groups have banned nanomaterials in foods, and there are number of international and national programs that have been requiring or funding the development of dossiers for nanomaterial safety evaluation.

But despite all this effort, there are very few health-based guidance values for nanomaterial, some that have been developed by private groups and some in occupational areas, but none that we could find for oral exposure and what is the reason for that? Is it because there is no need, is it too difficult, or have we failed in developing the right data? Next slide.

The challenge is that there are thousands of publications on nanomaterials, there are large sums of money that have been spent on developing all of this research and

supporting this research, yet many fundamental questions often remain. And it is often hard to see the forest through the trees, to see the big picture of what the key determinants of toxicity are and help to develop a health-based guidance value, the earlier talks did a nice job of laying out the issues, and the rest of the slide, please.

An example of these sorts of issues that we may encounter, you may have a question as to weather a certain nanomaterial is a reproductive toxin. For one I looked at, we had good guideline compliant studies, 70 nanometer size commercial nanomaterial that were negative and several studies with 5-10 nanometer sized particles with the same general material that found adverse reproductive effects, but only evaluated selective endpoints. Part of the challenge is the studies with the 5-1o nm particles only evaluated those limited number of endpoints, so it's hard to evaluate the data in a risk assessment context where we look for consistency across related endpoints, and a dose response, and they might also look at early biomarkers without a clear demonstration of the progression to an adverse effect. And even if these studies are high-quality, there remains the question of how to explain the differences between the studies with the two different types of particles? Is it related to size, what if the toxicity of the five nanometer particles is a substantially higher than that of the 70 nanometer ones, even if we normalize sing a standard metric such as surface area? And there is a broader question of what is the relevance of the five nanometer particles to my overall goal of protecting people who are exposed to the nanomaterials from some product? And how should I integrate the data? Next slide.

And framing the overall question, I wanted to go to a fundamental that I imagine most all of you are aware of, of the framework for risk assessment that was developed by the National Academy of Sciences. And typically we focus on the middle, in phase 2 and stage 2, of the risk assessment paradigm, where we see hazard characterization, dose-response assessment, and exposure assessment which we integrate to the overall risk characterization, but for the purposes of this talk, I wanted to focus on the problem formulation, where we're saying that the reason that we are doing the risk assessment is because we have a specific question that we are trying to answer, and the development of the risk assessment should be informing risk management decisions. And so that ultimately, we need to be tying the risk assessments to a specific exposure. And at the end, as it shows in stage 3 in the phase 2 section, we need to confirm the utility of our assessment, making sure that the assessment is related to the actual exposure that occurred, that we're tying together the dose-response portion with the exposure.

The key questions that we need be looking at are, what is the appropriate dose metric? Given the variety of forms and manufacturers within a given nanomaterial, such as carbon nanotubes, titanium dioxide, etc. can we group related types, such as looking at, we know that toxicity is different for rigid versus tangled multiwalled carbon nanotubes, but is that broad grouping sufficient, or do we need to look separately and at different subtypes, and what are the key determinants of toxicity? We've heard about surface coatings and functionalization, which are important for the use of the nanomaterial, but we can expect that they also have impact on biological activity. And how do we connect to the toxicity and exposure measurements?

I do want to note that there are people who are doing some of the hard experiments to address these issues, some of that in earlier talks today, and I also want to give a shout out to a very careful study that was done by Kelly Frazier and colleagues that was published at the end of 2020 that looked at how fundamental material characteristics of carbon nanotubes and nanofibers affect the toxicity and other cellular markers, and they concluded that the distribution of physical dimensions is more important than the means to predicting toxicity, so another complication in terms of how we characterize.

And we've heard a chunk about different aspects of nanomaterial characterization, and I wanted to highlight the second bullet here, of the characterization as it was, the animals and toxicities study that were exposed, so characterizing the hydrodynamic size and poly dispersity in the index when in liquid form, and looking at the aerodynamic diameter, characterized by the mass median aerodynamic diameter and the geometric standard deviation, for aerosols. And think about the implication of commercial versus laboratory synthesized nanomaterials and the challenge of interpreting the relevance to human exposure of noncommercial nanomaterials.

This slide illustrates these sorts of challenges that we commonly face in using nanomaterial toxicity data for risk assessment. To give some perspective, when I'm assessing chemicals, I only have one to a few repeated dose toxicity studies, and the guidelines of the would use 10 animals per sex per dose where a chronic study would use 50 animals, if conducted according to test guidelines. A nano material that I evaluated, where we had 14 some subchronic or chronic oral toxicity studies, using up to 40 animals per sex per dose, and some use 30-40 animals, well above guidelines for the sex per dose. From this perspective, we had a huge data set compared to what I typically have for bulk chemicals, but very little of the data set was actually usable for risk assessment purposes, and what I did have raised as many questions as they answered.

Of these fourteen studies, nine did not have adequate nanomaterial characterization, we've heard about some of the issues related to characterization, the concentration more simply as particle size distribution, nanomaterial concentration or dose, and the purity of the nanomaterial's source, even then many of the publications lacked that. 13 of the 14 evaluated only a subset of the standard study design, some did evaluate endpoints such as body weight, organ weight, and histopathology, as part of a mechanistic evaluation, but often lacked the standard array to be able to look at a weight of evidence characterization by using related endpoints. Many were focused on mechanistic endpoints, and such data are useful, but I would like to have that information directed at addressing the key questions and first helping us to understand the biology sufficiently to address the key determinants of toxicity.

As a risk assessor I focus on mode of action and key events. We had a nice discussion on key events and adverse outcome pathways. The key point here, we need those high-level key events, to describing the pathogenic process, we don't need to know the interactions that occur in a molecular pathways. Part of what's challenging in this is that there is an issue of animal welfare in a large number of studies that were conducted, a large number of animals tested for a less than desired payoff, so even with this data set, there was testing with nanomaterials from five different manufacturers, eight of the studies tested a noncommercial material,

size range was 5-80 nanometers, so it was nice that there was a wide variety of materials tested, and that could have been used to help us identify what they key determinants are and how various variables affect toxicity, but we couldn't do that sort of assessment because the studies were done using so many different test protocols and approaches. A more integrated testing strategy could have used far fewer animals, consistent with the 3Rs for animal testing, and could have been much more useful from a risk assessment perspective, and also the issue that three of the studies tested only a single dose, so we had no dose-response information. Next slide.

With the data set like this, we have a few options for developing a health-based guidance value, but none of them are very appealing. We could use the most sensitive or conservative value, protecting from a worst-case scenario, but how do we ensure that the result is meaningful? We could throw out the poor-quality studies and use the most conservative, but then we need to identify criteria for inclusion and exclusion of studies. We could use a weight of evidence approach on studies meeting the minimum criteria, and we would then need to define the nanomaterial characteristics for which the calculated guidance value applies.

On the next slide, we're going to shift now from talking about problems and challenges with assessments to talking about some work that has been done to address the sorts of issues that I'm raising, and what I like to see more of. Nice work from the National Institute for Occupational Safety and Health where they are comparing the mass dose in service area as dose metrics, and the left-hand panel shows the dose-response for the particle mass dose in the lung, versus the incidence of polymorph a leukocyte counts in the lung, for lung inflammation, the graph on the left shows several different particular compounds, silicon dioxide, several studies of titanium dioxide it different sizes and barium sulfate, and you can see each study has a different curve, the right-hand side, using the particle surface area as the dose metric, we have everything but for silicon dioxide, falls on basically the same line, indicating that particle surface area is a much better dose metric than particle mass dose, and that silicon dioxide is different from the others as far as what causes toxicity and it is higher toxicity.

Because the studies were done investigating multiple sizes of the nanomaterial and the bulk material, NIOSH was able to identify the dose metric and this is shown in the next slide, which shows how they developed a recommended exposure limit, next slide please, for titanium dioxide. I don't know if I've got a delay. No back one, thank you. I did have a delay, thank you. So, using that information on the dose metric, being the particle surface area dose in the lungs, NIOSH started with the relationship between the particle surface area, and the response, they were able to calculate a tissue dose-based benchmark dose, and then they used the tissue dose base value to extrapolate from the rats to the humans, taking into account differences in the lung surface area, in humans, and then they use a human lung dosimetry model to determine what working lifetime exposure concentration would result in the equivalent tissue dose that would result in the adverse effects of the rat, from that they develop the recommended exposure limit. But they are able to take into account, the recommended exposure limit was indeed based on milligrams per meter cubed, but they also took into account the impact of surface area by

developing separate recommended exposure limits, or fine, versus ultrafine titanium dioxide.

So, this is my proposal in an ideal world. We would want to have coordinated consortia designing fit for purpose testing, using well-characterized test materials, standard and standardized test protocols, and systematically varying one parameter at a time. I recognize that is hard to vary one parameter at a time particularly with nanomaterials, often when you are varying one parameter, the synthesis methods mean that other parameters are also varying, but there are mathematical techniques such as the Frasier study that I mentioned, that are available to look at a range of nanomaterials with varying characteristics that have been tested together and using these mathematical techniques to determine which parameters predict toxicity. I realize that this consortia testing has been done, there are several examples shown in the slide, and this work has involved many of the people who are in the audience right now. And if you click twice to reveal the rest of the slide, but the problem is we still don't have the information available. So, was the challenge? I've been told a chunk of the challenges that it has been hard to publish the results of some of these studies, because publications with negative results are hard to get out into the peer-reviewed literature, so as a risk assessor, publications with negative results are as valuable as those with adverse effects if the testing is done according to guidelines and up to a limit dose.

There's also the challenge that often funding organizations are against doing a full dose response, they will be encouraging the experimentalist to cut down on the number of doses tested, and it’s important that funding organizations recognize the need to do the full dose-response evaluation in order to both have the information to look at the consistency and is there a dose-response, and to use the dose response information for the quantitative risk assessment. I do recognize that these latter 2 issues are not unique to nano, I have colleagues in bulk chemicals, also talking about study sessions encouraging them to cut back on the dose response, but this information really is critical for the risk assessment process. And I do also want to acknowledge that progress is being made on these issues, to at least some extent, but it is still a barrier to doing good science.

So, if you remember only one thing from this presentation, I would ask that it be this slide, I very much appreciate the opportunity to speak in this forum, and I recognize that many of the people listening to the colloquium are the movers and shakers in these fields, in the area of nano and toxicity, toxicology and risk assessment. You are the ones who make the funding decisions, who sit on study sections and reviewing papers, so I appeal to you to think about these issues that I'm raising here, as you make those decisions. And I will get off of my hobbyhorse and move on to from hazard and dose response issues to the exposure area.

The evolution shown here provided by NIOSH, reflects the changes that are associated with going from the new nanomaterial, to what people are exposed to, and some of the earlier talks, and what people are exposed to occupationally or the nanomaterial from a product. And these sorts of processes are also very important at concentrations relevant to animal studies and to occupational exposure, and people fight to de-aggregate and minimize the agglomeration of particles, but then the actual particle sizes even in high-quality studies are often on the order of several microns,

and that sort of exposure may be occurring in occupational exposures, where exposure might result from a nano material that's part of a formulation, and the worker may be exposed to that, from the nanomaterial, but for consumer products exposure, releases are much lower, so there’s a lower probability for agglomeration, which means that it is harder to measure in order to determine if there is aggregation and agglomeration. Next slide.

When thinking about the actual nature of exposure, we need to think about the sorts of products into which nanomaterials are incorporated, and there's a difference in the exposure potential from a situation such as nano-enabled fabrics, or say a tennis racket, in which a nanomaterial has been incorporated in the handle, versus a situation where it is in a closed compartment such as an engine, where the nanomaterial is incorporated into a component of the engine, either way you need to have weathering or abrasion, so that there is a release, and that release of material needs to get out into an area where the person can be exposed. And in evaluating what is actually released from the product, we need to consider the matrix also that has the embedded nanomaterial. So, some very good work has been done in this area as part of the nano release effort followed by a ISO committee in addressing the release, and as shown in the second bullet from the bottom, there is a release and result in a mixture in a variety of different combinations of the matrix and nano materials in different forms, and we need to consider what is being released, weather what the sizes, and if that is even too large to be inhaled. So, we need to bring all these considerations of the nature of the exposure and combination with our consideration of the toxicity, and make sure that we are relating the two appropriately.

When we have developed a health-based guidance value, we need to consider how broadly can be applied, how should the exposure be measured, what is actually being measured, considering different size categories, family size categories are needed, what sorts of forms we need to apply to it, and all of it relates to what are the key determinants of toxicity, what matters in determining toxicity and what doesn't. Next slide, please.

So, the slides shows recommendations and a wish list going forward, so we want a systematic evaluation of these key determinants of toxicity, there has been good work and we need additional evaluation of these variables, a good systematic work has been done, it helps us with the hazard part a lot, and there are additional issues in considering the relevance, taking into account kinetics, and we need to have testing done was standardized set of assays, comparing cross studies and the need for coordinated testing strategy. And also, do want to recognize the desire to be getting away from in vivo testing, and better use of in vivo data. And previous information on considering of groupings, a path going forward.

One approach would be to evaluate every nanomaterial separately but that would be very labor-intensive and expensive, so need some way of grouping them, perhaps. There has been work done on grouping and it has been easier for the characterization but translating this to an in vivo dose-response is still challenging. Next slide.

In summary, nanomaterial characterization is key in evaluating study quality and comparing studies, the development of the health-based guidance values need to consider the appropriate dose metrics, consistent testing is needed to identify the key determinants of toxicity, and having a characterization, that are less will developed. And next slide shows the references, and acknowledgments for those I worked with in the assessments, the nanomaterials for CPSC, I thank you for your attention and look forward to any questions.

Canady: Thank you very much, Dr. Haber. The chat screens have delivered quite a number of questions that are all going to be very interesting to have the panel address.

What I would like to do now if we could, are there any questions for clarification actually? For Dr. Haber, before we turn to the generalized questions? We've had some general questions which we will turn to, but any specific characterization questions would be useful to get at this point?

Roundtable Discussion
Moderator: Rick Canady, NeutralScience LC3, Camano Island, WA
All speakers
Timothy Duncan, US FDA, Bedford, IL
Agnes Oomen, RIVM, Bilthoven, The Netherlands

Seeing none, what I like to do now, is turn to the conclusion slide set if I could. And next slide. So, what we're doing now is removing to a panel discussion, all the speakers, the four that you just heard, I will participate and in addition we have two added experts, Dr. Timothy Duncan, and Dr. Agnes Oomen. We will look at the questions developed by Dr. Oomen, and Dr. Duncan. If it's possible to put those up. Can you put the introductory slide set up and move to that slide within the introductory?

Colloquium Staff: Do you want me to go back to presentation number 1?

Canady: Yes, thank you, if you could put that back up. So, Dr. Oomen, I wonder if you could choose from the questions here just to start the discussion, and all the presenters, and the two added panelists, please turn your mics live, everybody else don't go live, if you have questions, please put them in the chat screens and we will collate them and feed them into the discussion. So, Dr. Oomen, please take the stand.

Agnes Oomen: Thank you. First, I would like to take this opportunity to thank the organizers for this very well organizes colloquium, and also the presenters for their great talks. This topic I think has been spot on with the issues like the challenges related to the characterization, and also related to how the nanomaterials are administered, whether in food, via drinking water, or gavage, and if this affects the local or systemic effects. But also, to what extent the administration affects the absorption or cellular uptake, and how the relationship between the degree of agglomeration and absorption is always very important, not just for one. So, in risk assessment, we have to deal with the situation, and like a realistic or worst-case

situation, it introduces a lot of uncertainty, and potentially a more conservative risk assessment than necessary, tempering the innovation potential, of nanomaterials and the benefits that we have from them, and also in other situations, the risk assessment can be wrong or inaccurate.

So, I think it is important to look at these fundamental questions that have been raised during this colloquium, I would like to those that work on these issues, but also with the people involved in this community, so yes, as a general appeal, thinking really about these fundamental questions, and which questions are important for risk assessment, and how these be assessed is really something that we should take into account.

And looking also at the question now on screen, I have another slightly more alluring question to start with for the presenters today. What if you had a considerable budget, not limitless, to spend on research that benefits the risk assessment of nanomaterials, where would you start and what would be the priority for you?

Canady: Wow, Anil, I wonder if I can turn to you to start the discussion, because you have directed large programs and developing evaluations. What if you have a large budget, not unlimited but large, where would you start?

Anil Patri: So, I'm thinking about this, it is quite challenging because nanomaterials are not single species, if I talk about liposomes, I can provide an answer, they are not applicable as someone posed a question about carbon nanotubes. And so, you know, if we have in the limited budget, which we don't, I would think that the appropriate exposure scenario has to be used, in other words if I make it about this first nanomaterial was certain coating, it doesn't have any influence on the data that I may get from that may not be really useful for real-world exposure scenarios. At the same time the material is used for like a sunscreen, for dermal, or it is not applicable to the data that you would get from an in vivo oral administration or IV administration. So, if I had the money, I would look at the possibility of mimicking real-world situations and concentrations, because some studies are presented at the SOT, for many years, that the doses that are used for toxicity measurements are immense, large, that we might not really see in human exposure. That is my take on that, thank you.

Canady: Yes, please.

Oomen: Thank you for your answer, Anil. Of course, in risk assessment, it's important to look the realistic worst case, the exposure conditions is important, but you also want to cover all relevant scenarios. How would you approach that? Looking also at the screen, do you think it’s important to look really at those well dispersed situations, in toxicity testing for this worst-case exposure situation as well, or do we need to be investigating other realistic situations that there are?

Patri: Maybe I will turn now to other colleagues, who can talk more about the exposure, because I'm not an exposure scientist and I don't want to misspeak or misrepresent, but as I mentioned, when we say nanomaterials, the question may be specific in someone's mind, maybe titanium oxide, and the carbon nanotube. And they are not applicable to the liposomal materials, so I would maybe ask other

colleagues to chime in on the exposure situation. But I would really like to say real-world situations, because for us at FDA, if you look at toxicological studies, they can be confusing. And we want to make sure that if nanomaterials are used in devices in food, or in drug products, then the assessment is properly considering the safety of the doses and things like that. Thank you.

Canady: I wonder if I could turn to Dr. Sayes, given that exposure is the focus that we seem to be turning to at this point, first understanding exposure than what we can understand with the toxicology, as Anil has pointed out, coming from the well characterized drug or diagnostic environment, to the Wild West of what gets out there, it is a challenge as you proposed some approaches and others did as well, I wonder if you could give your thoughts.

Christie M. Sayes: Yes, thank you, Rick. Of course, it is a challenge, and nobody has unlimited budget but taking together would all 4 the speakers said today, I think it's very important to design studies that can be as useful as possible for decision-making. There are different types of decision-making of course, for instance if I put on environmental toxicology hat, then I would want to do as Tara suggested in the chat window, which is test what we are really being exposed to. For instance, if we are not being exposed to well-dispersed nanomaterials, then maybe we ought not to do our studies and well-dispersed nanomaterials. However, if I put on my molecular toxicology hat, we need to do the well-dispersed nanomaterial studies so we can do the analysis and answer the question of why. So, this is kind of why I've been using this approach with my studies in my lab with my trainees, which is a systematic approach to be able to first do dose range finding studies of the actual entity for which we are being exposed to, which is often a mixture of sorts. And after that dose range finding, then we can narrow into a specific range, in that dose, where we can have more incremental exposures to be able to help us with some molecular bio signatures that are going on. Again, of the mixture, what we are being exposed to.

In parallel to that, and this is where budgets get to be pretty crazy, as Joanne and I often talk about, which is I would want to include the individual constituents, individually in the study design as well as the larger mixture, which we are exposed to, so that being said, if there was unlimited amount of resources, that's how I would do it, I would do a larger dose response, or a larger dose range finding study to understand which particular doses I want to focus in on for the molecular side. I would still want to do what we are exposed to, which may not be well-dispersed nanomaterials, but I want to include them, so I get individual predictors and determinants to be able to help us get to the prediction and the supervised machine learning that we have to get to eventually with the plethora of scenarios that were talking about.

Canady: I would like to move to Timothy Duncan's questions in a second, if we could, but I wonder, Dr. Brand, you provided an example of a 2-log difference between gavage and feed exposure, do you have any insight into whether specific dosing regimens are more useful in organizing our thinking and approaching these based on your evaluations? Walter?

Walter Brand: Yes, here I am, I have some problems unmuting myself. I think in principle, I agree with the other speakers that if you want to do a realistic or a worst-

case risk assessment, you need to have more knowledge about what happens in the intestinal tracts, but it is even more complicated, I think, because if you related to foods, and also maybe requesting whether it is important to have a well-dispersed exposure, it can be for food additive for instance, it is like titanium, titanium dioxide is a food additive, it is different if it is in a cake or in a muffin for instance, than chewing gum, for instance, because chewing gum, it is maybe an exposure over a longer time via saliva, so it is also a matter of exposure via food is taking place. So, it is also a complicating factor that in risk assessments, I think you need to know what can happen at these different circumstances. So, yes.

Canady: Thank you, so I wonder, Dr.—I'm sorry, go ahead.

Lynne Haber: This is Lynne Haber. I just wanted to add.

Canady: Exactly, that's who I was going to turn to.

Haber: Thank you. I think the previous three speakers have really addressed many of the key areas and I would agree with where to focus of the research, it is equally important though to also talk about what is not useful, and one of those areas that unless the exposure is relevant for some drug design, then what would raise questions about the non-physiological exposure routes such as subcutaneous and intravenous, etc., again, if it's relevant to a drug design, including that information is needed, but we should not be doing the studies just because they are easier to do, than the physiological exposures, and the in vitro data can certainly helping to initially address important questions, and helping us to focus the in vivo studies.

Canady: Thank you very much. From my perspective, as you pointed out, Dr. Haber, the current literature database is full of lots of studies, apparently for lots of funding, that develop information that is not fit for purpose for risk management. And the question becomes weather we have a different paradigm need for nanomaterials where we need to focus, more funding, and risk management, than we do at this point for supporting basic research. I think that's a question, I'm not saying it is something that I propose but it is a question.

Dr. Duncan, I wonder if you could respond to Dr. Oomen's question and transition to questions that you have, if that is possible?

Timothy Duncan: Hi, can you hear me?

Canady: Yes, we can.

Duncan: You want me to first respond to the question that was posed, about that, could you repeat?

Canady: If I could write you a huge check, what would you focus that, and where would you focus in order to address the translation from real-world exposures and product exposures to toxicology databases or vice versa?

Duncan: From my perspective, I would want funds, let me back up, my area of interest is in food packaging and potential exposure routes for nanomaterials that

would be incorporated into those. So, from my perspective, having more funding for real materials, and I think there's a lot of data out there on a lot of different things, whether or not they are really relevant to practical materials coming on the market, and I think that is an open question, and I think you mentioned, fit for purpose, if we are getting data on materials that either are not relevant to practical commercial products, or materials that are in isolation of the broader environment, I think that date is not as useful. So, being able to anticipate what practical consumer products are going to be, and what environments are going to be used in. And it's going to be impossible to do every one of those permutations, and it takes money, the more you want to do, the more money is going to take, I think if I were given a blank check, I would start to look at more practically relevant scenarios rather than some of these more abstract situations.

Canady: Thank you very much. So, I wonder if you can frame or pose questions to the panel from your perspective.

Duncan: Yes, so I do want to think you and the organizers of the symposium, I think it has been really interesting, and it's amazing listening to these talks, how broad this area is, and that's probably one of the reasons why it's so challenging, even without the nano element. And the safety assessment involves so many different areas from physical measurements, toxicological considerations, exposure considerations, and the broader risk evaluation. The organizers have done a really excellent job at bringing speakers to address these different areas, particularly the challenges that nano brings to each of these parts of the broader problem.

I'd like to ask a two-part question, and this is going to be embodied in questions four and five in the list that you sent out, I'd like to provide just a little context as to why created these questions. One of the things I've been interested in for a while is how nanotechnology influences the transmission of data between exposure scientists and toxicologists to ensure that safety standards are met, and a lot of times we think of these as two different disciplines, they are little bit, but they need to be well integrated, and communication needs to be really efficient, to do quality safety assessments. And nanotechnology introducing challenges into the communication pathway.

The presentations today have shown that nano introduces significant uncertainties, both the definitions of key terms critical towards evaluation both exposure, and biological effects, things like definitions of dosage and identities, and also introduces unique challenges to measurements, which we've heard about, certainly in the first talk. And significant strides in identifying what the challenges are in very broad terms, one thing where we've made less progress is in translating this knowledge into a practical operation framework, with exposure and toxicological assessment, for comprehensive safety evaluation of real products, coming from FDA, having to evaluate real products, submitted by manufacturers and real data. And making recommendations to manufacturers about what the appropriate date is, and what data do manufacturers need to provide and what is the appropriate way to measure them, FDA makes recommendations like this, in guidance and informal recommendations. How do we translate all the technical and situational information, on toxicology and exposure assessment, and it has been presented today, how do we turn it into practical assessment guidelines? It's an area I'm really interested in,

and I haven't seen a whole lot of progress in that area. At least not as much as we need.

With that preamble, in terms of the hypothetical scenario where a manufacturer wants used a new food ingredient, either non-nano or nano, to evaluate the risk of such a substance and acceptable daily intake or no effect level, which would be the toxicological assessment might be compared to an estimated daily intake or exposure assessment. So, to each of the panelists, in your view, what are the key challenges that nano introduces to comparison of acceptable intake estimates to measured intake assessments? That is part one and part two, what recommendations would you give for standardizing operational procedures to determine and record related nanotoxicology and nano-exposure data? Basically what are the key challenges here, high-level challenges, and how would you recommend that operational guidelines be formulated to do with those challenges? Thank you.

Canady: Let's start in reverse order from the way we answered the previous set of questions and start with Dr. Haber. Lynne, how would you respond to Dr. Duncan's questions?

Haber: I would suggest that a key challenge is connecting the form of exposure in the toxicity study to the form that people are actually exposed to. We've heard a lot about the impact of different formulations, and even if the, both are incorporated in food, there is a whole difference in the animal feed formulation, and how, or a potential difference, versus how nanomaterials would be in human food. And I'm hearing a lot of background noise. Thank you. So, that would be a key challenge that would need to be addressed, as I have noted, I'm not an experimentalist, so I'm not sure exactly the best way to address this, but would want the formulation to be as comparable to the human exposure as possible taking into the issues of application and agglomeration, and related to that, the issue of how do we measure dose in a way that can be meaningfully translated, both to toxicity considerations and to the risk management scenario and I will stop there.

Canady: I wonder if I could jump in, so Tim, the baseline question for me is how do we know what the exposure is to. And the presentation that Walter Brand gave, saw a tremendous difference in apparent dose-response between two different delivery methods. And in my mind, that raises the question of whether we can actually deliver a high dose in a sort of typical maximum tolerated dose or high dose to low dose extrapolation approach that we typically take for materials. So, the baseline question for me is how do we understand what the exposure is from a food or a packaging situation? And I don't know how to answer that question, because extracting it changes it. So, I just wanted to throw that wrench in the discussion a little bit, to me it comes down to formulating the problem and even in developing that problem we have trouble understanding how to deliver dose in ways that we usually deliver dose to understand low dose effects. Anyway, not really response but I wonder if I could turn to Dr. Brand, do you have a response to Tim's question about the relationship between that and dose-response with the primary issues to address?

Brand: Yes, I think also in addition to the things that you just said, I think that also in the exposures, in toxicity studies, and realistic dose exposures should at least be a part of the exposure range. That is important.

Canady: Agreed. Absolutely. So, finding the correct exposure range is a primary issue. Why don't we turn to Christie at this point? Given your presentation with regarding to grouping and mixtures analysis, does that provide a fresh look to Tim's question?

Sayes: Yes, I think it provides a fresh look and in fact I think it was Vladimir that mentioned in the comments, we also should be thinking not only about molar ratios of mixtures or potent rations, but also because surface area is so important in nanomaterials, we ought to think about equal surface area doses as well, when it comes to mixtures. I do want to say a comment to your comment, Rick, which is, can I say that if after 10, 15 years of this, if it's still very difficult and we're not happy with measuring the actual intake, what is taken up either into the gut or into the cells or whatever, can't we start using the actual exposure concentrations in some way to help with risk science and decision-making? I understand that that's not the traditional way of doing it and certainly the way that we have policies or guidance documents doesn't necessarily allow for that as acceptable exposure limits and time weighted averages, but perhaps it's time for us to start thinking about making decisions simply based on what we can measure, which is that exposure dose concentration.

Canady: Yeah, fascinated to hear responses to that. Obviously, we've tried that in the past, so somebody has their speaker on. So, the mega mouse experiment, where we tried to actually measure dose response at measures of exposure anticipated some products or environmental exposures. Took an inordinate number of animals to get to a pace where we could identify relevant risk evaluation levels. So, I think an approach like that probably is not practical, but I think what I'm reminded of when you raised your question was Lynne's comment when she said, what information is not useful? Start removing the information at, for example, high dose agglomerated exposures that don't provide information for realistic exposures, we would at least simplify some of the analyses that Dr. Haber needs to do in order to identify health-based guidance values.

Sayes: I just want to answer Lynne's question and then she can respond. Hopefully it's not too provocative. What I'm trying to say is, as an experimentalist in the lab, I know exactly what the concentration for which I'm giving my cells, my zebrafish, or my rodents. I know that dose. I am less accurate and more complicated for me to measure the actual dose that is internalized intake. The intake concentration. Therefore, if I am confident for what the dose is, or the concentration is at the time of exposure, perhaps we can have some studies or some projects that work with what I am confident with and accurately can measure and use this in some way to make decisions.

Haber: So, this is Lynne, and the reason I asked the question is, I'm still trying to figure out if we're speaking the same language, because the vast majority of studies that I see are expressed exactly as you're asking. So, inhalation studies are expressed as per meter cubed in air. Oral studies are expressed as milligrams per

kg per day. And that's where we start. And when we have in the rare circumstance that we have good information to address internal dose, we can use it, as you saw with the NIOSH example. And certainly, we can have some pretty good estimates of the deposition in the respiratory tract of different particle sizes, and particle size distributions. But the way we normally do risk assessment is the way you're suggesting, unless I'm not understanding what you are suggesting.

Sayes: I guess I'm picking up on what you just said. You just mentioned that, yes, normally the exposure concentration is reported, and you said that is a good starting point for us to determine other types of metrics. And then, you refer to as the other types of metrics as the actual good data to then go into the framework. I guess I'm just, again, trying to be provocative to get some more conversation, but can't the exposure concentrations for which we can measure, can those not be classified as good data to go into the frameworks?

Oomen: And can I jump in as well for a moment?

Canady: I think we would all like to, but please go ahead.

Oomen: I see two things. I think we are already working on nanomaterials for over 15 years, and we are still struggling. With the fundamental questions. So, I think it's, for all of us who are scientists, regulators, is key to really get things straight. And get to the research, and the questions that are really relevant. Because there is no, people want to have answers at the certain point. So, this becomes some kind of an urgency. But I also see that it is complicated, and there are a lot of questions that we still need to address to go forward. For example, with the real-world concentrations, and exposure concentrations, it is important to include those exposure doses as well with exposure studies. But also, look at the external concentration, and look at the high dose. Because we do need high doses for finding this health-based guidance values. Or to proceed them. But to understand what is happening this includes information on the characterization and internal dose, otherwise, questions remain. And we cannot really draw conclusions.

Canady: Yeah. I wonder if I can jump in again. I think what we are talking about, or circling around, is changes to policy about how we address findings that show no adverse effect. For materials that we believe, can ascertain to be, realistically exposed in the way that people would be exposed to them. It's a different policy approach, and perhaps, it leads to larger numbers of test subjects. Or larger numbers of test wells, or whatever the appropriate factoring is. In order to be able to see low-frequency events that we typically regulate at. It's a complicated question, but what it refers to is that we've seen in several of the presentations that as you increase concentration, you paradoxically change dose. And I just wonder if there's a way to address this in policy, or a way to address this in study design that we haven't yet explored. Because again, as Lynne pointed out, there are a lot of studies out there in the literature, that just are not useful in informing dose response. And others have pointed out as well. So, that wasn't really a question. I wonder if we could turn to the question. I'm sorry. So, Anil, do you have a response to the Tim's questions?

Patri: Yeah. Maybe briefly. For intentional exposures, meaning for intentionally engineered nanomaterials for drugs, for therapeutics, we have ways of measuring.

But we already know the way that the exposures should occur, even preclinical studies and clinical studies, and is not a problem. Even though we are struggling with a few things with how it showed how the measurements and poly dispersity and services were change in the potential toxicities. But overall, I would say we have more control over those kinds of studies than unintentional exposures. Whether consumer products or in other types of exposure scenarios. And so, if I get the dose, the unintentional exposure are not those that require preclinical data such as drug products. Then there is the question that, again, came up about the surface area as a metric. Or using the surface area for dosimetry. And I agree with Lynne that for inhalation exposure, that may be an appropriate scenario. But I don't know of any studies, or at least most studies using milligrams per milliliter, or things like that to do the in vitro or in vivo studies. And they don't usually measure the surface area, because the only way that you can measure that is with the BET. And the other way to model it and then the kind of come up with the surface area of nanomaterials. And that is still a challenge with the conglomerates, and poly dispersity and they bring additional challenges. But again, I would distinguish between intentional exposures and those that we don't know of. And still, very complex when it comes to three-dimensional material, whether it is carbon nanotubes, or graphene, those kinds of materials have different challenges. Thank you.

Sayes: Rick, if you are trying to talk, you're on mute. But while there is a silence, I would like to address question number five here. Which is, recommendations for standardization. Personally, I really value standard operating procedures that are published by OECD or ASTM, ISO, things like this. But I also really cherish the papers that are posted in the literature about method development and are very clear about the operating procedure that is used, in particular, dispersion of nanoparticles. Like we brought up a couple times, is very different than each particle type you are talking about. But personally, I really cherish those. And frankly, I would like to see more efforts, or at least easier paths, maybe, for scientists that are doing these standard operating procedures to be able to make them publicly available, and maybe be able to cite SOPs in our literature so that we can help with the some of the standardization of laboratory methods that we are doing every day.

Canady: Thank you, Christie, I could not agree more. In fact, moving more toward standardization for nanomaterials seems to be a path that is indicated by the differences between nanomaterials and traditional substances. Chemicals. I wonder if we could move to some of the questions from the chat screen for now. Unless anyone from the panel, please raise your hand or just jump in, would like to add to the current discussion? Walter?

Patri: Sorry, go ahead, Walter. I will jump in later. Thank you.

Brand: Okay. There was one thing that I would like to add here. Because we talked a lot about dose. But actually, we talked a lot about the amount. Or concentration. Because, also, exposure time is also a component of dose, of course. And I think this is also important in toxicity studies and standardization of risk assessment procedures. Because for a lot of nanotoxic-specific issues, you can wonder if the exposure times, which are used in studies, are well enough in representing differences, the lifelong exposure in humans. So, we should also talk about exposure

times, not only about exposure concentrations, I think. That's another point to take into account.

Canady: Yeah, absolutely, I agree. And again, it brings us back to low-dose exposures, and timing of low-dose exposures. Over a lifetime, that we model using approaches for traditional substances. But it's not clear to me that we have addressed that low-dose exposure, long-term exposure issue. Or extrapolation, or for nanomaterials quite yet. Anil, you had a comment? Or response?

Patri: Yeah. One comment I wanted to make based on the prior discussions. It's about not really having infinite funding for either of these studies. We break down to nanomaterials and may be different sizes. So, if you have to do studies on each of those materials, to conduct in vivo studies, and proper exposure scenarios. This is a challenge. It's a significant challenge, right? Early on, I guess it's been more than 15 years now, we started these databases to capture the data. So, you need have all the characterization data, and then, all the in vitro data and in vivo data. And then you can come up with a way to bring about common scenarios, and common themes as to how the surfaces, compositions, and other parameters influence biological behavior, safety, toxicity, and efficacy. There are many databases now both here in the U.S. and in Europe. But none of them are at the stage where they are user-friendly for an exposure scientist to go through. Or a toxicologist to go through and come with scenarios. I think that's one area. We have so many studies, thousands of publications. But we have to get to the point. We cannot keep doing the same thing, the same mistakes over and over again indefinitely. So, have to get to the point sooner than later.

Canady: Thanks very much. I wonder if we could turn to questions from the chat screen. So, early in the chat, we had a question regarding dose. Actually, two questions regarding dose. The first was whether systemic absorption is something that we are focusing too narrowly on. If nanoparticles support local effects, e.g., on the intestinal mucosa or induce dysbiosis, which can have important systemic effects, is systemic absorption relevant? And this is a question that I guess was stimulated by Dr. Brand's presentation. It's more of a question of what we should consider, rather than what we are not considering. Dr. Brand, do you have a response? Should we be focusing on alternate exposure conditions, particularly in gut. Rather than focusing on systemic absorption, as much as we are? I'm sorry, Dr. Brand, did you hear my question?

Brand: Yes. Yes, I've had some problems unmuting again. It's depending on the endpoint, of course, which you will be looking. Of course, for toxicological endpoints, which are the reasons of the systemic exposure, and absorption is important. But I don't agree that for circumstance of nanomaterials, also direct effects on the gut wall could lead to some adverse effects, at least for titanium dioxide. I know that it has been related to things like Crohn's disease. If you want to study that, I think it's also important to study that. And in that case, yes. Absorption plays a factor, of course. But I see that as a different endpoint.

Canady: Yeah, absolutely. Absolutely. It's a matter of database coverage rather than ignoring a particular endpoint. A similar question came in. Actually, this is more of an extrapolation question. And that is regarding protein corona. If you are using, I will

read the question. In vitro protein corona associate, for examples, with silver nanoparticles, is different if you are using human serum versus rat serum versus fetal serum. What are the implications, and maybe, Lynne, you could respond to this if you could? Because your needing to choose among studies in developing health-based guideline values. What are the implications if you're using animal data extrapolated to human data, particularly for systemically absorbed doses that have corona formulation? Do you have a response? Lynne?

Haber: Yeah. Yeah, that's an excellent question. And I guess my answer is that this is something that is important to have an understanding of the implications of. And this is where, I guess this gets back to Christie's question of, can we use the external exposure? And it's these sorts of issues where having an understanding of an internal dose metric, and differences between animals and humans, could be useful. That said, I haven't seen studies that really quantitatively address these issues. So, all I can say is, yeah, it's an issue that we need to consider. Really good question.

Patri: Can I jump in? Very quickly?

Canady: Yes, please.

Patri: So, my colleague at NCL has done some studies early on, Marina, about the protein corona incubating gold particles with human plasma. So, the challenge we encountered, this was, I think, more than two years. With 2D gel electrophoresis mass spectrometry, with actual human samples. Just isolated. We had at NCL access to fresh human blood. And that challenge was that the data for each time you do this incubation is not consistent. You have to see some proteins that are consistent, but then depending on the different patients, you see different types of proteins. And depending on, if you do the same subject, depending on a different day, depending on what they eat or their stress level, the protein corona compilation is different. From that study, I pointed to one of the publications that came out of the study. The challenge is to use that in vitro nanoparticle incubation protein data sets to do anything more predictive other than, okay, that is a protein binding. You could always say that is less protein binding, more protein binding quantitatively, and that has more implications in the audio system uptake, reticular endothelial system uptake and biodistribution. But beyond that, to get into anything more predictive, or from the data that is, in my opinion, is a significant stretch. Because when we think of a cartoonish representation of a nanoparticle, we have a protein binding, depending on what the initial proteins are bound to the particles, the secondary and tertiary protein bonding may be different. It is very difficult and complex to model that. That is the conclusion in the paper, and she has additional papers on that. But there is a lot of work on this protein corona issue, but one has to be very careful in using that as a tool.

Canady: Any other responses from the panelists?

Duncan: Hi, Rick, this is Tim Duncan. I think since we're interested in food here, it is worth pointing out that one of the real challenges with foods is that unlike, maybe, every sample has its own challenges. But the diversity of substances found in foods is really enormous. Foods obviously have proteins in them, all different types of proteins. But also, other substrates and biopolymers and small molecules that can

bind to the surface of particles and is becomes a really dynamic system. I think any sort of evaluation, these things can't be done generally. They have to be targeted to the specific use. And what matrix they will be in. Because it can really change the exposure endpoint, and also, most likely, the toxicological endpoint. And it also scales up the quantity of experiments that need to be done to get a sense of the exposure toxicology. When you talk about resources for food, particularly, I think the resource costs can escalate quite substantially, and also, the complexity of the evaluation, compared to other types of consumer products. Thank you.

Canady: Yeah. Other responses? So, this brings up a question. Agnes, do you have a response?

Oomen: Yeah. Just general. Perhaps you are getting a bit closer to understanding what variables are affecting the key processes. For example, the protein corona, and the degree of agglomeration and the matrix affecting the absorption, or the cellular uptake. But we don't understand yet to which magnitudes. So, we don't know which are the most important variables to be taken into account. But this is important, because in the end, we want to do some studies, either in vitro or in vivo, to cover those. And we cannot study all conditions possible. Food is too diverse. So, we want to go to some kind of realistic worst-case situation. To be used, and to also be indicated in guidances. How to do studies for risk assessment. So, we really need to focus on finding those variables that address the key processes, and getting insight on the impact, and what are really the most important ones to get to these realistic worst-case situations that can be covered by toxicity studies.

Canady: Yeah. If I could expand on that, that's getting back to problem formulation. I mean, Agnes, if I could interpret what you said, you're saying that we understand more than we did 10, 15 years ago. And so, we should be able to focus resources towards focusing risk management decisions a little more directly.

Oomen: Yeah.

Canady: And it becomes, fit to purpose, again to use that phrase. One of the questions, for example, I asked, if you're doing physical-chemical characterization for nonspherical carbon-based nanomaterials, you get a different set of priorities, and a different set of dosing and characterization challenges than you would for the spherical, or the metallic nanoparticles. So, prioritization comes down to what you are actually trying to address in the real-world context. Problem formulation becomes your primary task, as it always should be. I don't know. Are there insights with regard to standardized tests? Assays? Ways of approaching grouping or mixtures evaluation again that help us get to this sort of narrowed problem formulation? Anybody have any thoughts on that?

Duncan: Hey, Rick, this is Tim. I was really intrigued by this idea that Christie had brought up about mixtures toxicology. I think this is a really fascinating concept. I would like to see that incorporated somehow into standard operating procedures. But I don't know how you would do it. Maybe she has a comment on that. But I also wanted to ask, one of the things I thought about when she brought this concept up in her talk was that she presented this plot where, I guess, she had two or three component mixture. I think the difference between a mixture of, say, conventional

chemicals, if you want to call them that, with nanomaterials is that for conventional materials, your mixture is usually discrete. Whereas in nanomaterials, if your mixture, if your distribution of sizes, if you're considering each size particle as a different substance. Your mixtures are particles of different sizes. You have a continuous distribution. Your mixture is a continuous distribution there, versus a discrete distribution of a mixture. That becomes, in my mind, much more complicated situation, because you can't test every substance in the mixture. The best you could do is somehow try to replicate the size distribution. So, I wondered how, from a practical standpoint, I don't know much about the mixtures toxicology. Not being a toxicologist. But how, from a practical standpoint, do you adopt this mixtures approach to more continuous distribution that is characteristic of nanomaterials from a more discreet distribution of more conventional chemicals?

Canady: Before Christie answers, I want to throw two more things in there to confuse everyone more. I don't anyone can dose a nanomaterial that is not a mixture. Pretty much any nanomaterial will be mixture of sizes, shapes, coatings, and so on. And the second wrench is that the mixtures are dynamic, not just continual as you presented, Tim. But also dynamic in the sense that mixtures change based on concentration and external conditions. Anyway, turning it back over to Christie.

Sayes: Yeah, thank you for throwing a monkey wrench into my original answer. Now my original answer is not as impactful, because it doesn't account for either one of those things. But if I were to pretend you didn't say that, then what I would say to Tim is that the good news is, we're not having to invent something new. Because we can look at environmental toxicology literature that looks at persistent organic pollutants and environmental matrices, and often those pops are a mixture in the environment for eco-toxicology. Similarly, for the pharmacologists in the audience, they know that isobologram analysis is typically used in drug development. Specifically, to look for drug interferences, and things like this. What I would say, and I hate to be the person to bring up machine learning, but what I would like to do is establish some type of way where we start at the beginning with what an experimentalist can handle. Which is two different entities in a mixture, and build that out to three, four, five, six, as many as we can handle. And that data becomes training data to help us get to constituent number N. We can do this mathematically. I think it will be the easiest way to move forward. In the slide deck, I have the reference for the concentration addition models that are used. And frankly, it doesn't have to be just two or three, like we do in our papers. You can have up to N different constituents in your model, and it can handle that. You'll have a lot more error. The more constituents you have. But it's a good way for us to really be able to bridge this into, let's have a continual example, where we can get some data in the laboratory in a very controlled way, and be able to use it for predictive models when we get constituents to the value. I don't know if that answers your question, but this is an opportunity where we kind of go outside of nanotoxicology and enter the rest of toxicology. Because this is something that needs to be done, and currently, people are doing it. But for other types of toxicological situations.

Rick, I don't know how to address the whole dynamic part of that. Obviously, a lot of what we're talking about our study designs in instant time or moments of instance. And it's assuming static conditions. And we are trying to bridge that out to real world,

where the real world is dynamic. So, that's a separate research question. I still think it's doable, but perhaps the mixture that case is just one constituent, and how it changes or transforms over time, or over condition. And perhaps, there's training data that can be collected there, as well.

Canady: Yeah. There's other mathematical approaches that can deal with that sort of dynamic situation. We would probably have to turn to Taylor, or other kind of series. Anyway, we can address that with other approaches. You are proposing a simpler model that is static and addressing time and place and dosing can be added to those models at a later time. I wonder, does anyone else have a response to that? I think the approach that Christie is proposing is something that should be considered. Does anyone else on the panel have a response? Or thought to throw in? Just turn your mic off, and I will come to you.

Patri: Yeah, this is Anil again, Rick. We just had an SOT session a few weeks ago on the mixtures toxicology. And so, there's a bunch of discussions. I will use this as an example. And so, I'd say, maybe just going through that. Those of you who are interested in the mixtures toxicology. And they had to be real-world situations. You and easy throw in gold nanoparticles or TiO2, for example, because you can detect them. But what is the real situation? Real-world scenario of that exposure happening? Any studies, as I initially mentioned early on, have to be something that is related to real-world exposure, so that any data coming out of that is most useful.

Haber: This is Lynne.

Canady: Lynne?

Haber: Thanks. So, I like Christie's approach. I'm definitely intrigued by going to the N in the mixture. But just another monkey wrench for you, Christie. In thinking about how to approach this, I'm thinking about the publication that I mentioned, by Frasier and colleagues. Where they said that the mean of the distribution that is inherent to any nanoexposure. Specifically, they were about carbon nanotubes. But the mean is not the best predictor of toxicity but rather the high end of the distributions. That's another challenge, shall I say, in that mixtures approach.

Canady: So, we just have a minute left. But I wanted to throw one quick, final thought out there. It came from a couple of questions saying, basically, when do we start disconnecting from bench science? How do we do that? I think we have outlined through this discussion, and through various approaches in the discussion afterwards, that we need to consider when to throw out the studies. And when to better focus studies. One of the issues, for example, is artificial dispersion. To what degree is dispersion of nanomaterials, rather than letting them agglomerate as they would, at reasonable concentrations, when should we throw the information out? Or what kinds of information should we throw out, as Lynne alluded to? Anyway, we don't have time to really address that question. I'm sorry I threw it out there. At the end. Does anyone have a quick response?

Oomen: In my view, with the current information we have, this might be the worst-case situation with artificial well-dispersed exposure conditions. And if we don't know exactly what we have otherwise, this is, for now, the best situation for risk

assessment to go to. In the meantime, we should look at how we can address those fundamental questions. For the present situation, with well-dispersed titanium that we are exposed to, but we really don't know. So, take the worst-case approach there. That's my view.

Canady: I'm afraid we have to leave it there. Thanks very much for that final comment. So, Jason, Betty, are we having someone present final slides?

Jia-Sheng Wang: Yes. This is Jia-Sheng Wang from the University of Georgia. I'm pleased to serve on the SOT FDA Colloquia Organizing Committee. I'll take this opportunity to really thank.

Betty Eidemiller: Did we lose the audio for Dr. Wong?

Colloquium Staff: It looks like he's muted. Give us one second. We'll unmute him.

Wang: Okay, you can hear me?

Colloquium Staff: Yes, we can hear you now.

Wang: Okay. Okay, I don't want to repeat. Just thanks to the presenters, and special thanks to Dr. Canady for chairing and organizing the wonderful team here, but also the SOT and FDA staff to make this available to deliver internationally. So, finally, I want to remind all attendees to complete the survey for this colloquium. So, thank you all, specifically, thanks to the liaisons for that. So, pay attention: we have other topics coming for this year in December or next April. So, just keep watching the announcement. Thank you very much. This is where we close for this session, colloquium.

Canady: Thank you very much.

Eidemiller: Just a reminder, all the recordings are available on the SOT web page. It will take us about two weeks to get this one up. But it sounds like from the active discussion, some of you may want to come back to the slides and recording, and we hope you will do that.

Canady: Thanks very much everyone. Goodbye.

Wang: Okay. Bye. Thanks.

Oomen: Thank you, all. Bye.