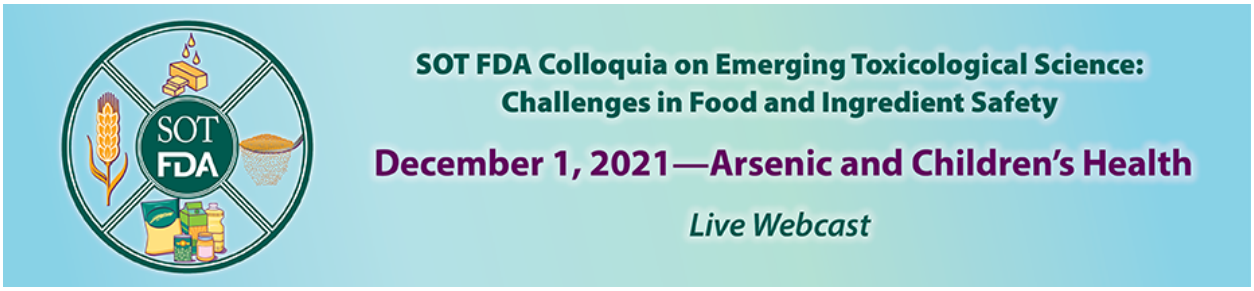

Real-Time Captioning

Note: This is not a transcript.

Schedule

9:00 AM–9:15 AM	Welcome and Overview Conrad Choiniere, PhD, Office of Analytics and Outreach Office Director, US FDA, CFSAN, College Park, MD
9:15 AM–9:20 AM	Speaker Introductions Aaron Barchowsky, PhD, University of Pittsburgh, Pittsburgh, PA
9:20 AM–10:00 AM	Current Understanding of Mechanisms Underlying Arsenic-Induced Developmental Toxicity Rebecca Fry, PhD, University of North Carolina, Chapel Hill, NC
10:00 AM–10:40 AM	Nutritional Manipulation of One-Carbon Metabolism: Effects on Arsenic Methylation and Toxicity Mary Gamble, PhD, Columbia University, New York, NY
10:40 AM–10:50 AM	Break
10:50 AM–11:30 AM	Systematic Review Framework and Dose-Response Methods for Identifying Reference Doses for Inorganic Arsenic Alexandra Larsen, PhD, US Environmental Protection Agency, Research Triangle Park, NC
11:30 AM–12:10 PM	Arsenic Mitigation in Foods and Ingredients Cheryl Callen, MS, Nestle USA, Arlington, VA

12:10 PM–1:00 PM	Summary: Brenna Flannery, PhD, US FDA, College Park, MD Roundtable Discussion Moderators: Co-Chairs Discussion: All speakers Additional Panelist: Jeffrey Gift, PhD, US EPA, Research Triangle Park, NC

Welcome and Overview
Conrad Choiniere, PhD, Office of Analytics and Outreach Office Director, US FDA, CFSAN, College Park, MD

Good morning, everyone, and welcome to the SOT FDA Colloquia on Emerging Toxicological Science Challenges and Food and Ingredient Safety, and today's discussion on Arsenic and Children's Health.

My name is Conrad Choiniere, director of the Office of Analytics and Outreach at the Center for Food Safety and Applied Nutrition at FDA. And I'd like to spend a few moments talking about this colloquium series. This is a partnership between US FDA, and the Society of Toxicology, and it's in its seventh year. It's designed to stimulate a dialogue among leading toxicology experts on future-oriented toxicological science relevant to food and food ingredients safety assessment. It's a forum to discuss the latest toxicological science in the context of food chemical safety, but it is not a forum for soliciting regulatory advice or discussing food or food ingredient regulatory issues. Next slide.

It's open to the public, and at no cost. And we have a global audience from all employment sectors. Immediately after the colloquium is done will have recording and slides available for all. Next.

I won't list these names, but these are the organizing folks that helped organize this series and today's discussion. Before we get into the discussion, I'd like to spend a few moments, talking about you know why this particular topic, arsenic and children's health, is important to FDA. As many as some of you may know, at FDA I do care our toxic elements working group; it prioritizes our efforts for reducing elemental contaminants in the food supply. Next slide please.

In April of this year, I made heightened attention, we released our Closer to Zero action plan. It's a plan to reduce levels of lead, arsenic cadmium and mercury in foods commonly consumed by babies and young, and young children. These contaminants exists naturally in the, in the environment, but they're also present in the soil, water and air from human activities such as industrial activities and energy production. The agency has prioritized lead, arsenic, cadmium, and mercury due to their relative toxicity, the prevalence in the food supply, and the relative contribution of food as a source of

exposure to these contaminants. We also prioritize exposures to young children, because of the impacts that exposures can have on their development.

Although we have made great strides over the years in reducing levels of lead and arsenic exposure from foods, we strive to make further reductions, because for these contaminants, we have not identified safe levels of exposure, particularly when talking about impacts on development among the very young. Although it will be, it will be difficult, if not impossible to get to a point of zero exposure, we believe there are steps, all of us can take to get us closer to zero. Next slide please.

At the heart of the Closer to Xero action plan is a cycle of continual improvement. It's a science based iterative approach for gradually driving explosions from foods, lower over time. For each of the contaminants we'll first evaluate the current science to develop a reference level. And although we may not be able to say that that reference level is a safe level, it is a level that we can rely on about exposures from foods to guide the development of action levels for specific foods and categories of foods. Action levels of the levels of contamination and food, that might lead us to take an intervention. We'll propose those action levels, and then get input from stakeholders on current levels of achievability and the feasibility for making reductions. We'll adjust the proposed action levels based on the current science for managing the contaminants. Are there practices growers can implement to reduce contaminants? Are there steps manufacturers can take to reduce the contaminants? Or are there nutritional aspects that we should consider, aspects about the foods that may be protective against those exposures? We'll take that all into account, and then finalize those action levels and articulate our expectation that industry will strive to do better.

Throughout this cycle we'll be monitoring progress, to see if levels of contaminants and foods have changed, to see if exposures to children have changed, and will take enforcement actions when warranted by routinely sampling and testing products and taking action when products exceed the action levels that we specified. We will be inspecting facilities and taking action against firms that are not putting in place adequate controls and we'll be making that information public for our stakeholders. All along we will also continue the research that we have going on within our center. We have research on analytical methods, toxicology, epidemiology, risk and exposure assessment, consumer and economic behavior, and risk communication.

Then we'll start the process again, plans will continue to develop will learn more about whether and how we can reduce exposures or reduce the impacts of those exposures through nutrition. We'll take that information, reevaluate and adjust our reference levels, then use that new reference level to adjust our actual levels.

For lead, we've already developed an interim reference level. It's a level of dietary lead that is based in part on levels of lead found in the blood of children. We're using that reference level and are busy working now to guide the development of action levels and foods. And so, for lead we're in the propose part of the cycle. Next slide.

However, for arsenic, we're in the evaluate step of the cycle. Today's discussion serves as part of our evaluation of the science around arsenic and its impact on children's health and development. This evaluation will ultimately inform the development of a reference level for arsenic exposure from foods. Next slide please.

Consistent with, consistent with the cycle continual improvement, this is not the first time we have evaluated the science on arsenic. It is also not the first time we assess the science related to arsenic's impact on children's health and development. In 2016 FDA published a risk assessment on inorganic arsenic and rice and rice products. Given the State of the Science of the time, the risk assessment quantitatively evaluated the cancer occurrence for longer term exposure to inorganic arsenic in rice products, and the potential risk management options might have on this risk and qualitatively evaluated certain noncancer risks and certain susceptible life stages, from inorganic arsenic in rice and rice products. In our assessment, we developed those response models for our next impact on bladder and lung cancers. And we're able to quantify the risk of cancer occurrence from longer-term exposure to inorganic arsenic in rice and rice products.

However, in 2013 when we initiated the risk assessment, we found no reviews of the literature regarding adverse health effects during pregnancy, infancy, or early childhood. So, we completed our own literature review to assess whether pregnancy, infancy, and/or early childhood our periods of greater susceptibility to the toxic effects of oral inorganic arsenic exposure. And if so, can these risks be quantified? Ultimately, we were unable to quantify the impacts on noncancer risk, but we were able to make two general conclusions. Next slide.

FDA concluded that there was a likely causal relationship between certain adverse pregnancy outcomes and oral exposure to inorganic arsenic, but important uncertainties remained. Although low to moderate levels of maternal intake of inorganic arsenic during pregnancy appeared to be associated with adverse health effects in the fetus, the uncertainty in the measurement of exposure to inorganic arsenic in the pregnant women studied, along with other weaknesses and confounders in the studies we reviewed, made it difficult to quantify the exposures that result in those adverse effects during this life stage.

When looking at other life stages, we knew that children, and we know that children, are particularly susceptible to neurotoxic effects as a result of low-level exposure to lead and methyl mercury. And at the time we completed our review there were data suggesting that children may likewise be particularly susceptible to neurotoxic effects of exposure to inorganic arsenic. Next slide.

But FDA concluded that there was a likely causal relationship between neurotoxic effects in early childhood and oral exposure to inorganic arsenic, but important uncertainties remained. In the studies we reviewed, not all sources of exposure to to inorganic arsenic from the diet were accounted for, or quantified, and deficit for measure that a single point in time and did not assess the long-term consequences in cognitive function. Next slide.

As you see many important questions remain unanswered. And the answers to these questions can be helpful as we implement are Closer to Zero plan. We look forward to today's discussion in the hopes that it could provide useful information to support our efforts to develop a reference level for arsenic exposure on children's development. Thank you. And now let me pass this on to Dr. Aaron Barchowsky to take us into the colloquium.

Speaker Introductions
Aaron Barchowsky, PhD, University of Pittsburgh, Pittsburgh, PA

Hello, I'm Aaron Barchowsky. I'm the co-chair of the colloquium and professor in environmental and occupational health at the University of Pittsburgh. And we have a great lineup of speakers for you today. The first talk is going to be given by Dr. Rebecca Fry. And Rebecca is a well-recognized molecular epidemiologist, who has spent a fair number of years in environmental epidemiology studies, as well as basic studies. She is the Carol Remmer Angle distinguished professor in children's and environmental health and associate chair at the Gillings School of Global Public Health at the University of North Carolina in the Department of Environmental Sciences and Engineering. She's also the founding director of the Institute for Environmental Health Solutions at UNC, and the director of the UNC Superfund. She's going to share with us today her expertise in basically mechanistic understanding of the toxicity of artistic development toxicity. Rebecca?

Current Understanding of Mechanisms Underlying Arsenic-Induced Developmental Toxicity
Rebecca Fry, PhD, University of North Carolina, Chapel Hill, NC

Good morning. Thank you, Aaron, thank you for the introduction and thank you for the invitation to be here. Next slide please.

I have no conflicts of interest to report. In terms of the objectives for my talk, I'd like to highlight the critical window of developmental exposure to inorganic arsenic, for example, exposure in utero, and how that is related to both early and later in life health effects. I'll talk about the epigenome as a potential link between the exposure to arsenic, and the early and later life health effects. We'll talk a little bit about some of the studies that estimate dose response for these epigenetic effects. And those also highlight sexual dimorphism in arsenic induced health effects. Next slide please.

We're very concerned about arsenic contamination around the globe. Almost every continent is affected by naturally occurring aquifers of inorganic arsenic that can contaminate drinking water, as well as food sources grown in contaminated regions, so it continues to be the king of poisons and major contaminant of concern for us in the United States but also globally. Next slide please.

And we know that exposure is associated with both cancer and noncancer endpoints; arsenic is classified as a group one carcinogen so established as a carcinogen and humans by IARC, it's associated with cancers of the skin bladder lung, liver, prostate, kidney. But it's also associated with noncancer endpoints; for example neurological disorders, reproductive affects, cardiovascular disease, and diabetes. Next.

And of relevance to our colloquium, we know that in organic arsenic exposure is associated with early life health effects, so for example, infants being born at lower birth weight, immune dysfunction, increased mortality, and cognitive impairments. Next slide please.

Some of the work that really supported me and influenced the trajectory of my own research laboratory is shown here, research that was done on the left in mouse models and on the right in human populations, which on the left, you're looking at some citations, published by Mike Waalkes who was at the NCI and the NIEHS. And Mike performed some of the first studies where he exposed pregnant mice to inorganic arsenic and then followed the trajectory of the pups to show that that early life exposure was associated with carcinogenesis later in life. So that established that link between in utero exposure to arsenic, and cancer in the mouse model.

On the right, we're looking at a graphic that comes from one of Alan Smith's articles that focused on a human population and Antofagasta, Chile, where because of a unique exposure situation in Chile, Alan could study the effects of in utero and early life exposure in humans, whether or not that was associated with increased risk of dying of cancer noncancer endpoints, and the answer was yes to both of those. And so, both the mouse model research and the human population research really was pointing to this fact that early life exposure was associated with long lasting health effect changes. Next slide.

And so for those of us who are interested in understanding developmental origins of health and disease, or DOHAD, had arsenic really is a model contaminant for this study. Next slide please.

And arsenic has complex mechanisms of action, some are shown here, where it can for example interact with sulfur, phosphate induce ROS, at high concentrations induce genotoxicity, alter DNA repair machinery, alter signal transduction pathways. And one of the areas that we focused on is this impact of inorganic arsenic on epigenetic dysregulation, for example, DNA methylation but not limited to DNA methylation. And of course, all of these mechanisms contribute to turning critical genes on and off of critical pathways in various cells. And so, understanding these mechanisms is really important as we try to identify solutions for arsenic associated disease. Next.
And so one of the questions that we can ask is, which of these mechanisms is relevant to the developmental toxicity of inorganic arsenic? And what's the biological chain of events that links arsenic to its developmental toxicity? Next slide.

As I had mentioned one of the areas of focus for our lab has been to understand epigenetic dysregulation as a link between arsenic exposure and human health effects.

And there are many types of epigenetic modifiers; the methylation of cytosines is known as CPG methylation. And when this methylation occurs in regions where for example transcription factors need to bind, can serve as a silencing mechanism. And then there are also other modifications that can happen to histones, for example, that influence chromatin accessibility, or small microRNAs, and all of these conserve as a switch, turning genes on or genes off again and these critical biological pathways. Next slide.

So, the first vignette that I'll share with you are some studies that focused on inorganic arsenic exposure. Its exposure happening in utero and the links between CPG methylation. Next slide. And one of the outcomes that this research focused on was the birth weight in infants. Next slide.

Several studies around the world have established that prenatal arsenic exposure is associated with lower birth weight in infants and here I'm showing you a graphic that displays this in populations in Bangladesh, in Chile, in Taiwan. And the same phenomenon has been observed in Mexico and in the United States. So again, what you're looking at here is this trend for lower birth weight in infants associated with in utero exposure to arsenic. Next slide. And in fact, the same trend has been shown in mouse models exposed to arsenic in utero. So, a very strong toxic ecologic endpoint associated with in utero exposure. Next slide.

One of the ways that we've studied this is to follow a pregnancy cohort in Mexico, that I launched several years ago with funding from the NIEHS. The region is known as Gomez Palacio, and I couldn't have done this research without Gonzalo Garcia-Vargas who's pictured on the lower left. And this was a region where we knew that water was contaminated with arsenic, but no biomonitoring studies had been done before. So, we launched the study which we call BEAR for “biomarkers of exposure to arsenic” and looked at trends between arsenic exposure of the pregnant women, and the health outcomes of their infants, and on the right-hand side you're seeing a graphic that shows one of the major findings from one of our early studies, which highlighted that as arsenic exposure increased on the x axis, the birth weight of the infants decreased.

And in fact, one of the, one of the findings that was fascinating was that this was really dependent on how the pregnant women metabolized arsenic and you'll hear more about that in later presentations in the colloquium. So, the, the, finding here was that as these developing infants were exposed to in utero arsenic exposure, their birth weight decreased. Next slide please.

So, the strategy that we use to try to understand a mechanism that may underlie this lower birth weight effect was to collect DNA from the infants from Gomez Palacio who had experienced these different levels of exposure in utero to arsenic, to collect DNA, to look at DNA methylation so that methylation of cytosines, but then also collect samples that allowed us to look at the genes, as the, the end result of the epigenetic modification and so really asking, not only was there a methylation mark, but was the methylation mark associated with a change in gene expression?

And in addition to linking these two biological measurements we also required that the methylation and the gene expression changes were associated with the birth weight of the infant. So, by requiring all of these aspects we were able to identify some really fascinating genes that appear to be potential mechanistic links between arsenic exposure and birthweight. Next slide please.

And one of the genes that rose to the top through these analyses, it is a gene known as KCNQ1. And here you're seeing an area of chromosome 11 in humans on the bottom. We're also seeing similar chromosome in, in mice that are extremely homologous, these areas are a hot, this is an area that's a hotspot for imprinted genes. These are really fascinating genes that do not follow Mendelian laws, I'll talk about that a little bit more in detail, but what you're seeing here is this imprinted cluster of genes on chromosome 11. So, you may recognize some of these other genes, for example, insulin growth factor 2 is on the left hand side, bottom, left hand side, and insulin growth factor 2 is an example of a gene that displayed differential methylation in relation to prenatal famine exposure and has been tied to later in life health outcomes of populations exposed to famine in utero.

So, again, one of the genes that we identified was KCNQ1, which is an imprinted gene which displayed altered methylation, altered expression, and both of those biological features were associated with the birth weight of infants. One of the things that we know about imprinted genes is their expression depends on the allele which you inherit from your mother or your father. And that genes that tend to be maternally expressed tend to be growth restrictive and genes that tend to be paternally expressed are growth promotive. If you, if we look at the next slide, you'll see that this has been described as a genomic tug of war between mothers and fathers over the use of maternal resources by the fetus and so in a way, these genes can serve as switches, controlling the size of the fetus at birth. But our research would suggest that arsenic is modifying the switch and influencing the size of the fetus at birth through arsenic’s effects on DNA methylation of these key imprinted genes. Next slide please.

One of the interesting features about imprinted genes, as well, is that their methylation marks are laid down, prior to zygotic formation. And so, one of the things that we were really interested in looking at was, and that these data suggested, was since we saw these imprinted marks in the methylation marks and the imprinted genes, we wondered whether or not this could be an indicator that arsenic was having its effects due to preconception, not just in utero, but preconception exposure; of course very complicated to study in humans, but I'll show you in the next slides that we've investigated this in mouse models. But for, in terms of this first vignette where we're focusing on prenatal exposure to arsenic and the weight of infants at birth, by using, by combining gene expression, DNA methylation, and epidemiologic analyses, we can identify genes again that we believe are targets for this linkage between chemical exposure and outcomes in infants. Next slide please.

So, just again coming back to this idea that based on the human population findings where we identified imprinted genes, as the marks that we were seeing in the DNA of infants, and the fact that we know that those imprinted marks are laid down prior to

zygotic formation, it led us to ask whether or not preconception exposure could influence the health of the offspring. And these are two recent studies that my collaborator Merrick Styblo and I have been working on, where we asked and answered that question, where we looked at preconception exposure in, in mice and showed that that preconception exposure was associated with phenotype, in this case, metabolic dysfunction in the offspring.

The other thing that we observe in the mouse models, and is also observed in human populations, is the sex dependent effect of the early life exposures, meaning that the sex of the offspring really determines the types of outcomes that you're seeing. There can be various reasons for this that I won't have time to get into in this talk. Next slide please.

One of the questions that we've been asked is, at what dose do we observe some of these effects? And so, I was delighted to work with Julia Rager on asking this question. Could we apply a benchmark dose approach to the epigenetic data sets? And so, here, you're looking at classic dose response curve, knowing that what is happening here is mathematical modeling of an exposure or the dose on the x axis and the change in the incidents or severity of a response. And so, what we did was to use the y axis and the response as the epigenetic indicator. So, for example, using the data that we had on DNA methylation as our outcome or our response and looking at these classic dose response measures, for example the LOEL, NOEL, or benchmark dose, to determine at what point, we saw these effects. Next slide.

And so, we analyzed the data, for example for the CPG methylation marks, within the population in Gomez Palacio to ask, what was the concentration in drinking water at which we saw these the benchmark dose or the benchmark dose lower bound? And the answer was 86 parts per billion, or 55 parts per billion. Again, this is probably highly dependent on the population of study, and it's just one of the first where we're looking at dose dependent effects on epigenetic dysregulation as the outcome, but an interesting technique where we can use classic benchmark dose approaches. Next slide please.

And so really the end goal here would be to identify the concentrations at which the contaminants induce the epigenetic effects with biological plausibility as causal factors in some of the infant health outcomes that we're looking at. Next slide please.
So, I've highlighted some of our work, focusing on CPG methylation which is one form of epigenetic modification. As I mentioned, there are others, and this next vignette I'll walk you through some of the findings related to microRNA expression as it relates to immune dysfunction. Next slide please. And so again in the first vignette I walked you through findings of the association between CPG methylation and birth weight. And here we'll look at microRNAs as potential drivers of immune dysfunction or increased risk for infection in children. Next slide please.

Some really powerful studies have come out of the lab of Margaret Karagas at Dartmouth and Margaret has studied populations who are exposed to arsenic, through drinking water, and the effect that that has on the offspring and developing fetus. An example of one of her papers is shown on the right-hand side, where Margaret and her

team looked at levels of arsenic exposure in pregnant women on the x axis, as it related to the number of infections that needed to be treated with prescription medications and infants so you can see that as that prenatal exposure increased, risk for increased infections increased. Next slide please.

And so, one of the questions that we asked was, is, could micro RNAs be a potential link between this prenatal exposure and immune dysregulation? microRNAs are really fascinating. They are, the micro stands for, or miRNA stands for micro which means small. They range from 18 to 22 nucleotides in length shown on the left in in red, and the microRNAs target transcripts, they have a sequence that aligns with transcripts and the genome. And then through a whole sequence of complicated molecular machinery, once they've found the messenger RNAs, the messenger RNAs to get degraded or translation is blocked. So, in both of these cases as microRNAs would increase, their transcripts would decrease, so again this is a description of how microRNAs can serve as a gene expression switch in the cell. Next slide please.

And so again, working with the data that we had from the Gomez Palacio population, we looked in a dose response manner, so from you're looking from the left-hand side of this graphic to the right, you can see that on the left-hand side would be infants who had lower-level prenatal arsenic exposure, infants on the right-hand side would have higher prenatal arsenic exposure. And then, the heat map that's in red and blue is showing you the identification of nine microRNAs, we tested for hundreds in this analysis, but nine displayed Statistical Association with arsenic exposure. And as you look from left to right in the red and blue heat map you see that generally moves from blue to red, which is showing us that as that prenatal arsenic exposure increased, these microRNAs and their expression increased in the cord blood of influencing Gomez Palacio.

And then the heat map that's in green and yellow below is showing you the target genes of these microRNAs, so one microRNA can have many different transcriptional targets, which is why you could have nine microRNAs, and yet 66 genes that are in yellow and green, and the green color is showing you repression. So as the microRNAs increase in this dose response manner from in relation to prenatal arsenic exposure, the messenger RNA transcripts are being shut down.
And as we look at the biological pathways that are enriched in these genes that were identified, they include toll-like receptors, toll-like receptor five and nine, and genes that play a role and interferon gamma pathway. So, we know that these genes are critical for innate and adaptive immune response. And we can see that they're silenced in a dose response manner; as prenatal arsenic exposure increased, these genes which we know or responders to bacterial and viral invaders are being silenced. And so, these data are providing evidence that microRNAs may serve as a link between prenatal arsenic exposure and the dysregulation of the immune response and children. Next slide please.

So again, highlighting both of these results are highlighting first that CPG methylation could be an integrator between that prenatal arsenic exposure. And here that microRNAs are controlling the transcript levels in children. Next slide please.

So, we continue to try to understand this link between the exposure to arsenic early in life in humans, and various health effects and the role for epigenetic dysregulation; some of the research that we're doing now is really focusing in on the placenta as a target organ for the toxicity that's being observed. We have recent studies that show that arsenic exposure can modify and serve as an endocrine disruptor in placental cells, and that this is happening through epigenetic mechanisms, and again the reason to focus on this research is to, if we identify these mechanisms, we may be able to identify solutions that could control these and be used as interventions.

So, in summary, next slide, what I've shown you is that epigenetic mechanisms may tie early life arsenic exposure to both early and later in life health outcomes. I shared with you our date on CPG methylation and microRNAs. We have increasing information that the placenta is a key target organ for developmental exposure to arsenic, and more research is needed here, and some very new data in mouse models that highlight concerns about preconception arsenic exposure and offspring health. Next slide.

I have listed some of the references that are relevant to the research that I've presented here. And in my next slide, want to acknowledge my lab; this research wouldn't be possible without an amazing team, and the Institute for Environmental Health Solutions and the Superfund research program. Our Superfund research program focuses on protecting populations in North Carolina from arsenic. So, thank you so much for your attention and happy to answer questions later on.

Aaron Barchowsky: Thank you, Rebecca. And I guess we have some time for questions. And they can be submitted through chat. And Jason, are there any questions that you've received so far?

Jason Aungst: None yet.

Barchowsky: I think we may have lost Jason. So, please feel free to send in questions. Rebecca, outstanding talk, and I think one of the striking things is that you are really trying to find a good scientific marker for dose response, if you will, for the response, a real mechanistic marker. And I think that's wonderful. Do you think that the microRNA or DNA methylation changes in genes is tight enough to really be a disease endpoint marker?

Rebecca Fry: Definitely. Aaron, we've focused on these right dose response estimates of both microRNAs and CPG methylation in relation to prenatal arsenic exposure. One of the early studies that we did focused on gene expression as potential biomarker for inorganic arsenic and we used some really sophisticated computational prediction methodologies to determine that.

I do think that there's value in thinking about several of these epigenetic indicators as biomarkers, but I think we have to be cautious that the analysis has included the change in gene expression, as well as the association with the health outcomes. So, that was really important for us to be able to identify, for example, the imprinted gene, KCNQ1, that then led us down the path of the mouse model experimentation.

I think if we had just, if we hadn't required that linkage to gene expression, and to the health outcome, we would have had a much larger set of potential biomarkers that would have been hard to narrow down.

Barchowsky: And related question that's in the chat is, I believe you put up a reference, but has the benchmark dose work on the epigenetic dataset been published?

Fry: It has, yes, the first author is Julia Rager and we can make sure that we provide that reference to it on there.

Barchowsky: I think you had it on there, yeah.

Aungst: Aaron?

Barchowsky: Sorry, Jason.

Aungst: Oh, can you hear me now? Sorry, maybe I couldn't get through before. So yeah, some of the questions. How was arsenic transported across the placenta? Are there known transporters involved?

Fry: There are. There are transporters, for example, transporter known as aquaporin that's expressed in the placenta. And, and arsenic travels, passes through the placenta very well. And so the exposure, mom's exposure, is significantly associated with fetal exposure as well.

Aungst: Thank you. And do all heavy metals, share similar ADME?

Fry: Depending on the metals that we're talking about the, the mechanisms can be quite different. Arsenic is, as you probably noticed in the slide where I talked about the different mechanisms by which arsenic can cause disease, it is, what would you say, promiscuous in terms of its biological mechanisms whereas, you know, I would say some of the other metals, excuse me, have a much more specific and tight mechanism of action.

Barchowsky: Although I would have to say that arsenic is not a heavy metal; it's a metalloid, and that actually gives it so it's unique properties that would be different than a lead or a cadmium.

Aungst: And how is benchmark response determined for this benchmark dose analysis.

Fry: Yeah, so we used the definitions for benchmark dose, benchmark dose lower bound, we use classic definitions. So, for example, that there was a 10% difference in the methylation change, so we use the same quantitative metrics that are used for, you know, estimating dose response for other classical health outcomes as the end point but here we used, for example, methylation level as what was the response on the y

axis, or when we looked at microRNAs, we looked at their abundance. So that, and that is all detailed in the, in the article that I cited. Yeah.

Aungst: OK, thank you. Those were the questions in the chat.

Barchowsky: OK. Actually, there was one earlier. Can you talk about interaction of arsenic with other DOHAD had toxicants?

Fry: There's certainly evidence that, well, as we know, we're likely exposed to many environmental chemicals in mixture based format, and that those mixtures, related exposures, likely have synergistic effects is absolutely an area that we need to be focusing on. Some of the research that we're doing really ties into arsenic's role as an immunosuppressant, and then suppressing the immune response that would then allow for other chemicals to, right, to have a synergistic response with arsenic. So, it's absolutely a concern that coexposures to other, you know, that that arsenic could suppress for example the immune system, and then disrupt the body's natural responses and how they would respond to other chemicals, so something that we definitely need to be thinking about in toxicology.

Aungst: In a related question, were there any of the people taking p glycoproteins inhibitors.

Fry: I don't think so. I assume that's related to the Gomez Palacio cohort and I don't, I don't think so.

Barchowsky: OK, well Rebecca, thank you very much, and we'll move on to our second talk.

Nutritional Manipulation of One-Carbon Metabolism: Effects on Arsenic Methylation and Toxicity
Mary Gamble, PhD, Columbia University, New York, NY

This is by Dr. Mary Gamble, who's a professor in the Department of Environmental Health Sciences at Columbia University. Dr. Gamble comes to toxicology in a unique way, and I think it really has been a great driver of her research over the last decade or so, in that she is actually a nutritional biochemist who is now looking at the nutritional aspects of susceptibility to arsenic, and also has done incredible work in helping us understand arsenic metabolism. And so, she will be giving a talk on the, let's see, the nutritional manipulation of one-carbon metabolism and the effects of arsenic methylation and toxicity. Mary?

Mary Gamble: Hi, good morning, everybody. Can you hear me?

Barchowsky: Yes, we can.

Gamble: Good morning, and thanks, Aaron, for that kind introduction. I want to thank all the organizers for inviting me to talk to you about my work today on nutrition and its influence on the metabolism and toxicity of arsenic. Next slide please. I have no conflict of interest to declare. Next.

So, as you heard from Dr. Fry naturally occurring contamination of arsenic and groundwater is a major problem worldwide. Several regions throughout the world including parts the United States have naturally occurring arsenic in well water used for drinking. Current estimates indicate that roughly 140 million people in over 70 countries are exposed. In Bangladesh the problem started in the 1960s when UNICEF and other NGOs, encouraged a massive switch from drinking microbiome contaminated surface water to drinking well water in was really a milestone effort to reduce infant mortality, due to diarrheal disease. Unfortunately, it wasn't until 20 years later, that it was discovered that many of these wells were contaminated with arsenic.

Well water arsenic concentrations vary widely, and many are far in excess of the maximum contaminant level of 10 micrograms per liter advocated by the World Health Organization. This map of Bangladesh shows that there are some regions in which most wells have arsenic concentrations exceeding 50 micrograms per liter which is the Bangladeshi standard. And yet, other regions where arsenic is not really so much of a problem. Our study site is located roughly 30 kilometers east of Dhaka. Next.

So, as you heard earlier arsenic is associated with increased risk for fetal outcomes such as reduced birth weight, with neurological impairment and children and adolescents. In adults, arsenic is associated with increased risk for health outcomes such as cardiovascular disease and respiratory disease. And one of the earliest and most obvious arsenicosis is the appearance of characters that skin lesions which I'll mention briefly later. In addition, arsenic as a group 1 human carcinogen is associated with increased risk for cancer stem cell lung, bladder, liver and skin. Next slide.

So, although the mechanisms of action for arsenic have not been clearly defined several have been proposed, and the mechanisms of mechanisms are likely multifactorial. So, for example arsenite is known to inhibit many enzymes through binding to hydro groups in their active sites, leading to irreversible inhibition. Other studies have shown that arsenic exposure is associated with altered DNA repair, chromosomal abnormalities, oxidative stress, and endocrine disruption, and much evidence indicates that arsenic induces epigenetic modifications, including alterations in the methylation of both DNA and histones. Next slide.

Arsenic and contaminated drinking water is inorganic, but once it's ingested it undergoes methylation. Next. And this generates mono and di methyl arsenical species, which are referred to as MMA and DMA. And the dimethyl form shown in the lower right has a much shorter circulating half life and is rapidly extracted in urine. So, for many years, arsenic methylation was considered to be a detoxification pathway. Then in the 1990s in vitro studies by two independent groups demonstrated a very high degree of genotoxicity and cytotoxicity of the intermediate trivalent form of MMA, raising the possibility that methylation was actually a form of bioactivation, leading to increased

toxicity, but much data indicates that if you get all the way to BMA, that you are reduced risk, and I'll show you some data to support that. The enzyme that capitalizes both of these methylation reactions as arsenic 3 methyltransferase, shown in the middle, and the methyl donor shown on the left is S-adenosylmethionine, or SAM, and the synthesis of SAME is dependent on the B vitamin folate, and we'll come back to this. People vary a lot in their capacity to methylate arsenic, so for example in humans, the percent of total urinary arsenic extruded as DMA ranges from roughly 40 to 90%. Next slide.

So, starting in the late 1990s, Rick Finnell's group conducted an elegant series of studies on arsenic-induced neural tube defects, employing mice for various folate binding proteins. These studies demonstrated that for all genotypes studied, including wild type, dietary deficiency caused a reduction and total urine arsenic excretion primarily due to a reduction in DMA. Furthermore, folate binding protein 2 null mice were more susceptible to arsenic induced neural tube defects, and this phenotype was further exacerbated by a folate deficient diet. Next slide.

So, David Thomas's group at the US EPA has demonstrated that genetically diminishing arsenic methylation capacity by knocking out the arsenic three methyl transfers enzyme results in prolonged and elevated tissue retention of inorganic arsenic in mice; as indicated by these black bars, less arsenic is created in urine show in the lower left panel. And this greatly exacerbates systemic arsenic toxicity and damage to target tissues. Next slide.

So, there's also a growing body of evidence from epidemiologic studies, indicating that people who are poor at methylating arsenic are at increased risk for several arsenic related health outcomes. This is a forest plot that the estimated relative risk for health outcomes associated with percent MMA in urine. And it illustrates that people who have relatively higher amounts of MMA and lower amounts of DNA are at increased risk for a wide variety of arsenic related health outcomes, including bladder cancer, lung cancer skin cancer, as well as some non-carcinogenic outcomes. One limitation common to some but not all of these studies is that the measure of arsenic metabolites was taken after the time of diagnosis, so they can't fully rule out the possibility of reverse causality, i.e., that having the disease itself alters arsenic methylation patterns. The inverse associations between MMA and outcomes related to metabolic syndrome is one example in which I think we can't at present fully rule out reverse causality or confounding. Next slide.

So, SAM biosynthesis is regulated by one carbon metabolism. And, as you can see this is a very complicated biochemical pathway, but all you need to know for today is fairly straightforward. Next slide. Here's a simplified cartoon of one carbon metabolism, showing that methylation reactions are ultimately dependent on folate. This is because full weight recruits a methyl group from searing basically passes it along. It's used to remethylate homocysteine to form methionine, which is activated to form SAM and SAME is used to methylate arsenic, and DNA, and histones and many, many other substrates. Next.

So, if folate levels are high, this lowers homocysteine concentrations which is good because this pathway can then move forward. Next. Conversely, if folate levels are low, then homocysteine levels build up and methylation reactions are inhibited because this reaction is readily reversible and SAH is a potent product inhibitor of almost all methyl transferase enzymes. So, you need folate to pull this pathway forward. So based on our basic understanding of the underlying biochemistry, we hypothesize that nutritional status would be associated with our second methylation, and toxicity, and that this could be manipulated, simple nutritional interventions. Next.

So, at this point I'd like to highlight the major consumers of SAM. In particular, the synthesis of creatine, which consumes roughly 50% of the SAM that normally turns over on any given day, and I'll come back to why this is important later. Most methyltransferase, including arsenic methyltransferase consumed quantitatively very little SAM. Next slide.

OK, so this slide shows a satellite image of our study site in Araihazar, Bangladesh. The green areas are vegetation and the arsenic concentration of 600 wells is superimposed. So, each of these dots represents a well color coded for arsenic concentration. And you could see that there are some areas in blue, where most of the wells have very low concentrations of arsenic, and there are regions where most are high shown and dark red, and some areas where when it's very heterogeneous, and we've learned from our colleagues in geochemistry at Lamont that much of this heterogeneity relates to the depth of the well. Deeper wells here tap into an aquifer tends to be lower in arsenic. Next slide.

So, from an initial survey, we learned that this population has a very, very high prevalence of fully deficiencies turning red at nearly 60% of males and 40% of females, and consequently they have similar high homocysteine shown in blue. The prevalence of vitamin B 12 deficiency, shown in green bars, it's a bit lower at 10 to 12%. Next slide. So, why is there so much folate deficiency, when folate is relatively ubiquitous in the food supply in Bangladesh, we don't know for certain but traditional methods and food preparation there involve prolonged cooking, which is known to destroy up to 50 to 90% of naturally occurring food folates. Of course, this is a practice we don't wish to discourage because it also kills foodborne pathogens. It's also possible that public Bangladeshis simply don't eat enough of these foods. The main staple of their diet is nonfortified white rice, which is not a particularly good source of folate. Next slide.

So, in our initial full light trial, we wanted to test the hypothesis that folate supplementation to folate deficient adults, increases arsenic methylation. Next. So, we conducted a clinical trial, in which we recruited 200 folate deficient adults and randomizes them to receive either folic acid at 400 micrograms per day which is the US Recommended Dietary allowance, or placebo for 12 weeks. Next slide.

Shown here are the effects of folic acid supplementation on arsenic metabolites in the urine. Folate supplementation resulted in a decrease in inorganic arsenic at 12 weeks, a decrease in MMA significant already after only one week, and continuing to decrease

at 12 weeks, and increase in DMA also significant after only one, when we can continue to increase at 12 weeks. Next. Shown here is a frequency distribution of percent DMA in urine, at the time of enrollment. And you can see there's a wide range of variability of present DMA with different individuals' DMA ranging from less than 46 to 85% of total urinary arsenic. So next week plot in the yellow, the distribution of percent DMA after one week of folate. And in beige after 12 weeks and folate supplementation. As you can see the entire distribution shifts. Next slide.

At this point in time, advances in technology and our trace metal court lab allowed us to measure arsenic and its metabolites in blood. This allowed us using both samples from the same initial trial to test the hypothesis that folate supplementation lowers blood arsenic concentrations. Next. This figure shows the percent change in total blood arsenic, by treatment arm and illustrates that folate treatment on average lowered blood arsenic concentrations by about 14%, as compared to placebo. Next slide.

So, we are particularly interested in the effects of folate treatment on blood concentrations of MMA, as this is an intermediate metabolite with high toxicity. This is a bit of a complicated figure, but I like it because it shows every single data point. So, I'll walk you through it; let's focus first on the left panel. So, first what we do is we sort the data on blood MMA, and we plot that, and what you see is a smooth curve showing every single person's blood MMA concentration in the blue dots. And you can see that blood MMA at baseline ranges from one to 15 micrograms per liter. Then you add each individual's blood MMA after the intervention shown in green. And when you see this, you see that when you do this you see that almost every single person in the folate group shown on the left, experienced a decline and blood MMA, and that the decline was greatest for those who had higher blood MMA to begin with. The average decline in blood MMA was 22%. In the placebo group on the right, you see that half up, and half went down and that's simply regression to the mean. OK. Next slide.

So, the next piece of the story that I'm going to tell you, wasn't originally hypothesis driven at all, it just fell out of a finding that was completely serendipitous. So, I'm going to tell the story as it happened, the finding first and hypothesis that followed.

First you need to know that when we measure arsenic concentrations in urine or any other urine metabolite for that matter, we express it per gram creatinine in order to just adjust for fluctuations and hydration status. Otherwise, if you're dehydrated, everything in urine looks concentrated. Next slide. So, here's the finding: urinary creatinine is a strong predictor of arsenic methylation; it's associated with decreased inorganic arsenic and increase DMA percent in urine in six out of six independent studies. It's true in adult, children, pregnant women, and both males and females. It's true in Bangladesh, to study separate study in Mexico, and it's been replicated by other investigators. These are simply Spearman correlations, but you can't make this go away. No other covariate that we've adjusted for seems to influence the strength of this association. Of course, the big question is why. Next slide please.

So, what is creatinine. Creatine, the precursor to creatinine, is a small peptide that plays critical roles in many aspects of energy metabolism and tissues with high energy

requirements, such as skeletal muscle, heart, and brain. It's used to regenerate ATP and serves as a source of rapidly available high energy phosphate bonds. For this reason it's commonly taken in supplement form by athletes, you can buy creatine and health food store. Because creatine gets created degraded to creatinine, which is excluded in urine at a constant rate over the course of a day, urinary creatinine is used to normalize for fluctuations in the concentration of urine, however urinary creatinine concentrations are strongly influenced by other factors which include muscle mass, age, hormones and renal function. And next, next, and dietary, or supplemental creatine consumption, which importantly down regulates endogenous creatine synthesis through repression of AGAT. So, the more creatine that you consume, the less you need to make; dietary sources are almost exclusively from meat. And so, the creatine that we measure in urine originated from both dietary sources, and endogenous synthesis. Next slide.

This brings me to our next clinical trial, which I'll refer to as the folic acid and creatine trial, or FACT. Next. So, the first hypotheses we wanted to test was that folic acid supplementation lowers blood arsenic in a mixed folate deficient and replete study population. So, this has public health implications because if it's only effective in folate division individuals, then a more targeted intervention may be more appropriate than for example of food fortification initiative. We also wanted to know whether a higher dose would lower blood arsenic, more than 400 micrograms. Next slide.

So, the FACT study is a little bit complicated, but I will walk you through it. 610 participants were recruited, and we're randomized to one a five treatment arms shown on the left, placebo 400 or 800 micrograms of folic acid, creatine, or creatine plus folic acid. And we chose to employ a dose of three grams of creatine per day which is roughly one and a half times the average daily requirements for creatine normally provided through a combination of diet and Nagina synthesis. So, the first phase was 12 weeks, and the dots represent the time points at which urine or blood were collected. After 12 weeks have to half of the folate groups, which switch to placebo to evaluate potential rebound, while the other half continued folic acid. The creatine groups were all switch to placebo to maintain the study blind. For ethical reasons we were obligated to do our best to lower arsenic exposure. And so, to this end we provided parsnip removal filters shown on the right. Next slide.

So, our first observation is what we refer to as the new toy effects of water filters. Over the first 12 weeks you can see that as expected, urinary arsenic went down, but so did urinary specific gravity, and so did urinary arsenic adjusted for specific gravity. Clearly, in the beginning they liked the filters, they drank a lot of low arsenic water, and then the novelty wore off. This was unfortunate, but from a scientific perspective, luckily this didn't different between treatment arms. Next slide.

In contrast to water filter use we know that pill compliance was excellent both through pill counts and based on folate status; plasma and red cell folate concentrations increased in both folic acid groups with a greater increase in those receiving the higher dose and levels went down after switching to placebo. Next slide.

So, our primary outcome was total blood arsenic, and we see here that the 800 micrograms of folic acid group had a significantly greater decline in mean blood arsenic than the placebo. At the end of weeks one six, and 12, and although both groups reverted towards baseline values due to reduced water filter compliance, blood arsenic was still lower and those taking folate supplements. Not shown here, there were no significant effects on total blood arsenic in any other remaining treatment groups, overall, next.

So, on average, in this treatment group blood arsenic concentrations declined by 12%, which is very similar to our earlier trial that focused only on folate individual deficient individuals. Next slide. So, we next switched to test the hypotheses that folate supplementation increases arsenic methylation in participants who are not necessarily folate deficient. And that a higher dose may increase arsenic methylation more than 400 micrograms. Next slide.

So, now let's look at the change in methylated arsenic metabolites in urine over time. Shown here is the relative stability in arsenic metabolites in the placebo group. Next slide. When we look at all treatment groups, we see that all of the groups that received folate at either dose and with or without creatine had lower inorganic arsenic, lower MMA, and higher DMA in urine, as compared to those groups that did not receive folate. Similar to our earlier trial the 800 microgram dose increased arsenic methylation capacity already after only one week. Next slide.

So, for the creatine arms we first wanted to determine whether creatine supplementation down regulates creatine biosynthesis in humans as has been shown in rodents. We then wanted to determine of course if creatine supplementation increases arsenic methylation. Next slide.

So recall the creatine synthesis is a major consumer of SAME, and we hypothesis that creatine supplementation by inhibiting AGAT with lower creatine biosynthesis. If successful, this would be reflected in lower GAA concentrations and increasing SAM would also lower homocysteine concentrations. The ultimate goal here was to quote unquote spare SAM for the methylation of arsenic. So, in data that I don't have time to show you today, next, I can tell you that creatine supplementation did lower GAA and plasma by week 12 as predicted. And next, that this furthermore. Next slide. This furthermore, lowered homocysteine concentrations. Okay, next.

In this table, I'd like to focus on the creatine treatment effects. Creatine supplementation significantly lowered percent MMA in urine, as compared to placebo. At weeks one, six, and 12. Although the effects on inorganic arsenic and DMA were in the expected direction, they did not achieve statistical significance. The magnitude of the effects of creatine were less than what we had predicted. And we believe that they may have been tempered by long range, allosteric regulation of one-carbon metabolism, that regulates hepatic SAM concentrations. Next slide.

Moving on to the second half of the trial, the two folic groups were split such that half were switched to placebo and have continued their folate supplementation. Next slide.

This is study design allowed us to ask whether total blood arsenic concentrations would rebound after cessation of folate supplementation. And whether arsenic methylation capacity would decrease 12 weeks after cessation of folate supplementation. Next slide.

So, in the second phase we again see that 800 micrograms of folate shown in blue for 24 weeks lowered blood arsenic to a significant greater extent than placebo shown in green, and there were no differences in blood arsenic lowering between those that remained on folic acid, and those who switched to placebo shown in the blue dotted line. The apparent increases over time did not achieve statistical significance and they parallel the plots where I show the new toy effect, and so, these reflect decrease in compliance with water filter use. Next slide.

When we look at urinary metabolites in the second phase of the trial, we see some reversal of arsenic methylation patterns. When comparing those who continued folate shown in red, versus those who switched to placebo. Those on placebo shown in gray had increased percent MMA, and decreased percent DMA between weeks 12 and 24. So, while total blood arsenic did not significantly rebound, we do see this tendency for arsenic methylation patterns to revert. And given the known role of arsenic methylation and artistic excretion and elimination, possibly had we followed the study participants for longer period of time, we may have also seen a more significant rebound until the blood arsenic. Next slide.

So, we were also interested in the influence of our treatment honors arms on arsenic metabolites and blood. Here I'm only showing the first 12 weeks, and for simplicity and I'd like to draw your attention to the primary and secondary methylation indices or the PMI, and SMI. So, the PMI is simply the ratio of the concentration of MMA in blood to that of inorganic arsenic, indicating incomplete methylation, and a secondary methylation is the ratio of DMA to MMA indicating increased methylation. When you look at the data in the red box, you see that compared to placebo, all of the treatment arms increased arsenic methylation, as indicated by decreasing PMI, and increasing SMI. I will note that these data are not yet published and we're still analyzing data on blood arsenic metabolites from phase two of the study. So, that's all I have to share with you today on that FACT study. Next.

And coming a bit back to the theme of the course. We know that children are increase our increased risk for arsenic related health outcomes as Dr. Fry has already described. This child on the right is showing us that she has arsenic induced skin lesions. But there are only a limited number of studies, linking nutritional status to arsenic methylation capacity in children. I will mention that we have recently completed a trial of folate and B12 supplementation and eight to 10 year old children. Unfortunately, I don't have the data to share with you yet from that study but stay tuned. Next slide.

So, for reasons that are poorly understood, children in general are far less likely to be folate deficient and in fact their folate status tends to be fairly high, and they're known to be more efficient at methylating arsenic. So, this is just a brief summary of some of the findings in the literature; it's by no means comprehensive and there's certainly not studies that I have not included.

So, in cross sectional studies in Bangladesh on six-year-old children, nine-year-old children, and adolescents at least among males, folate was inversely correlated with percent inorganic arsenic and positively correlated with percent DMA in urine. We did not observe this among adolescent females, but we did find that red cell folate was inversely associated with total blood arsenic concentrations and female adolescence.

In a case control study of developmental delay in Taiwan a combination of high plasma folate and high B12 were associated with lower inorganic arsenic and higher percent DMA in urine, and with reduced odds of developmental delay. I should note that a number of studies in Bangladesh children by Joe Graziano and colleagues found detriments in cognitive function that were associated with arsenic exposure but were not actually associated with arsenic methylation capacity. Next slide.

So, we know remarkably little about one-carbon metabolism early in life. We know that pregnant women preferentially deliver folate to the fetus even at their own expense, I mentioned that folate status tends to be higher in children than adults. We also know that folate biomarkers decline progressively with age from newborns and children through adolescence and up into adulthood. Clearly, the demands on one-carbon metabolism, to provide several necessary ingredients to support growth, are very different from those of adults. These include for example increase names for DNA synthesis and increased use of amino acids, such as methionine for protein synthesis. Given these metabolic differences, I think it's important to exercise some caution and interpreting cross sectional studies of arsenic methylation in life stages where these biomarkers that likely in a state of flux, and may not similar to reflect overall One-carbon metabolism in the same way as they do and adults. Next slide.

So, in conclusion, folate supplementation increases arsenic methylation and lowers blood arsenic and both folate deficient and folate sufficient individuals. Arsenic methylation patterns tend to revert to baseline 12 weeks after sufficient of folic acid supplementation. Together these two findings indicate a need for widespread and long-term public health interventions, such as food folate fortification programs, as opposed to supplement use at the individual level, which relies on long term compliance which may be difficult to maintain. And data from to Nesta case control studies that I didn't have time to show you today, risk factors for arsenic induced skin lesions include both folate deficiency and high homocysteine. Creatine supplementation lowers creating creating sentences and lowers MMA. However, I feel that additional research is needed to more fully understand the strong cross-sectional relations consistently observed between urine creatinine and arsenic methylation. Next slide.

So, this map. This is a map of folate fortification programs, and it looks a bit more promising than it is because the countries shown in lighter blue only have voluntary programs which in many regions means none. And some of these programs are not fully enforced. In red dots are showing the 10 countries with the most significant problems of arsenic contaminated drinking water. And these 10 countries did not have mandatory folate fortification programs. As it's been demonstrated Western countries such as the United States and Canada, folate fortification can nearly eradicate folate deficiency and many of its associated consequences. In arsenic endemic countries, it

may have the additional benefit of facilitating a partial reduction in the extensive public health burden of arsenic exposure, as one component of comprehensive arsenic mitigation remediation programs. Next slide.

So, the number of people involved in this work are too numerous unfortunately to mention I'd like to acknowledge support here from the NIH. Next. And I would be remiss if I didn't mention some key players. Vesna Ilievski, who runs my lab. Xinhua Liu, who's an amazing biostatistician. Faruque Parvez who was instrumental in overseeing fieldwork. Vesna Slakivich who did blood and urine bloodwork analyses. Joe Graziano who ran our NIH Superfund program and trace metal core lab for many years, and I've been most fortunate to have outstanding PhD students, including Rick Pilsner Megan Niedzwiecki, Brandi Peters, Caitlin Howe, Anne Bozack, and Ahlam Abuwad, and my colleague from EIP Megan Hall, who contributed to this work on so many levels. And Lex van Geen from Lemont to provided, among other things of the well water data. Next slide.

And finally, thanks. Many thanks to our amazing field stuff and study participants without whom none of this work would have been possible. Thank you for your attention. I'll and be happy to take questions.

Barchowsky: Thank you, Mary. I don't see any questions in the chat yet.

Aungst: We have a few here.

Barchowsky: Okay.

Aungst: People are welcome send us to me. And it's mainly related to the dose response. So, the first question was, the dose of 800 micrograms per day of folic acid, and given those significant results, is a consideration and going higher?

Gamble: No, I don't think so; I think the public health implications of doing a higher dose interventions are not really very practical. 400 micrograms is the US recommended dietary allowance. You certainly wouldn't fortify foods to a level that's going to be exceeding something like the RDA. So, I guess that's the answer.

Barchowsky: Is there folate toxicity?

Gamble: There is no known folate toxicityl it's an interesting question—it's something that's actually been at the forefront of folate world because folic acid is a synthetic form of folate. It's not the same as what's naturally occurring in foods; we do metabolize it to 5-methyl-tetrahydrofolate, which is the form found in foods, but not 100% of it is converted to that natural form and so now we all have detectable levels of folic acid in our blood and some people are questioning whether or not that's a problem.

Aungst: Yes, that relates to another question so just in your opinion, with the US with so many white rice products being fortified with folic acid. Do you think we still need to keep it at that level or change it?

Gamble: I think the food folic acid fortification programming United States has been remarkable; it's reduced the incidence of neural tube defects very successfully. And I don't think that we over fortify but people are asking those questions. I actually have another grant to look at metabolomic effects of folic acid supplementation to see are there any unpredicted effects that we wouldn't have expected, good or bad? I think that answers that.

Aungst: Okay, yeah. Did any of the, I'm sorry, can folic acid supplementation be used to treat arsenicosis?

Gamble: So I, I think of folate as a preventive measure it clearly increases arsenic methylation, which should reduce some of the health outcomes that are associated with incomplete methylation. It may protect against some other arsenic mechanisms of actions such as altered DNA methylation or histone modification to chromosomes stability. It has all those beneficial—a lot of potential for beneficial effects in that regard. In terms of treating people who have disease, I haven't seen evidence for that. There's one study in West Bengal where they treated one area with low arsenic water and the other was low arsenic water plus folate, and they seemed to have some symptomatology improvement. But that's the only study that I'm aware of. And so, I think we really need more research before we could say that. Furthermore, I would not be inclined to consider folic acid treatment for people who have arsenic related cancers, who may be on chemotherapy agents which are anti folate drug, some of them are anti folate drugs, so I hesitate to consider using it as a treatment at this point in time.

Aungst: Next question is what's the level of seafood intake in Bangladesh trial population; we're assuming it's pretty low or absent?

Gamble: It's fairly low; they have some farmed fish. But I wouldn't, I would not consider that they consume a lot of fish. And if the questions related to fish related arsenicals, I would not think that they would be very high in that. And we have measures of arsenobetaine, which is a biomarker of that. And they're not particularly high.

Aungst: Okay. Is the creatine relationship to arsenic that we see in humans also seen in mice?

Gamble: I don't think anybody has ever looked at mice; and I wish somebody would—that's a great question.

Aungst: Yeah, it's interesting. Additionally in development where would disruption of one carbon enzyme be the most detrimental for example in maternal liver, placenta, fetal liver?

Gamble: I think of disruptions in one carbon metabolism from something like systemic, like folate, but probably affect most organs, which would be most detrimental. I guess those organs that arsenic targets.

Aungst: That's all the questions we had from the chat. Thank you.

Barchowsky: Okay. Mary. Thank you very much for an excellent talk. And at this time, I guess, we're a little bit ahead of schedule, but we have a scheduled break. And I guess we'll reconvene at let's say 10:45.

Systematic Review Framework and Dose-Response Methods for Identifying Reference Doses for Inorganic Arsenic
Alexandra Larsen, PhD, US Environmental Protection Agency, Research Triangle Park, NC

Brenna Flannery: So, my name is Brenda Flannery, and I'm a toxicologist with the US FDA. I'm co-chair along with Aaron, and I would like to introduce our next speaker. Her name is Dr. Alexandra Larson. She received her PhD in statistics from North Carolina State University, and she is now a statistician with EPA. Joining her is Dr. Jeff Gift. Dr Gift is an expert in dose response modeling and chemical risk assessment, also with the EPA, and he will provide his perspective as well specifically for answering questions. And so Dr. Larson is going to speak to us today on the systematic review framework and dose response methods for identifying reference doses for inorganic arsenic. Thank you, Dr. Larson.

Alexandra Larsen: Thanks, Brenna, it's great to be here. And thanks to everyone for joining us today for this presentation. We have no conflicts of interest to declare, and the views expressed in this presentation, are those of the authors do not necessarily reflect the views or policies of the US EPA.

So, today I'll be presenting on the systematic processes used, and the IRIS assessment of inorganic arsenic, to identify data or reference dose calculation here at EPA. I'll be walking through our systematic review process, study evaluation procedure, our approach for judging strength of evidence, and systematic dose response methods including Bayesian meta regression. Next slide.

Our systematic review protocols aim to identify studies reporting effects of exposure to inorganic arsenic, and we focused on health outcomes suggested by the National Research Council in 2013. These outcomes included bladder, lung, and skin cancer, skin lesions and ischemic heart disease and tier one. So, these were outcomes in the in our that the NRC found to have the most well-established causal association with arsenic exposure. We also conducted study evaluations for tier two and three outcomes to try and investigate the presence of a causal association. These tiers include endpoints particularly relevant to this audience, birthweight, neurodevelopmental effects, and feed a loss, stillbirth, and neonatal mortality. All study evaluations and risk of bias determinations were conducted using the OHAT approach. Next slide.

We conducted our strength of evidence synthesis across epidemiology studies and based our conclusions on findings from other assessments or from conducting new systematic reviews. Since the tier one outcomes like bladder, lung, and skin cancer and skin lesions are accepted hazards, we consider the strength of evidence for these outcomes to be robust and carried out no new evidence synthesis. However, we did

conduct specialized systematic reviews for these accepted hazards to identify studies for dose response analysis, and I'll go into this in further detail and later slides. For all other health outcomes under consideration, new systematic reviews were conducted to characterize the strength of evidence for potential hazards and vacation. Next slide.

Very briefly, this is the PECO statement that were used. So, the populations, exposures, competitors, and outcomes of interest. We did consider a number of oral exposure metrics, not just drinking water, for all outcomes, and we did explicitly look at pregnancy and neural developmental outcomes. Overall, we focused on epidemiology studies. Next slide.

Our study evaluation criterion presented here. We use the OHAT individual level domains for epidemiology studies. These include selection, confounding performance, attrition, detection, and reporting bias. Our risk of a bias evaluation protocol included questions for each of the six domains, which were informed by arsenic specific clarifications. They were carried out by two independent reviewers. Based on the responses under each domain, a risk of bias or ROB rating was assigned which translated into an overall study rating of high, medium, low, and uninformative, which we excluded. Next slide.

These diagrams outline our systematic process to iteratively identified the most relevant studies. On the left we have the initial literature search, which yielded a very large database, upwards of 46,000 papers. We used AI based screening to narrow this down to 7500. And these went on to title and abstract screening, and then finally to full text screening. The flow diagram on the right details the update we conducted in 2018, which follows the same steps. This update resulted in a total of 467 epidemiology hazard ID studies. Next slide.

That final set of studies we apply the hill criteria to evaluate evidence for causal relationship, which involved examining risk of bias, study sensitivity, consistency, strength and precision, biological gradient, or dose response and coherence. Next slide please.

We summarize our findings in evidence profile tables for each hazard, the example shown here is for neuro developmental outcomes. In addition to summarizing key findings, we noted factors that either increased or decreased certainty and recorded a judgment on the strength of evidence for causal relationships of either robust, moderate, or slight. Next slide.

So, for pregnancy outcomes of fetal infant morbidity and fetal loss, stillbirth, neonatal mortality, our systematic review yielded robust events in favor of a causal relationship with arsenic exposure. Next slide. We found moderate evidence of a causal association with neurodevelopmental toxicity. And both robust and moderate evidence outcomes were considered for dose response analysis, which I'll talk about more next. Next slide.

To start our dose response analysis, we conducted relative risk exposure to background or RRB stream, where we aim to identify studies and endpoints suitable for dose

response modeling. For each study we focused on the single best model to derive point of departure, EPA's benchmark dose software was used to estimate the exposure that would increase risk customers by 20%. We derived our RRB values for all 12 robust and moderate endpoints. And we're able to use results to compare arsenic potency across hundreds of databases for multiple endpoints. Next slide.

All endpoints except for immune and developmental neurocognitive supported RRB modeling and were considered for further dose response analysis. The figure here shows the distribution of RRB values for each of these 10 points, and we can see that we got better coverage and outcomes, with more than 25 data sets versus things that have less than 25 data sets. Next slide.

To conduct the actual dose response meta regression, we employed a Bayesian hierarchical model as the best approach for pulling information across multiple studies. In our approach we converted all exposures to a common estimate of intake, micrograms per kilogram day, we conducted a multiple study hierarchical dose response analysis, under a logistic model framework. We investigated low dose non-linearity was fractional quality models, which are extensions of the logistic model. And we use the live table analysis to estimate lifetime of extra risk. In the event the Bayesian meta regression approach was not feasible for an outcome, we conducted our dose response analysis based on a single best study.

As mentioned previously we used a logistic framework where we assumed a logistic perspective like our hierarchical structure assumes study specific logistics slope parameters for beta coefficients, and these are normally distributed around the beta mean and standard deviation Beta Sigma. Both of these parameters are assigned priors and updated, and this hierarchical structure explicitly accounts for heterogeneity across studies. The figure on the left shows the posterior distributions and medians for both food estimate, and studies specific betas for one of our outcomes.

The live table analysis takes background exposure into account to estimate extra risk of disease and target population, which for our purposes, we focused on the United States. The table on the right shows pooled meta regression estimates of lifetime extra risk bladder cancer incidence at various doses for 10,000 and drinking water exposures using MLE dose estimates from our desk conversion analysis. Background rates of disease for this analysis are soon to represents zero extra risk from arsenic exposure. And based on the data for the US, we assumed a mean background arsenic of 0.071 micrograms per kilogram day. Our approach can be used to estimate, both risk at dose, and dose at risk values.

Pregnancy outcomes can either be dichotomous, or continuous. Dichotomous outcomes like fetal mortality, for example, are amenable to our Bayesian meta regression approach. However, more work is needed to be able to apply like table analysis to these kinds of data. For continuous outcomes like birth weight, we would require an entirely separate method for dose response. So, there's a need for further work to develop alternative modeling strategies for pregnancy outcomes. Next slide.

These might include the use of other already developed meta regression methods, for example, those that are made available in the R package metaphor. Urine biomarker studies, could be used for meta regression approach, but for the fact that PBPK models are not often parameterized for pregnant women, or fetuses. Under these limitations, until these limitations are addresses, relying on single best studies may be necessary. Ideally, we would have study reported beta coefficients to calculate BMDs and BMDLs.

For neurodevelopmental outcomes dose response screening only identified two studies appropriate for further dose response analysis. One of these two is summarized in the figure here. Both of these studies reported effects as continuous outcomes, which is not conducive to our meta regression approach. Next slide.

So, here we list some alternative modeling strategies. Of the two studies we identified for neuro development, only one was conducted in the US, focusing on this study for dose response analysis may prove beneficial. Since we could reduce uncertainties in extrapolating effects across diverse study populations. Dose response for full IQ score perceptual reasoning, verbal comprehension, and working memory are non-monotonic, which could introduce complications. This is something that we also want to consider.

Using raw data would allow for the analysis of effects when arsenic exposure is treated as a continuous variable. Apparent nonmonotonicity may take, they make toxicity value derivation difficult. So, a default to a NOAEL or LOAEL approach may be necessary.

So, to summarize, We implemented systematic review methods to inform our hazard identification and dose response analysis for arsenic related health outcomes. Multiple outcomes were considered for dose response analysis with Bayesian methods effects on children are exposed fetuses were considered for RFD derivation, including developmental neuro toxicity and birth outcomes. However, not all endpoints were suitable for Bayesian modeling. So alternative methods may be necessary for RFD innovation.

And next slide. I'd like to conclude by acknowledging the IRIS Inorganic Arsenic Team: Janice Lee, Allen Davis, Jeff Gift, Ingrid Druwe, Alex Larsen, Bevin Blake, and Martha Powers. And again, I'd like to thank the colloquium organizers for inviting us here to speak today. Thank you.

Flannery: Thank you, Dr. Larson, for your presentation. I really appreciate the systematic approach that's being used. So, now let's go ahead and take some questions, if you all have any questions, please put them in the chat to Jason Aungst and so I actually have a first question for you and also for Dr. Jeff Gift, who will be assisting with questions. How did you all estimate the background exposure to inorganic arsenic? I know you said from United States data, but I'm wondering how specificly that was done. You're unmute, Dr. Larsen. Thanks.

Jeffrey Gift: Would you like me to respond??

Larsen: That'd be great.

Gift: Okay, so we estimated the background levels in the United States, based on a review of literature and determination that the dietary background was around 0.5 micrograms per meter cubed and the water concentration background was around 1.5 microgram. I'm sorry. I said the 0.5 dietary background was in terms of micrograms per kilogram day. The water concentration background was estimated to be 1.5 micrograms per liter, and that translates to using our EPA handbook estimation of median consumption rates to 0.021 micrograms per kilogram per day so adding those together we got a background, we estimated a background level point 0.071 roughly micrograms per kilogram per day, and that was our, our background assumption for US population. And I forget the actual paper citations, I can provide that.

Flannery: Thank you. Are there any questions, Jason, from the participants?

Aungst: Yeah, the questions mainly focus on publication. So, is the meta regression work published yet and it's just dose response work published or undergoing peer review? I think people are interested in seeing this come out publicly.

Gift: So actually, we do have publications for publications that describe various methods relative to dose response and they're 2020 publications, the publications on the meta regression methods are Allen et al. And then the publications for the screening approach that Alex referred to his Hobbie et all. HOBBIE, I believe. Why I should be sure about that. And then there's another publication that we use to determine whether the studies in Taiwan, Channel et al. 2020, 2010 in particular, could be used, could be relied upon for extrapolation to the US. So, we did a model averaging approach, and we found from that model averaging approach that there was a significant amount of extrapolation that needs to go on with that study, and so we determined that the, that that is part of the reason we determined to use the meta regression approaches that we use in describing the other papers and that paper is Mendez et al. 2020.

Aungst: Thank you. Next question, can estimated dietary exposure be extrapolated to other areas of the world, for example, where rice is a major proportion of the diet?

Gift: Well, certainly, we took that into account in our meta regression analyses, so each population received a specific estimate in the meta regression of dietary contribution. And then when we, in the end, estimated the US relevant point of departure we used the US relevant estimate of dietary exposure. And in combination with life table estimates of the background risk. Does that answer?

Aungst: Thank you. But another request here, made for Dr. Larsen: What were the two epidemiology studies specifically that were identified as critical for the neurodevelopmental dose response?

Larsen: So, Jeff can give a little more detail on those studies, but they're both by Wasserman group spaced a few years apart, and one of them focused on a Bangladesh population. And again, the one that we talked about today was focused on the US population.

Gift: Right. That was the main study that was referred to earlier by the other speakers.

Flannery: And I have a question for you actually about that as well. If you can answer it. So, the studies that you identified for the neuro development. You know, you had discussed the nonmonotonic response. And I'm wondering, is that something that you're seeing that is consistent across the literature or at least across the two studies you identified or is that just specific to the studies that were deemed the best quality to use?

Gift: I'll let you respond to that first, Alex, and then I'll take a stab.

Larsen: I actually think, Jedd, that you would be best to answer. How often we're seeing that across the studies.

Gift: Okay. This is not something that we're necessarily seeing across studies, it's something that just was unique to this Wasserman study, and we're not exactly sure why the phenomenon is occurring; as Alex pointed out, it caused us to not be able to do a reliable dose response. Unless we assume biologically untenable dose response curves, which we decided not to do. We needed to use a NOEL LOEL approach for that study. It's unfortunate, but that is the best study, given that it's a relatively large US population, we're looking at, and we, the Bangladesh doesn't give us that issue, but it used many, much higher doses, and therefore the extrapolation back to US doses was untenable and unreliable, we determined.

Flannery: Thank you. So, Jason, are there any more questions from the participants on the chat that need to be addressed or? It's a little early but we can move on to Cheryl's talk.

Aungst: Yeah. We're good.

Flannery: All right, well thank you very much again, Drs. Larson and Gift. We really appreciate it, and we look forward to discussing this more with you during the roundtable.

Arsenic Mitigation in Foods and Ingredients
Cheryl Callen, MS, Nestle USA, Arlington, VA

Flannery: So, our next talk is by Cheryl Callen. She is the Senior Director of regulatory affairs and scientific affairs for Gerber Products Company. She also, which also operates as Nestle Infant Nutrition. She has been with Gerber for the past 15 years. Cheryl has a master's degree in food science from Rutgers University and a Bachelor of Science in food and nutrition from West Virginia. And today she's going to speak to all of us on arsenic mitigation in foods and ingredients. So, thank you, Cheryl, and welcome.

Cheryl Callen: Thanks, Brenna. I want to make sure everyone can hear me because my, my headset did die.

Flannery: We can hear you.

Callen: Okay, perfect. Anyway, so thank you. I wanted to just display my next slide, my conflict of interest, that other than being an employee of Gerber Products Company, I don't have any other conflicts of interest to disclose. We can go to the next slide.

So, the objectives for my talk today are really twofold is to talk about nutrient intake for infants and young children and we'll talk a little bit about nutrient intakes for folate and vitamin B 12 as well as choline and the food sources of these nutrients and the diet of young children, and then we'll move to arsenic mitigation, the tools and systems that companies, growers, and suppliers to use to manage contaminant risks, as well as other risks including arsenic in foods. We can go to the next slide.

So, the nutrient data will be based on the feeding infants and toddlers study or FITS. FITS has been conducted three times on 2002, 2008, and 2016 and is a 24 hour dietary recalls study of all foods, beverages, and dietary supplements consumed by children from zero to 48 months. The survey collects data on foods and amounts consumed as well as lifestyle behaviors and impeding practices. And over the course of the three studies, we've surveyed nearly 10,000 parents and caregivers, about the diets of young children so is a very robust data set for this population group. And we can move to the next slide.

So, this is the usual nutrient intakes for instance from six to 11.9 months, and we pull this data out and we're just focusing on three nutrients, but we, we have a whole host of nutrients that we can focus on. And we focused on this age range in particular because this is the age when complementary foods are introduced into the infant diet and become increasingly important source of nutrition, as well as feeding skills development for young children. Prior to the six-month mark, the diet's primarily composed of breast milk and or infant formula So, as you can see I just pulled out two data points here one is the 50th percentile, and the 10th percentile and then in the copy above, we talked about the total infant population that was surveyed. And we can, this shows you that a 10th percentile we have a significant number of children above the AI, that's the adequate intake, level for folate and overall, 98% of infants are about the adequate intake level. Similar for vitamin B 12, 97% of infants are of the AI. And for choline not quite as, as, not quite the same but we do have 61% of infants above the adequate intake level for choline.

So, it also allows us to look at the foods that children are consuming that supply these nutrients. So, these are ranked in order. And we looked at the major and minor food groups that contribute 2% or more to total intake of these nutrients. So, for folate, again for the same age range, you can see that infant formula is number one but for food sources infant cereal is number two, followed by non-infant cereals. It's also probably important to point out that mixed dishes, which includes baby food mixed dishes as well as baby food fruits and vegetables, do contribute folate to the diet for this population group. And they do play an important role.

B12 is a similar story with infant formula providing a significant amount to the intake of vitamin B 12 for this population and an infant cereal for food sources is number one. We can, I think, what I probably just want to call out here is given the importance of infant formula and infant cereal to these intake levels, we thought nutrient adequacy should also be looked at by milk feeding type which you know what does nutrient adequacy look like for exclusively breastfed child, formula fed child, or mix fed child meaning consuming both breast milk and infant formula? And we also wanted to look at the cereal and noncereal users. So, if you could go to the next slide.

This really just shows a little bit of the data that we've collected related to milk type and cereal use and nonuse, and this is for breastfed babies so exclusively breastfed babies, whether they are consuming infant cereal or are noncereal infant users. And as you can see here, for folate, and the turquoise lines is about 98% of breastfed babies consuming infant cereal meet the adequate intake level for folate weight, where 50% of non-cereal users are meeting the AI. Vitamin B 12 100% of breastfed babies consuming infant cereal meet or exceed the AI, 67% of non-cereal users meet the AI. And for choline, again the trend continues with about 65% of breastfed babies consuming cereal meeting the AI and 44% of without infant cereal. So, from this we can see that infant cereal does play an important role in ensuring adequate nutrient intakes, especially for the breastfed baby. We can move to the next slide.

So, that's sort of led us, despite the fact that infant cereal does play a significant role in nutrient intakes, they don't have a standardized required on nutrition composition. With one exception and that would be infant cereals that participate or want to be eligible for the USDA Women, Infants, and Children supplemental feeding program. The infant food package requires infant cereals to have 45 milligrams of iron per 100 grams. Other than that, there are no required fortifications for infant cereal.

So, what I did here is just pulled four infancy roles that are in the marketplace and compared they're a subset of their nutrition values in this chart. And you can see that for the first three cereals iron is 60% of the daily value and that really is the comparable level to 45 milligrams of iron per 100 grams, so these three cereals are eligible, based on their iron content for the women, infants and children's feeding program. The fourth cereal with 45% DV iron would not be eligible. And you can also see then previous nutrients that were sort of focused on today folate, vitamin B12. And then I added zinc because it's certainly another important nutrient and infancy and choline, nutrient values do vary. Folate it could be 15% of the daily value up to 25% of the daily value. B12 is the same among all three cereals, zinc is very similar, and only one cereal is fortified with choline. And then of course the last cereal with the blank data does not have any additional fortification, other than iron. So, I think it's just an important to think about it fortification infant cereal would change or not be consumed or consumed with a nonfortified that could affect dietary and takes of those nutrients. We can move to the next slide.

Okay, we're going to move to talking about toddlers This is our 12-to-24-month population group. We really pulled up the same three nutrients. And you can see the intake levels are very similar, we have 98% of children this age range, about the AI,

almost 100% above the AI for vitamin B12, and for choline, similar to infants but a little bit lower, we have 51% above the AI or adequate intake level and I should just point out that those are estimated average requirements for folate and vitamin B 12 not AIs. Okay we can move to the next slide.

Even though the intakes are similar the percentage of children median AI is similar the food sources is very different, and so with this 12 month mark, children are typically transitioning to cow's milk from breast milk or infant formula, and often moving away from consuming products that they would have consumed in infancy, like infant cereal or baby foods and going more to the foods that the rest of the family is consuming. And you can see that reflected here with both folate and vitamin B 12 food sources. And again, these are the major and minor food groups that contribute 2% or more to total intake of these nutrients. And you can see the number one contributor is non infant cereal.

So, these are ready to eat and hot cereals that are fortified with folic acid. They are by far and away the biggest contributor, but I also want to highlight the other categories under folate that are in red, so breads and rolls and crackers and pasta and rice. All of these are grain-based foods and most likely their presence here reflects the folic acid enrichment that was put in place by FDA back in 1998, that when you have enriched pasta or enriched flour, you do add folic acid to it and so I think that is probably what's driving the presence here. Infant cereal still is on the list but very, very small amount of intake contribution.

For B12 it's no surprise that cow's milk is the dominant source of vitamin B 12 in this diet as I mentioned earlier, children are typically transitioning to cow's milk around this age range, and a lot of children do consume a fair amount of cow's milk and meat, of course, is also a top source of vitamin B 12. And while, this isn't unusual it probably is just worth mentioning that as more consumers move to plant-based milks or plant-based meat alternatives, we could see an impact on vitamin B 12 intakes us younger children are consuming those the diet more often. We can go to the next slide. Oh, okay.

With that we're going to move into arsenic mitigation and really, this isn't specific necessarily arsenic, but really a broader look at how contaminants are managed in foods, so we can to the next slide. So, the first step is always consideration for how do you establish an internal standard? Certainly, the very first place, you would look would be where do they exist regulatory requirements or standards, including action levels, whether they are final or proposed? That would be you know our recommendation is always to, you know, you know be compliant with proposed requirements as well as final ones and of course regulatory compliance is not something that's negotiable.

Then you need to also consider where your products are sold; certainly for our company, we sell many products in the United States but there we also export products to other parts of the world, so understanding what those regulatory requirements are is also important to make sure that you maintain compliance. And of course, if there's no standard exists, no requirement from a regulatory standpoint, that's where internal

standards often get established. So, in that case you're looking at potentially what global standards might exist, such as those in the European Union, or other markets. And then of course you're using your own basis of, you know, what does the scientific literature say, with respect to controlling a contaminant? And then on an ongoing basis, you want to monitor the scientific literature for emerging contaminants, what new things do you need to be monitoring for in your ingredients are in your foods? And then of course changing findings: when does the science start indicating that a contaminant is more problematic than previously thought, or where exposure levels are found to be to be different than previously thought as well? And then specific to Nestle. In 2010 we did adopt an internal global standard that affects you know many contaminants and it's based primarily on EU regulation and of course these would be in addition to any local regulatory requirements. We can move to the next slide.

And this is just a quick comparison of FDA, European Union, and the Baby Food Safety Act for inorganic arsenic. And I'm not going to spend a lot of time on this slide, I'll just call your attention to the infant rice cereal line that's about midway down. You can see here that the US FDA action level is 100 parts per billion in inorganic arsenic, for this product, EU limits are 100 parts per billion, but the basis is different in the EU, the basis is 100 parts per billion on the incoming rice used an infant cereal, where on the FDA requirement is for the finished product, making the FDA requirement, more, more restrictive. And I'll explain a little bit more about why that is later on. And then the Baby Food Safety Act levels, this is legislation has not yet been enacted into law but the level four in that legislation is 15 parts per billion for inorganic arsenic. We can move to the next slide.

So, the next, you know, as you heard my background is not toxicology, I'm not a toxicologist, more of a nutrition person, and so historically you know when we've worked with our recipe developers, we you know we usually think about it from a nutrition perspective, the whole grains, brightly colored or dark fruits and vegetables, how do you increase the nutritional value foods that you're making for young children, but having gotten more involved in the contaminant issues, I, I can see the other side of that so this is a little bit of an exaggeration on the slide, but it's meant to just illustrate the fact that, you know, previously, you know few years ago probably would have said whole grain brown rice is, is the ingredient we should be using. And now, we probably would not say the same thing right with the toxicology hat on.

Same for sweet potatoes versus white potatoes. Sweet potatoes are an excellent source of vitamin A and beta carotene, and usually would, you know, for most people would say, you know sweet potatoes are preferable to white potatoes from a nutrition standpoint, but from a toxicology standpoint, maybe not so much. And of course, spinach, you know the favorite food of Popeye, packs a real punch with respect to nitrates and cadmium, and so you do have to manage that both in your ingredients and the level and your recipe and so all of this was really meant to say you know recipe design is another way to manage contaminants in foods, by looking at the incoming ingredients as well as the choice of ingredients, as well as the amount of ingredients that you use in your food. We can move to the next slide.

There are other recipe considerations; fortification, we talked a lot about infant cereal earlier. So, fortification does help address nutrient gaps in the population and we do use the bits data to help us assess when fortification is appropriate for foods for young children to help them meet their nutrient goals, but it's also important to look at maintaining intake; so we saw that infant cereal does contribute quite a lot to adequate intakes in the population. And so, changing the fortification of infant cereal could have an impact on that and has to be considered as you're thinking about changing fortification or removing certificates from a product.

At the same time, you have to look at these fortificants, especially those that say like calcium that comes from the ground and what potential it has to contribute contaminants to your food and so again looking at both the positive and the and sort of the contaminants that go along with fortification has to be balanced in your recipe and really it's just an indication that every, every ingredient that you're using, in a recipe for a product has to be considered with respect to its, its nutritional value as well as it's, it's contaminants that's coming along with it.

And then of course we heard a lot earlier today about water, and the importance of water quality I would say to heavy metal intake. And so, water sourcing and testing is also important when you're making food products; are you using a local water supply that's making the drinking water standards and it's routinely tested? Are you looking at those test results? If you're using well water for your manufacturing location, do you have a monitoring program in place? And how are you ensuring the quality of the water is maintained to drinking water standards? And then of course you can look at our reverse osmosis or our water treatment aspects that you could put in place in your factory or other treatments such as carbon filtration.

And then the last is processing. Processing can do two things: it can help remove contaminants, such as the washing and peeling of fresh produce, so washing helps remove the, the soil that might have contaminants in it from the produce and of course peeling helps remove those contaminants that might concentrate in the outer part of the fruit or vegetable. The processing can also concentrate contaminants via loss of moisture. A good example of this would be a fresh apple which might have about 85% moisture and you dehydrate that Apple down to make an on the go, dehydrated apple snack, you're going to lose a lot of moisture, but you're also as a result concentrating both the nutrition and the contaminants that are in that apple.

I mentioned a little bit earlier about the FDA standard for inorganic arsenic, being more strict than the EU one and one of the reasons is because of the loss of moisture. Incoming rice when we process infant cereal does lose some moisture, which doesn't affect, contribute to slightly increased level of the contaminants that are in the rice. And so, you have to take that into consideration when you're sourcing your rice and using it in your recipe. We can move to the next slide.

So, all of that really leads us to ingredient selection and sourcing. Specifications for ingredients and finished products are of course, a very important part of this. Specification is really the agreement between, say the manufacturer and supplier of the

ingredient for what are the key elements that you want and that ingredient, what are the things you're looking for to have a positive standpoint, and what things need to be controlled, perhaps from a negative standpoint, whether those are microbiological or other contaminants, such as toxic elements or snake or lead, for example. And then of course I mentioned already, but you need to consider what's really relevant for the ingredient you're, you're purchasing. What contaminants might be present, and how, how do you need to limit them in your ingredient in order to ensure your products meet your internal standards or regulatory requirements? And that requires knowledge of ingredients and what could be possibly present in them.

And of course, working directly with suppliers to meet your requirements. Producing baby foods we have certain requirements contaminants, then say, going into a product for an adult person, and explaining that and working with suppliers on understanding those requirements and why they are what they are, it's really an important part of the ongoing work with, with ingredient suppliers. And then of course, audits and testing of ingredients is also an important part of sourcing and in the. We talk a lot about you know finished product testing and ingredient testing, and I usually think that you know testing of ingredients, you want to try to test and control your ingredients as close to the source of potential contamination as you can. So, for many ingredients especially agricultural ingredients, the level of, you know the place of contamination happens, typically, where the crop is grown, so at the field level. So, as far as you can get upstream in that process, test your ingredients to make sure that they your requirements is ideal. Because basically you want to you don't want the ingredient coming into your facility if it's not going to meet your requirements, so that's why testing of ingredients is a very important part of the control process. Testing finished products is a great confirmation and verification that your, your process is working, but it shouldn't replace on testing of ingredients.

And so, some specific things that Gerber does. We do work directly with many growers at the fresh produce, particularly at both the farm and field level and this allows us really to really understand the growing conditions, and the soil that is being used to grow produce that we use in our baby food. And then we hold annual grower conferences, that's led by our agronomy experts in Gerber. And this really allows us the opportunity to share changing requirements and best practices, as a group, and of course sponsoring research is also part of what we look to do. We can move to the next slide.

So, I want to spend just a couple minutes talking about factors that affect soil contaminants. And I'm not an expert in this by any means but I know it's one thing that we're always looking at the soil, you know what, what is the heavy metal or what's the toxic element that's in the soil that needs to be controlled for, and what could be affecting that? So, we look at adding say a new supplier or a new grower to, to our business, thinking about what the land was used for prior to growing the produce that we're looking at, what was what was on the land before? Was it used for cotton or tobacco which potentially could have resulted in the use of arsenical chemical pesticides? The second one is a new one little bit of a new one for me; I just kind of found out about this a few weeks ago: arsenic in chicken feed and the fact that if a land was used for chickens, or chicken farming them, chicken production is I should

say that it's possible that at least back in the 40s that arsenic was in the chicken feed could have wound up in in the soil as a result. And then of course, arsenic and other toxic elements, occur naturally in the soil and in the rock. And those may have nothing to do with some manmade activity, and then geographic location also plays a role, especially with respect to percentage of inorganic to organix arsenic in ingredients.

But soil levels isn't the only factor that affects what's in the crop and so this is really I think an area where more research is needed. We do monitor soil levels and we know, you know what the, say the, the arsenic or the, the lead level is in the soil for some of our produce. But that's not the only thing that affects uptake. You could have the same level of a, of a say lead in the soil but the uptake by the crop could be very different depending on things like environmental conditions, was the plant stressed during its growing season? Of course, you won't know that till after the growing season, was there a drought or was there some condition that stress the planet that might cause it to uptake more from the soil? The overall health of the soil in general, soil pH and organic matter, are factors for some, some ingredients. And then there's other factors and I'm not sure again that I know all of these but certainly things like the cultivar, does that make a difference and I think there's some indication of that, the fact that in some cases, called certain cultivars may be more susceptible to uptake and others.

And then for rice specifically we know that irrigation plays a role. In standard flood conditions arsenic is high and cadmium stays low. If you alter the flood conditions of the field, arsenic levels may decrease; the cadmium tends to increase. So again, when you're managing multiple contaminants and multiple things with your ingredients, keeping an eye on all of those is really important. You don't want to trade off, say, one problem for another and so keeping those all in perspective as you're working on ingredients sourcing is an important aspect as well. And of course, the, the more research, I have to have to emphasize, that I think the more we, the more we do and the more we learn will allow us to get better at lowering these elements in in food products. Move to the next slide.

I'm not going to spend a lot of time on this slide I didn't want to acknowledge this is coming from the University of Arkansas study, but I wanted to say the x axis is total arsenic, and the y axis is the percent that’s inorganic, and you can see that it's, it varies widely, right, so the example might be if you have 300 parts per billion of total arsenic, your percentage of inorganic is about 30%. If you move lower on total, it's a much wider range of what could possibly be inorganic arsenic and so the whole point of this is just saying lowering total arsenic may not result in a comparable drop percentage wise to inorganic arsenic levels so keeping an eye on both of those and really understanding you know what's happening with your ingredients is also important. We can move to the next.

And then just a quick knowledge of what you know what we're doing for infant rice cereal. We do source rice from a long-term strategic supplier. This allows us to for the supplier to work directly with growers to the field level and help maintain the identity and this really allows us to work with, with our supplier and their growers to incorporate

different practices to help lower inorganic arsenic in the rice that we use for infant cereal, and this has been a very successful program that we've been working on for quite some time and so does the whole point of mentioning this is it's a, you know, this, this takes time sometimes to find the solutions at the field level. And it's important to that we keep, we keep working on it.

And finally, the last slide, I guess no discussion on mitigation is complete without discussing, you know, education and variety. It's a very difficult subject to talk to consumers about things like arsenic and lead, but they really do want that practical information and guidance, so they can make better choices for their families. And so, one of the key things obviously that we need to continue to work on is lowering levels of substances like arsenic and lead. But at the same time, we need to make sure that we try to lower exposure by encouraging a variety of healthy foods in the diet, and both the American Academy of Pediatrics and FDA do a great job in talking about the need for a varied and nutritious diet, your children shouldn't just be eating rice and infant cereal; they should be eating a variety of grains. And so, we do try to reinforce that message as well when we're talking with consumers and in our educational materials. So that's the end of my slide, and we can go to the next.

So, some acknowledgement Trenton Roberts from the University of Arkansas graciously allowed me to use a couple of his slides and his information. Todd DeKryger, who's our regional manager of sustainable agricultural development, and Susan Pac who's a part of my team in the regulatory and scientific affairs group at Gerber products and then kind of buried after Todd's name is Kristin Finn, who's also in our department, and a nutrition scientist. So, I'd like to acknowledge their help with this presentation today. Any questions?

Flannery: All right, Cheryl Thank you so very much for your presentation, the information is extremely useful to all of us. And so, we really appreciate you taking your time to, to give it to us. So, now I'd like to welcome everybody. If you have questions for Cheryl to please send them to Jason Aungst in the chat. And so, I will start with the first question, and I was wondering, Cheryl, is there a way to process out arsenic, like whether through certain processing steps or filtration, is there a way to process it out of the product?

Callen: I don't think we found a way to process it out. I know there's been some reports of soaking rice and that type of thing when you're cooking at home on, that doesn't work for infant cereal. What we have found is probably, you know, I, again, not the expert here, necessarily, but the things that work are sourcing, going to the field level and finding the, the rice that's lower in inorganic arsenic, and then, oftentimes that sometimes means trading off whole grain versus non-whole grain rice. That does make a difference as well. When you remove that bran layer, it does reduce the arsenic and so you do have to, kind of, sometimes, you know, use that as a solution as well.

Flannery: Thank you, Jason. Are there any questions for Cheryl from the participants?

Aungst: So, what other type of sourcing is there for baby food ingredients like root vegetables and spinach? Is there other specific arrangements with growers similar to rice?

Callen: Yes, so we do work with a lot of growers directly in sourcing so it's a similar like process that we would do with, with rice, a little bit different because we work directly with the growers in produce, with rice we're working through a supplier who works with growers, so we do have a very similar but a little bit different relationship so we do work directly with, with growers on the produce side. Not every fruit and vegetable that we have that program for but we do have it for many of our fruits and vegetables and of course what we don't have that type of grower relationship, that's where your specifications and your suppliers become very important, in making sure that they're that you're giving them clear guidance on how to control the contaminant and then that they work with you to meet those requirements.

Aungst: Thank you. Does ambient temperature have a significant effect on level of arsenic and rice grain, and so what are those implications?

Callen: I don't know the answer to that I could, I could try to find out. Are you talking about in the field, or in the product?

Aungst: I'm assuming this would be in the field. I have a feeling this is related to increase in temperature for due to climate change.

Callen: You know I don't know that we've, we've seen that happen. But I could certainly, you know, ask about that and try to get an answer, answer back.

Aungst: Thank you, and next question is, what would you recommend we balance the benefits of a food product such as whole grain rice for infants, and the cons such as higher arsenic exposure? So, whole grain rice offers more nutrients; does this outweigh the possible exposure of arsenic?

Callen: Yeah, that's a tough one, and I just tried to touch on that a little bit with my you know my nutrition toxicology slide, I think it's, it's probably a balancing act right. We don't want to exclude nutritious foods from the diets of young children. You know things like spinach and rice whole grain rice or even rice that's fortified so that variety messages incredibly important, but at the same time, you want to try to get levels and slow as possible, so I think it's always a bit of a discussion about when, when does the whole grain, whether it's rice or another grain, become important and how do you manage the contaminant that may come along with it? And so I don't have a real clear straight answer other than it's a, it's a balancing act and you need to try to use those really nutritious whole grains, if you can get the contaminants down low enough to meet your standards. If not, you probably need to be taking other steps.

Aungst: All right, thank you. Those were the questions we had in the chat.

Flannery: Alright. Thanks, Jason. So, I have one more question, Cheryl. So, you discussed many strategies that Gruber uses to mitigate arsenic in its products. I'm wondering, is there any one strategy that's just the best bang for the buck? The best strategy? Or is it really just you have to approach it with all of these different strategies?

Callen: Think it's probably all of the strategies I think for rice in particular and for instance you have found that working, you know, with our supplier is really critical to ensuring ongoing supply and to lowering levels further. So, I think that's where we have found a lot of benefit to that relationship into that process and having a partner. And not just a supplier but a partner in that process, that they understand the importance of what we're, you know, we're all working toward. And that has been very successful, and I do think it's important, but it's really all of the things: you have to do the testing, you have to have the specification, you have to have the supplier relationship, with the grower relationship. And then you have to design the food that that's going to meet your standard and ideally move the standard lower over time.

Flannery: Thank you. Thank you.

Aungst: And we're having a couple more questions if we have time.

Flannery: Yeah, we have plenty of time actually.

Aungst: One question is about, is there ability to separate from epidemiological studies contributions from food as complete as compared to water? And with adverse health outcomes. I see that being very difficult because of the water is one of the sources to get into the food, but I don't know your thoughts.

Callen: I don't know that I'm probably the best person to answer that, maybe some of the other folks might have a better sense of whether that can be teased out from an epidemiological study. I was curious earlier that you know when we're on the studies that are looking at water and the contamination of arsenic in the water, I was also curious if there was also arsenic in the in the foods that were being eaten in these in these groups in Bangladesh and in Mexico, so it's also, I was curious about that as well but I don’t know whether you can tease that our in an epidemiological study.

Flannery: Cheryl, I did write that down for the panel.

Callen: I'd love to know the answer, but I don’t.

Flannery: All right, Jason, are there any other questions?

Aungst: That was it. Thank you.

Flannery: All right, well, thank you again Cheryl. We really appreciate your presentation

Summary: Brenna Flannery, PhD, US FDA, College Park, MD

Roundtable Discussion
Moderators: Co-Chairs
Discussion: All speakers
Additional Panelist: Jeffrey Gift, PhD, US EPA, Research Triangle Park, NC

And so, we're running about 20 minutes early but we're going to go ahead and move into the roundtable discussion. So, before, before we do that or while we're getting everybody's cameras turned on for this session, I would just like to provide a recap on the speakers' content. So, I invite you all to please submit your questions for the speakers for the roundtable session, we do have a few already, but into the chat box to Jason Aungst.

And so, I would like to thank all the speakers for their contributions today. We have heard the latest knowledge on arsenic and children's health, with two research presentations from academia, one from government, and one from industry. We began with Dr. Conrad Choiniere, who emphasized why it's important to understand the adverse effects of arsenic and children, and how this relates to FDA's Closer to Zero initiative.

Next, Dr. Rebecca Fry showed us that in humans, prenatal arsenic exposure can alter gene expression through epigenetic mechanisms, and that this may result in reduced fetal growth and increased risk of childhood infections. Her research also suggested that through changes in gene expression of imprinted genes, preconception exposure to arsenic could have lasting effects on offspring in mice.

Then we moved on to Dr. Mary Gamble, she discussed one-carbon metabolism and how her research shows that nutrients such as folate and creatine influenced one carbon metabolism leading to changes in the arsenic metabolite profile and reductions in arsenic concentrations in blood, which can then reduce arsenic toxicity.

Dr. Alex Larsen and Jeff Gift shared information from the EPA IRIS draft protocol for arsenic for systematically developing a reference dose for arsenic. She also shared ongoing work related to developing those response curves for children's health effects including neurodevelopmental toxicity and pregnancy outcomes such as fetal and morbidity.

Finally, we had Cheryl Callen provide us an industry perspective and how arsenic is mitigated in foods produced by Gerber; these strategies included but are not limited to funding agricultural research, providing grower education, having internal specifications for contaminants with the goal to minimize, and selecting suppliers that meet internal specifications.

And so, with that, I would like to invite all of the speakers to turn on their cameras. And I would also like to ask Aaron if you could please advance the slides to the roundtable discussion part. Wonderful.

And so let me go ahead and see if everybody is on here. Alright, so again I invite everybody to put their questions into the chat box to Jason specifically, and I will actually start with the question that was just asked during Cheryl's presentation, and the question that Cheryl had asked as well. So, I'd like to ask this to all of our panelists, is there a way to tease out arsenic exposure from water versus arsenic exposure from food in the epidemiological studies and thus the adverse outcomes? And how does one do that if there is a way to separate that?

Jeffrey Gift: I guess maybe I can start. I don't see how you can do that without studies that look at the increase in responses relative to dietary increases outside of water. I'm only aware of one that would have been used in our meta regression analysis, if it hadn't been focused on principally dietary, and that is a study out of Japan so auto study. I'd be interested to know if there are other studies that might be useful in that regard but that's the only one that comes to my mind.

Flannery: Thank you. Anybody else?

Mary Gamble: We mentioned there [inaudible] had a study in Bangladesh where they had good information on concentration of arsenic in the water and good information on the concentration of arsenic in rice and tried to look at the relative contribution of the various sources. But I am not aware of any studies that have been gone on to link that to health effects in children.

Gift: Right, and the study I referred to was also in adults.

Rebecca Fry: And I was going to comment. Similar to Dr. Gamble that the where, where you have to differentiate between the exposure sources is measuring what's in the water, and then trying to understand what's in food, because once it's in the body, we can't distinguish between food based or water based inorganic arsenic contamination.

Flannery: Okay. Great. Thank you. So, next question. How and this is more directed towards Cheryl and Mary, though I think everybody's opinion is very valuable.
How could consumers be educated on selecting foods that improve health and reduce the effects of arsenic exposure?

Cheryl Callen: I think what I mentioned sort of on my last slide was the, the idea of a variety of foods and I know with rice cereal in particular for infants, stressing the importance of a variety of grains, whether its oat, or wheat, a multigrain product, barley there, there are many choices in the marketplace and so I think making sure that, and they're all fortified the same, you're not trading off on the you know the fortification for these infant cereals among manufacturers, so it's I think getting that message out about

variety and diversity which is sort of true starting, you know, obviously we talk a lot about that for the adult diet but making sure we're equally reinforcing the importance of the diet for young children, and I know with a you know the Dietary Guidelines for Americans and birth to 24 months is now part of that, is really reinforcing the importance of nutrition at these very young age ranges and making sure that you're providing a variety of foods to help achieve both nutrient intakes as well as minimize exposure.

Flannery: Do you have anything to add to that Mary?

Gamble: No, I don't think so. Thanks.

Flannery: All right, Jason. Are there any questions from the participants for the panelists?

Aungst: Just one so far and general one for the whole panelists there. So, how can we best use a toxicologist to help figure this issue out and make a good decisions?

Fry: Can you restate that question? Sorry.

Aungst: I think we've heard a lot about epidemiology and nutrition, and I think the question is how does a toxicologist fit in here, moving forward and evaluating arsenic? And, of course, this is going to be relative to other metals and contaminants.

Gift: There was a question, relative to Alex's, Alexandra's talk that didn't get answered, I think, having to do with threshold. And while our assessment is undergoing agency review or someone will undergo go agency review, I can't get into the specifics of what EPA feels about that, but certainly toxicological considerations need to go into the determination of whether for a given effect there might be a threshold. And I should point out the methods that I that are described in the papers that I mentioned the Allen et al. 2020. Those meta regression method methods, there's two papers actually, one which describes the inputs, and one describes the modeling. They, they do talk about deriving extra risk values, and we need to do that because we don't know the contribution of arsenic to background risk. But there are considerations, I think, from a toxicological perspective on how those methods were developed. In addition to the determinations going forward from the results of the model. And the derivation of RFDs and slope factors. So, yeah, I guess that's just started answering question.

Fry: I would agree that we're going to, we need to focus in on dose response assessments, adverse outcome pathways, thinking about that chain of events that links the exposure to the various outcomes, and toxicology is so important to understanding mechanisms underlying arsenic induced disease, so it’s absolutely essential.

Aungst: The questions are coming in here. Now. The next one is about absorption rates of integrating arsenic, from food and water, whether you know the values and are there certain commodities that contribute more to absorption and body burden?

Gift: I guess I'll start again. I guess I will just say that that's not my area of expertise, but we do have a couple of papers that describe EPA arsenic PBPK model. And I'm sure that that question is addressed in there at least to some extent. And I, the papers are, I'll think of it in a minute, who's the primary author on that, maybe Alex knows, but I'll remember it soon and get back.

Flannery: Thank you, Jeff. Anybody else? All right.

Aungst: So, going along with that, are there any studies have been conducted looking at, iron, as protective nutrients?

Gamble: Not that I'm aware of.

Aaron Barchowsky: Mary, there's a little bit of literature on Selenium, isn't there?

Gamble: Yes, there's more than little literature on selenium. It's a whole other, more complicated story.

Aungst: Interesting. So, the next question, how can effects of dietary exposures to arsenic through mothers during pregnancy on outcomes in the fetus be compared to exposures of, compared to direct exposure of foo, to young children, so we can compare or understand relative effects?

Gift: Before answering that question, I'll say that I remember the author first author on the PBPK model paper, it's El Masri, EL MASRI, and that's an adult PBPK model, it's not a, a pregnancy model. But they may address the question that was earlier mentioned.

Aungst: Thank you, Jeff,

Flannery: Can you please read that question again?

Aungst: I think the question is getting at comparative effects between fetal exposure and direct exposure to the children.

Gamble: Yes, I could say that the correlation between blood arsenic and blood arsenic metabolites between mother and cord blood is nearly perfect, if that helps to explain the exposure to the mother equals the exposure to the fetus. As for comparing toxicity, Rebecca, do you have any thoughts on it?

Fry: We've done a little bit very recently, where we're looking at gene signatures in placentas is exposed to inorganic arsenic, mono methylated and di methylated forms, and see some differences where perhaps not surprisingly MMAs seem to have a strong effect in the placenta as well, but as you say, and I think related to the question that I see in the chat, do all the forms cross the placenta, the answers yes, but they may have differential toxicity to the placenta.

Flannery: And this is Brenna, and I'm going to give my opinion if I'm allowed to do that too. So, another thing when I think about the studies comparing toxicities as well, one thing is that when you're looking at the exposures in children, is that they also will have may have been exposed in utero too and so how do you tease that out then from the adverse effects that you see from children's exposure? And so that's another, I guess, challenge I would, I would say as well. Do you, Rebecca or Mary, have any comment on that, about how that can be done can it be done for epi studies, or there's really?

Fry: I mean the one, yeah, you're bringing up a good point, which is that it's hard and human populations to isolate these very specific developmental windows of exposure. When you likely have in utero and childhood exposure in the, the story, or the science that I presented from Alan Smith's group gets closer to that, where you know there was sort of a natural experiment of high-level arsenic and then that changed and was reduced in Antofagasta, Chile, but still not able to isolate the difference between in utero and childhood exposure necessarily in that study. I think, the way that we try to address it is using translational science and mouse models to, you know, to really point to the precise developmental windows of concern. Mary, I don't know if you have other thoughts.

Gamble: I know that there have been studies in Bangladesh in children's cognitive function where they because they were part of a large cohort, they knew maternal arsenic exposure over many years, and tried to segregate that out, maternal versus postnatal. But in the end, it's, it is a very tricky thing to accomplish.

Flannery: Thank you. I guess going off of that question then, that was just asked. For all of you, experts, where are the data needs in terms of arsenic exposure or inorganic arsenic exposure and adverse health outcomes in children, and in utero? What don't we fully understand yet, where are the data needs? And then we can even go a little bit further to ask about potentially all forms of arsenic, and the data needs for all forms of arsenic and adverse effects? I was wondering if each of you could please comment on that.

Fry: I can, I can start and say that some of the data that I presented at the end of my slides, point to this concern about preconception exposure. And one of the things that we don't yet know because we in the mouse model research we exposed, both the mother and father. So, we're working to isolate whether or not there could be paternal, we have reason to believe through sperm-based biomarkers that paternal exposure could be associated with offspring health. So, to me that's really important and significant scientific gap, we're talking here about, you know, maternal exposure that can influence the developing fetus but the idea that paternal exposure could influence the health of offspring I think is really fundamental change. And then, you know, I think about the fact that we don't measure or ask questions about arsenic in potential drinking well at least we don't in North Carolina in the clinic and pediatric clinics, so I see scientifically I'd like to try and fill this gap related to paternal exposures. And then, related to translation, think about how we can begin to monitor better or ask questions in the clinic that could help protect and identify exposures for children.

Gamble: From a different perspective I would add that it's become increasingly clear that we don't have a lot of information on the effects of arsenic exposure through food and how linking directly to health outcomes and study populations that don't really have high arsenic in their water supply.

Gift: I'll add a couple of things. One is a bit of a pet peeve of mine in that is that I feel like epidemiologists need to make a more concerted effort to identify intake of arsenic as opposed to the simpler thing to do, which is to measure the relationship between the effect and water concentrations. But that, in especially in the United States, that's difficult for me to wrap my mind around, because in the United States we have an educated, relatively educated population, they hear that arsenic in their well is potentially contaminated, there are so many other sources of fluids, and the information is out there and much more really obtainable by them; they're not like Taiwanese farmers that they can only get their drinking water from a drinking water well, that they, that's nearby. So, I think it's important, especially in places like United States for epidemiology studies to get a handle on intake, whether that's from surveys. Probably it would be from surveys, and I know that that is fraught with difficulties, but I think that that attempt needs to be made. The other thing I was going to mention is with respect to the one study we have on neurological cognitive effects that were will say one study, that's the one study we're focusing on in Maine, the Wasserman study, it'd be nice to because of the fact that that study is not we're not able to do a dose response analysis on this study because of the non-monotonicity of the data, we'd like to see a repeat of that type of study and looking at older children. Following the children, longer than they did. I think they only looked at up to 10, 12 years of age I can't remember, but it was a.

Flannery: You're interested then in the study looking at older children to then look at more, I guess their cognition as they get older, or even their IQ as adults? Is that what you're particularly, you would be useful?

Gift: Right.

Flannery: Thank you. Cheryl, are there any data gaps, I guess on from, from your perspective in terms of, it would be, you know, in terms of I did ask the question in terms of adverse childhood effects, but also one could ask the question regarding mitigation in terms of what, you know, are there any data gaps? And what would be useful for you all to know and understand?

Callen: Okay. Yeah, I did have a question to us on the studies that, like, if we do another study, would it be important to, I guess I'm going to ask a question to the panel myself. Would it be important to be looking at you know, total nutrition adequacy of the diet? I mean, just kind of building on sort of what I've been hearing today which has been fascinating. Do we need to be looking at the nutrition intakes of the children as well and saying, you know, do they have any gaps in nutrition adequacy relative to, as well as arsenic exposure, I mean, would that be important information? I guess not necessarily doing this research myself it just seemed like an interesting aspect to be thinking about, you know, just looking at, say, arsenic in the blood. What else is happening with the child, what does the total picture look like, especially in the United

States where it's different, it's a different population, as was mentioned earlier? But to answer your question about mitigation, you know, I have to give it some thought. We've spent a lot of time on arsenic with respect to juices and rice, those have been our focus in the areas where we've really tried to lower arsenic levels, and I think it's probably trying to do more research I would say set at the agricultural level, what other techniques can be used? We've found success with the irrigation strategies and those haven't, you know, we're just now putting some of those practices in, and in place and so continuing to monitor the effects of growing conditions and different agricultural techniques in mitigating inorganic arsenic, either the ratio of it or the uptake of it through the growing practices specifically to rice.

Flannery: Thank you, Cheryl. And, Cheryl going back to your question then, that you just posed, do we need to be looking at nutritional nutrition adequacy? How would, this is more geared towards, towards Jeff. But I guess how would want to count for something like that in a dose response? Can it be accounted for? How would one do so? And is that something that needs to be done?

Jeff: I'm sorry, Brenna. Can you repeat the question?

Flannery: Sure, sure. Yeah, so I'm going back to Cheryl's, I guess the question she posed about do we need to be looking at nutritional adequacy in children and when I think about that, I guess what I'm what I'm asking is, is, how would one incorporate something like that nutrition, whether it's you know calcium status or folate status or various things in dose response curves or I guess account for that, I guess? Yeah, I guess account for that for in the adverse effects that you that one sees?

Gift: From my perspective, there's not a real good way to do that at the back end if it's not done in the study. The study looked at folate as a factor and adjusted their dose response relationship for that factor. Then, we could use that information. You know there's, but we would want to make sure that there was that arsenic doesn't affect the folate levels. For instance, you know they're, I'll give an example from the cardiovascular effects paper, Moon et al. They looked at diabetes as a confounding factor for cardiovascular effects. And that model was one that we were reticent to use because arsenic can actually cause diabetes. So, you know factoring it out might not be appropriate. So, folate, you know, for instance, if there's a study that actually factors that in as an influencer, and that we would use, I think,

Flannery: I got you, what needs to be incorporated into the design of the epi study you're using. And so perhaps maybe that's where an answer to Cheryl's question, is do we need to be looking at nutrition nutritional adequacy? Well, perhaps we do but we need to do it early on and that the studies that are that are used.

Gamble: I can comment on that. We have, not of children but in adults to nested case control studies of arsenic and do skin lesions where we have folate status and homocysteine levels that were measured two to seven years before they developed the skin lesions. So, I guess I would ask Dr. Gift is that not, it's Bangladesh work and of

course it's adults but similar studies and children would that be useful. Or is that hard to model and the kind of work that you do?

Gift: I think that'd be useful. Yes. And the difficulty is, do we have enough quality studies to do an evaluation that that's not using meta regression analysis but the question was asked earlier about why we haven't developed the meta regression method for continuous data, and that the answer to that is more that there aren't enough continuous data studies out there to do a better regression analysis, to sort of, justify the probably the resources to developing or applying a continuous meta regression approach. But I guess getting back to this. Yeah. I had have go back and look at the folate paper that you refer to it and see whether it would be amenable to an analysis, similar to smoking versus nonsmoking. We try to do an analysis that is specific to sensitive subgroups, if we can. So, we will do that in our, we, we do enormous amount of sensitivity analysis and our assessment as it is. And if that's a possibility, we will look into it.

Flannery: Thanks, Jeff. Jason, are there, I've noticed there's a couple of questions in the chat I'm wondering if more questions came in for the panel.

Aungst: Yeah, I think one was addressed about mitigation soil. Another one was large epidemiology studies in the US and Japan showed no increased risk of cardiovascular disease or cancer which rice intake. One of the Japanese studies actually showed a decreased risk of cardiovascular disease with increasing rice intakes. So, any comments on this?

Gift: Well, again, our agents your review draft is still going through the review process. So, I can't get too specific but we do have a number of other studies of arsenic in water, where water is principal exposure metric that do show an increase in cardiovascular effects and cancer. They, I think I know the study you're referring to, with respect to Japan, I'm not sure I know this is being referred to otherwise, in the US. But, when in fact there, there were more than one study I think that that show either a flat or decreasing dose response and, but for bladder cancer for instance we have 12 studies so that would be just one study of the meta regression. I guess that's as far as I want to go at this point, probably.

Fry: I guess I would, I would also say that, you know, disease risk is influenced by so many factors, your nutrition, your genetic ability to metabolize arsenic influenced by your poly morphic variants and arsenic three methyltransferase, so much of, so much of our associations, is influenced by numerous factors that make some of these results complex.

Flannery: Yeah, and I'll just build on what Jeff and Rebecca are saying in that, you know, that's why it's very important to look at the totality of evidence that's available in the literature, versus, know, selecting a study for example, and trying to draw conclusions from one particular study. I think that's the strength in the systematic approach that is used to be able to draw conclusions about the adverse effects of

arsenic. And so, it's just yeah critical to take the systematic approach, and to look at the totality of evidence, like you were saying, Jeff.

Gift: And speaking of the totality of evidence. I'll make another a plug for our paper Alan et al. 2020, where we describe the met the attempts that we made, the methods that we used in order to be able to combine as many studies as possible with case control, we modified the response metrics to derive effective counts which allows us to use case combined case controls and cohort studies into our meta regression. We also converted those metrics from the multiple exposure metrics that are used in epidemiology studies, it takes consistency on that but two to one dose metric one intake dose metric estimate, so that we can combine multiple studies together that's unusual relative to other better regression space in the literature. And that allowed us to look a little bit more closely at the totality of the evidence as Brandon was saying.

Flannery: Alright, thank you. Thanks, Jeff. Jason, are there any more questions from participants?

Aungst: No, not yet.

Flannery: Okay. Aaron, do you have any questions, or rather the participant or rather, also the panelists, do you all have any questions for one another?

Gift: I guess I'll reticent to get too deep into this topic but the question about creatinine and its impact on metabolism of arsenic. I'm curious as to whether anyone's working on any more. I know, I think it was, was it Rebecca that said, there was other new studies, that there needs to be more research in that area. I'm just wondering if there's anybody working on the research in that area?

Gamble: Jeff, this is Mary. I'm not aware of other studies on ongoing with creatine and arsenic metabolism. I would like to see more studies; I feel like we don't fully understand the, the observational data. There are some hypotheses floating around that we plan to pursue. One is that we did see changes in our methylation with creatine supplementation, but it wasn't as much as we expected. And so, maybe part of the effect is unrelated to the effects of creatine on SAM biosynthesis, maybe creatine in urine, creatine comes from meat right meat sources so maybe it correlates highly with for example methionine or other amino acids that might influence one-carbon metabolism. So, we're currently working on a metabolism, study, we get we're going to be looking at our treatment effects and one of the things we'll be looking for.
We're doing a lot of targeted analyses but one of the things we'll be looking for is whether there's an effect an effect. And I also wanted to add some, one of the questions after I talked about creatine was, are people looking at this in mouse models. And I just to point out that my mice are extraordinarily efficient in methylating arsenic so it's a little bit hard to extrapolate our human studies to mouse models because we're not as efficient as mice are; there is now a mouse models a humanized arsenic methyltransferase, that might be quite useful for that kind of question.

Fry: I agree and I, I really failed to mention the fantastic work by Beth Collar and Merrick Styblo to establish the humanized mouse model that now metabolizes in a similar manner as humans, so I agree with you, Mary, that'll be a great opportunity for us to understand many of these things better.

Barchowsky: I guess, Rebecca, that's a fantastic development in the field of arsenic toxicity research. One question is whether, I mean, notoriously, we in the mouse world have been accused of using too high doses of arsenic in our studies, and trying to do back extrapolations and cross, cross species modeling of concentrations etc. but is that humanized model, allowing people to really drop their mouse exposures down into the low human range?

Fry: Yes, yes it yes it will, it will allow for lower exposures. That would be similar to what we would find in human populations.

Barchowsky: And I guess the other thing is, how is it, how's it going to affect our ability to figure out what's the equivalent dose between mouse and man? Just trying to get better science.

Gift: I've always been concerned about when I learned that NTP's feed head was high in arsenic, been concerned about the generational effect of feeding animals high arsenic levels, and how that perhaps built up some kind of tolerance and those strains. And I hope that someone's working on, you know, developing a strain that that has a reduced arsenic level or at least an arsenic level commensurate with background levels in the US.

Gamble: Now along those lines, since we're talking about mouse models, typical show diet in mice really high and folate. So, if people don't, don't realize that there's actually talk about reducing that, because it's like way higher than they require. So, if you tried to look at a folate treatment effect and they're already getting a ton of folate in their chow, it may be harder to see.

Barchowsky: I guess that it always reminds me that a lot of the compositions of childhood is dictated by Ethical Treatment of Animals, and I'm sure you get into this with your epi studies as well. You're talking about manipulating diet; is it ethical not to get people folate if you know that the folate's important? Your study was very interesting that you tried to give them good water. And maybe see when things would revert, but they went back to the bad water, but there seems to be some pretty tough ethics, with doing the human epidemiological studies overall.

Gamble: And that's an excellent point. I think at this stage of the game, we would like to have data showing that folate is protective against a health outcome but you're right, we can at this point, at this stage of the unethical to do a big, long term clinical trial where you have a placebo-controlled arm particularly populations where you know there's folate deficiency. The best we can do is nested case control studies where we happen to have bank samples from years prior to diagnosis.

Flannery: Alright. Excellent, excellent points. Are there any other questions, Jason, or any other questions, panelists for each other? Otherwise, we can move into closing.

Aungst: Nothing in the chat.

Flannery: All right, all right, well I'd like to thank all of our panelists, again, for speaking today and thank my co-chair as well, Aaron, for his expertise, and for the participants as well for participating. It's very valuable to have your questions and to ask the experts and so now I'd like to welcome Udayan, the chair of SOT Organizing Committee for this session to go ahead and provide us a closer.

Udayan M. Apte: Thank you so much, Brenna. I don't know if I have these slides but I'm going to basically thank, everybody here. Before we talk about the next one, I want to first of all thank all the speakers for their time and effort. This has been a wonderful, wonderful symposium and a great learning experience. In fact, I'm an academic toxicologist and I've gotten so many ideas that I need to bring to my students that were discussed here in one form or other, so I'm sure this will be very helpful to the FDA staff and, and the broader public listening to this symposium.

So, thank you to all the speakers. Special thanks to Aaron and Brenna. They have been working on this for a long time. I mean, this process started months ago, and they are really the reason this thing has come together, they have thought through the process what they need to focus on, they've thought through the speakers and, you know, worked with the speakers and have really, you know, put a tremendous amount of work in making this a huge success, so thank you both. Really appreciate your efforts and your time here.

Again, thanks to my fellow committee members for helping us shape this out and flesh out a very good symposium that looks like it's gone really well, and so thanks for their efforts as well. Special thanks to Betty Eidemiller, our help from SOT, who keeps the committee and everybody else and the speakers on toe and, and make sure we get our slides together and everything is done on time and all the paperwork is done, especially all the T's and I's are crossed etc., so thank you, Betty. And thank you all this on the part, you know, all the audience for attending.

I would like to, you know, point out that our next colloquium will be on toxicogenomics and its relevance to adverse toxicological effects, and as you know toxicogenomics has been a major topic within Society of Toxicology and other places. There's a lot of discussion, a lot of new science has come up, a lot of that has actually been in this space of environmental exposure and so the focus of this particular colloquium is going to be how toxicogenomics can be used for food and ingredients safety. So, this is a new, new topic as we have been discussing about this, we have realized that this is something that is really less used in the food area and so we are really looking forward to kind of getting some experts together and discussing how we can use toxicogenomics to our benefit and to improve our ability to do better risk assessment for foods. And the tentative timeline right now is April, end of April, early May, something

like that, after Society of Toxicology meeting. So, look out for announcements and more information on this topic, which will be good for coming very, very soon.

With that, I'm going to end. I just want to remind all the attendees that you will get a survey, please do the survey. Your feedback is extremely important, we actually look at that, we consider what you said. And that helps us shape the next, next, colloquia in the series, so please do take the survey. I really appreciate that.

And at this point, thank you so much for attending. I would turn it over to Betty if she has any final comments. symposium.

Betty Eidemiller: Nope, I think we’re good to go.

Apte: Alight, thank you, everybody. I really appreciate it, and have a great rest of your day. Thank you.