

Health Effects and Mitigation of Arsenic Current Research Efforts  
and Future Directions workshop panel discussion webinar series

Part 2: Arsenic and Susceptibility

Speakers

Danielle Carlin; NIEHS; Superfund Research Program Administrator  
John Cowden; US EPA  
Michelle Heacock; NIEHS

Participants

Andrea Allan; University of New Mexico  
Eric Ditzel; University of Arizona  
Craig Steinmaus; University of California at Berkeley  
Karin Engstrom; Lund University  
Carmen Marsit; Dartmouth College  
Molly Kile; Oregon State University

Danielle Carlin: So the goal of these panel discussions is to highlight new techniques and identify data gaps to help guide future research directions.

The panelists each proposed answers to specific questions that were posed to them and the panelists will discuss their answers towards the goal of a consensus answer.

I also want to remind each of the speakers that they have 12 minutes to answer their question and we'd also appreciate the speakers to try and keep their answers to approximately five to six minutes and I will warn the speakers when they have about two minutes left to speak.

We encourage you in the audience to submit your written questions and comments during the webinar as well and later during the webinar we have Dr. Michelle Heacock here with the Superfund Research Program that's going to be moderating those questions and comments.

The session moderator will tell you more about the format – or more information about the format of this webinar discussion. Information from the workshop from this webinar series will be captured in a report that is being written by Dr. Marisa Naujokas from MDB, Inc.

The session moderator for today's webinar is Dr. John Cowden who is a biologist in the – at the US Environmental Protection Agency's national center for environmental assessment. In this capacity, he provides expert scientific review during the development of human health risk assessments. Thank you, John, so much for moderating this session and now I'm going to turn it over to you.

John Cowden: Thanks Danielle. Before I get started I just want to make sure you all can hear me. Is everything okay on your end?

Danielle Carlin: So far, so good.

John Cowden: Great. Great. First of all, thanks again to the panelists and the organizers for putting this together. For those of you who traveled to North Carolina for the meeting, I just want to assure you that today its 92 degrees and sunny outside.

The title of this session is Arsenic and Susceptibility. At the end of the discussion we would like to have consensus answers to the questions. Answers can be lists or description of major points, techniques, concerns, or concepts. Although consolidating complex information can be difficult, it challenges us to discern the most important points relevant to these questions.

We will be following a strict time schedule as shown in this slide. If allowed, we can revisit questions that might benefit from additional discussion toward the end of the webinar. As Danielle said, she will mark time at six minutes and 10 minutes into the discussion, which will end promptly at 12 minutes.

Moderators and panelists will participate verbally in the webinar. (conference instructions).

NIEHS will collect all write-in questions and comments for consideration in the final report.

Panelists were tasked with a single question and their proposed answers will be shown in the slides to serve as a starting point for discussion. We can discuss changes to the proposed answer and from this discussion we hope to capture data gaps in order to guide future research.

I will begin by briefly reviewing the questions asked of the panelists and also introducing the panelists for today's session.

Questions for this panel discussion:

1. What types of mechanistic data are needed to identify novel susceptibility pathways for inorganic arsenic exposure?

And our panelists for this question were Andrea Allan from – a professor in the department of neurosciences at the University of New Mexico and Mr. Eric Ditzel a graduate research assistant in Dr. Todd Camenisch's laboratory at the University of Arizona, the College of Pharmacy, Department of Pharmacology and Toxicology.

2. What types of data on susceptibility are needed to inform the dose-response relationship for human health effects related to inorganic arsenic exposure (for example, variability in the response to a particulate)? What types of susceptibility information are needed to inform cumulative risk for individuals or populations?

And our panelist for question number 2 is Craig Steinmaus who is an associate professor at the world's finest university, which is the University of California at Berkeley. As an alumnus, it requires me to say that every time I introduce CAL professors. He's also an assistant professor at the School of Medicine and Global Health Sciences at the much lesser University of California at San Francisco and he's also a Public Health Medical Officer of Epidemiology at the CAL EPA

office of Environmental Health Hazard Assessment.

3. What methods or data are needed to identify susceptible individuals or populations? Alternatively, what types of data are needed to consider a mechanistic event, a biomarker of susceptibility.

Our panelist for this question is Karin Engstrom who is a post-doctoral fellow with the molecular biology group at the Occupational and Environmental Medicine at Lund University in Sweden.

I don't know what time it is in Sweden but it's probably pretty late so she's not attending the webinar today, but she did submit her proposed answer that we can use as a starting point for discussion and she was also gracious enough to submit some comments on the other questions that I will try and contribute to the discussion.

4. What mechanistic data are needed to inform susceptible life stage exposures, particularly the late onset of health effects following early life exposure?

Our panelist for question four is Carmen Marsit who is an associate professor in the department of pharmacology and toxicology, the section of biostatistics and epidemiology in the department of community and family medicine. He is also a co-director of the program in cancer epidemiology in the North Cotton Comprehensive Cancer Center at Dartmouth.

Our fifth question, what is the impact of differential susceptibility factors on epigenetic regulation? Which factor or factors have the biggest impact on arsenic susceptibility?

Our panelist for question five is Molly Kile, who is an assistant professor at the college of Public Health and Human Sciences at Oregon State University.

We will now proceed to the first question and I will turn over the control to Dr. Allan and Mr. Ditzel.

Andrea Allan: Okay, can everybody hear me?

Danielle Carlin: Yes.

Andrea Allan: Okay, great. First, I want to thank Danielle and all those that are involved in the webinar. I'm pretty honored to be included in these discussions.

The question that Eric Ditzel and I were asked to consider, the mechanistic data and novel susceptibility pathways, I saw it as two parts really, to this question. One is identifying and generating mechanistic data, and then the other part of this is to identify novel susceptibility pathways that are targeted by inorganic arsenic.

I generally am a proponent of more targeted hypothesis driven research so my answer to this question about identifying novel pathways is a bit out of character but there's much value to be gained in using a whole transcriptome shotgun sequencing approach. Particularly I'm interested

in the RNAseq and maybe some global proteomics approaches.

I think both of these approaches are likely to identifying novel arsenic targets. Targets that we don't typically think of. The use of next generation sequencing basically will provide a snapshot of the RNA present and the quantity from the genome at a given point in time, which will be helpful for arsenic because then we can define the amount given and the time point that we take samples from so that you can evaluate prolonged versus acute types of arsenic exposures.

There is an ability also to look at alternative gene splice transcripts and post transcriptional changes this way but also RNAseq will allow you to look at different populations of RNA including total RNA and some of those small amounts of coding RNA, like microRNA. The link between the RNA changes and protein changes in response to arsenic exposure using like a high through-put proteomic technology is also kind of a shotgun approach but you can then follow it up with peptide computational tool – database tool analysis like cQuest.

Pulling both of these together and using a pathway analysis following those would – applying that to both of the data sets would help identify some differentially expressed isoforms between arsenic and control samples. The pathway analysis can help – actually help us interpret the impact of arsenic and the expression changes that happen that are related to arsenic phenotypes. And so it's possible that this – a pathway analysis followed with these more target – genomewide looks would give you new outcomes basically to look at.

The other benefit also is transcriptome and the proteome in the cell are very dynamic and they're going to be highly responsive to the environment. It's not to say that genomic DNA work and array work on DNA is not important, it's just that if you're going to look at something that's changing in response to arsenic, I think the RNAseq and proteomics work will be more responsive and more dynamic. And there are a few recent publications out that have used some of these techniques with arsenic in a limited fashion, Waalkes new paper in Toxicological Sciences looked at microRNA in the prostate epithelial and stem cells, Rojas Lab has a paper in Gene where they looked at a mixture of metals on microRNA expression, and Wang's lab at UC Riverside also had a very nice study looking at proteomics and MMA, monomethyl arsenic acid.

There are a few – several -- recommendations that I would make here. One is that I think it would be helpful to look across multiple concentrations of arsenic and also during different developmental time points. It's fairly common for toxins to display an inverted U or J shape dose-response curve and so low doses could activate a pathway moderate in higher doses might inhibit that pathway.

I think it would be helpful to look at the intersection particularly if we identify novel pathways that are differentially altered by high and low dose arsenic. I think that would be particularly helpful to look at the two concentrations and where those pathways overlap could be a target.

The importance of tissue specificity, I think that was mentioned yesterday by Dr. Argos and particularly pertinent really with gene expression, microRNA expression particularly because it is tissue dependent and gene expression is not uniform throughout an organism's cells. It's strongly dependent on tissue type and cell type, so that's going to be critical. These are expensive

studies, so I think we have to pick those tissue targets very carefully.

The intersection between tissue target is important but also metabolic species of arsenic is critical to think about. I think one thing that illustrates this well is Dr. Jian Lu here at University of New Mexico, he has looked at arsenic interfering with zinc finger proteins and what he had originally identified in a pure population where a cell type derived population was that arsenic displaces zinc from the zinc finger proteins and his conclusion was that it really only targets the cysteine and histidine – three cysteines and one histidine or four cysteine motifs.

But when he then did a more recent study looking at MMA, he actually found that it was the C2H2 motifs that were mostly targeted by methylated arsenic. And as we all know, it's the methylated version that's seen in the mammalian population when it's taken in. We can get distracted if we use too refined a product to study these things.

I want to also say that the time dependence during cell lifetime, these things will change. So it would be good to have these – I know we're getting into really expensive stuff here, but not just a single sequencing experiment but maybe across a time period might be quite valuable.

The second part of this question really is mechanism and I think one of the key things is that we have to be able to reverse or block the damage by interfering with arsenic's effects. I'm not trying to over advertise Dr. Liu here, but that's one of the things that he did where he showed that high levels of zinc could actually then displace arsenic from the zinc finger proteins. To demonstrate that that is a target of arsenic, it's really important to see that it reverses and then reverses the outcome.

For RNAseq one of the ways we might do that if we microRNA changes would be to use viral overexpression or the lock nucleotide versions of microRNA and reverse the effects of arsenic and that would be the mechanistic side of the RNAseq type of analysis. Let's go to the next slide.

There's been very little work on transgenerational effects of arsenic. Human populations most of the epidemiological work there is probably on populations that have had multiple generations exposed to arsenic and somatic cells, epigenetic patterns early in the fetal development, occur and then they get re-established following fertilization. So the embryonic and the fetal development represent a critical period for looking at these environmental stressors like arsenic.

I think this could be a target for the epigenetic reprogramming and arsenic altering that reprogramming. I think we're going to hear more about this from Dr. Steinmaus. The impact also of the one carbon metabolism is very interesting obviously Dr. Marsit has looked at DNA methylation and methylation changes and the correlation with urinary arsenic species.

Again, the real critical finding there is to follow it through with targeted DNA demethylation to prove the mechanism. Arsenic could be altering methylation of the whole host of DNA promoters but to identify an end point and then demethylate that promoter, reversing the damage of arsenic would be a critical step.

Danielle Carlin: Andrea, can we keep it to maybe one more minute?

Andrea Allan: Yes, I can actually end here. That would be fine.

Danielle Carlin: Okay, okay and let's turn it over then to Eric, please.

Eric Ditzel: Okay, well I was covering a lot of the same ground that Dr. Alan has covered.

I think that it is critical for us to take these wide shotgun-type approaches in order to understand the potential metabolomic changes that arsenic exposure may be resulting in because currently in terms of studies that we have available have generally been focused on adults. A lot of that work has been focused on blood plasma, some of it has been done in the liver, but a lot of these tissues seem to have been overlooked or just the resources have not been there for them to be explored.

As this type of data becomes increasingly available, we'll realize that it's important and different in vivo, in vitro studies to be able to look at what very subtle metabolomic changes may be occurring as a result of arsenic exposure. I think that some of the data we've seen in the past few years using this type of approach has found subtle changes that we may be able to look to the past to provide a really good stepping stone into this type of followup studies that Dr. Alan was just referring to where we find a potential hit, we want to follow up with potential rescue studies figuring out exactly what is occurring mechanistically.

And as sort of a jump off point from that, a lot of these types of approaches have been, as I said before, approached with adult studies. I think looking at these potential metabolomic changes that are occurring as a result of early life or utero exposure I think we really need a more complete coverage of those early life exposures and how they may result in changes later in life as well as getting a look at what type of immediate metabolic changes are occurring directly after those exposures early on in life as well as looking at later on down the road what may be a more persistent effect.

I remember yesterday, I think it was Dr. [Earnest] was talking about how we may want to begin to incorporate more of these types of studies. It has, and in general, sort of as looking at how we're looking at more targeted approaches, we also want to see how these metabolomic changes may be potential biomarkers so that we can look at a bigger human population studies. Having this type of data to back up a more targeted approach could help us develop a better set of biomarkers so that we can use this tool box later on in other studies.

Generally, this type of data will help us get a more complete catalog of the type of metabolic changes that we see with arsenic exposure. Could we go to the next slide, please?

And building on that, we've seen a lot of studies with epigenetics of arsenic focusing on – as it relates to the development of cancer later on in life, but I think it's also critical to realize that these early life exposures – just like Dr. Alan was talking about, can result in changes later on in life due to the potential disruption of epigenetic imprinting early on in these early exposures.

This is an area that's ripe for looking at how an early life exposure may be resulting in potential disruption of metabolism, how we may have cardiovascular or potential diabetic effects. In fact,

it's also important to be looking at the transcriptome, like was covered, just a couple of examples from what we've seen at the laboratories at the University of Arizona, we've seen persistent activation of [heteromeric two] of potentially being mediated by arsenic disruption of P97 as well as work that's been done in our lab showing that TGF-beta family receptors and their SMADs transcription factors have been disrupted by arsenic.

There's multiple ways this could be occurring, disrupted localization, potential steric inhibition, and in the case of P97, persistent activation of heteromeric two, which [has] important potential and a development of cancer later on. Using these shotgun-type approaches can help us find a lot more targets that might be out there.

One of the things when people ask me, why is arsenic bad for you, well I think my favorite way of saying it is, it is extremely dirty. Anything with a cysteine can potentially be disrupted, it causes reactive [inaudible] damage and with a compound like this that can cause a wide variety of disruptions, I think using a shotgun-type approach to detect these disruptions is a very good tool in the tool box, although unfortunately we can all hope that as this technology becomes more mature, more widespread, it is not cheap at this point but it's something that's becoming increasingly cost effective and more viable.

It's a very good way for us to identify potential generalized mechanisms as well as maybe identifying it once we have MutliSeq modeling of protein or protein interactions. What might be a more susceptible candidate? For instance, I want to make a point here of looking at same thing – disruption -- that Dr. Alan talked about on how that identification is something that we've taken to heart in our lab.

We're interested in, looking at how on those potential targets, looking at other potential candidates that might be disrupted by arsenic and that type of work.

And with that I think I'll turn it back to the panel.

Danielle Carlin: Okay, for the sake of time, John, we have to move onto the next question.

John Cowden: Okay. That sounds fine. I will turn it over to our next speaker who is Dr. Craig Steinmaus. Go Bears!

Craig Steinmaus: Go Bears! You can hear me okay?

John Cowden: Yes. Coming in loud and clear.

Craig Steinmaus: My question is what types of data on susceptibility are needed to inform dose-response relationships for the health effects of inorganic arsenic and cumulative risk.

I took the approach – I kept in mind the fact that US EPA is working on a risk assessment for inorganic right now, so I took the approach of what sort of data – not necessarily may help us in five or 10 years but what sort of data may help us – help US EPA do this risk assessment that's on-going right now?

As we all know, there's a large body of literature on different factors that may influence a susceptibility to inorganic arsenic. Obviously I couldn't review all of that, so I tried to narrow it down to five major factors, which I thought were the most abundant in the literature and had the most consistent evidence based on human studies.

The first one was arsenic metabolism. Usually arsenic in drinking water is in the inorganic form and when we ingest that goes under goes a series of reduction and methylation steps to monomethyl and dimethyl metabolites. And originally we thought that was a detoxification pathway but more recently we found that there are probably some toxic intermediaries, specifically, MMA<sub>3</sub>, that may be the primary toxic species because they do seem to be at least in vitro studies to be more toxic than inorganic arsenic.

And there has been a fairly consistent, I would consider, a fairly consistent body of evidence that's shown that people with high proportions of MMA in their urine or high MMA/DMA ratios probably have increase of most or a lot of arsenic-related health effects including bladder cancer, lung cancer, and skin lesions.

So it seems fairly clear to me that differences and individual differences in arsenic metabolism are an obvious susceptibility factor and it would be nice if future studies that look at dose-response relationships do incorporate data on methylation. I know a lot of studies do. A lot of the more recent Taiwan studies and the studies from Bangladesh are incorporating information on not just arsenic exposure but arsenic metabolism.

Another factor is genetics. There's a wide variety of genetic polymorphisms that have been linked to not only arsenic metabolism but also arsenic-related health effects. For example there was a recent study in Argentina of arsenic exposed women where they linked [SNIPs] and N6AMT1 to increases in percent MMA and I've probably seen maybe hundreds of individual publications that have linked various SNIPs to various arsenic-related health effects or arsenic metabolism.

But what I haven't seen is a good taking a step back and a good summarization of all that literature and specifically trying to identify which SNIPs, which holo types do we have the most consistent and strongest evidence and quantifying how much risk – increased risk – is associated with each one of those SNIPs or holo types. I haven't seen an overall good summary article on that.

The other factor is diet. Various dietary factors have been linked to arsenic-related susceptibility. Most commonly that I've seen is folate, selenium, and factors related to overall nutrition like body mass index and things like that.

With regards to diet, in my opinion, its – in the past I've seen some people suggest that some of these studies that we do in Bangladesh or West Bengal or some of the older studies from Taiwan and populations where their nutrition isn't as good – I've seen in the past that some authors have suggested that dose-response relationships in those study groups with poor nutrition aren't necessarily relevant to the United States.

But I'm not so sure that's true because we have populations here in the United States that have low folate levels or low or high selenium levels or have overall poor nutrition. Obviously those people need to be protected from arsenic toxicities so just because our average nutrition in the United States is good doesn't mean that we don't have these potentially susceptible groups.

An area that we're working on is early-life exposure and in utero exposure and specifically the effects of those exposures on adult risks, specifically cancer and heart disease and other illnesses. In Chile we found that people that were high exposed in utero or early-life had 18-fold increases in bladder cancer, mortality, seven fold increases in lung cancer, two fold increases in myocardial infarction, a variety of different health effects.

Similarly in mice, Michael Waalkes and his collaborators have shown that even though arsenic induced cancers in mice are difficult to induce when arsenic is given in adult hood. He can induce cancers when arsenic is given prenatally suggesting early life susceptibility.

The other factor is co-exposures. I think there's fairly good evidence that there's a synergistic relationship between arsenic and smoking but more recently, I think an expanding body of evidence is linking other potential co-exposures to these similar synergistic relationships.

Specifically, there was a 2011 study in Bangladesh by Melkonian et al. that showed evidence of synergy between arsenic in drinking water and fertilizer use. And we've found evidence in Chile of synergistic relationships between arsenic and drinking water and various occupational exposures like asbestos and silica. So incorporating those sorts of things into risk assessment and into future studies I think would be beneficial.

To summarize, I just want to hit a few specific points on what I think is needed. I think we do already have a lot of data on these potential susceptibility factors but what we – to date I haven't seen that they are necessarily incorporated into any sort of risk assessments. At least not formally and I think we can get the data to quantify how much of a specific susceptibility factor causes how much risk. If we had that data, then they can be incorporated into the risk assessment process including the US EPA risk assessment process.

In other words, it would be nice to know how much percent MMA causes how much risk – increased risk. Now we have that in individual studies but what I haven't seen is an overall summary of all the studies that have looked at percent MMA and try to come up with an overall quantifiable number of, again, how much change in percent MMA causes how much increased risk in lung cancer, or something like that. So those types of reviews and summaries would be nice.

What I'm going to say next I think is heresy amongst a lot of people but I don't think we necessarily need to focus or limit ourselves to low dose studies. I know that's kind of the hot topic right now is what are the effects of low doses but I think it's important to keep in mind why – the benefits of the high dose studies. The whole issue of the [vapor here] criteria. The whole issue of less likely the entire excess relative risk it due to bias or confounding or the increase power that you have with higher dose studies.

I think there's very good reasons why a lot, if not most, of our major environmental regulations are based on dose-response data from higher exposure studies. I believe there's a very good reason for that. We shouldn't just solely focus on low exposure studies.

The other issue is the results that are coming out on early-life exposure point towards the importance of having good exposure data over subject's entire lives. I think studies that just have a single measurement of urinary arsenic or single measurement of water arsenic – that may miss the most relevant period of exposure. If you collect that exposure index during adulthood you may miss what their childhood exposure was and that could be an important exposure period.

Studies with a single measurement of exposure, they can be okay for assessing associations whether arsenic causes an outcome but as far as having accurate data on dose-response, I think it's better to have – see if we can have data on, and it's difficult, but see if we can have data on people's lifetime exposures.

Okay, that's it.

John Cowden: Well, thanks Craig. Go bears again!

Craig Steinmaus: Yep, go bears.

John Cowden: I'd like to open it up to the panel for discussion and I think this is where we miss Karin a lot because she works in Marie Boner's lab and she did a lot of the polymorphism work, particularly on the methyltransferase gene.

I was fortunate enough after the meeting to ask her that specific question about what's the data that we would need to say are these biomarkers susceptibility? And she said the data that we have right now are two types.

We have data that demonstrates that polymorphisms AS3MT are affecting the levels of MMA and the second type of data that we have are that increased levels of MMA lead to increased health effects but what we don't have is a demonstration of particular populations and having this allele then subsequently developing a higher level of health effects.

I hope that captured sort of what her comments would be and I also wanted to put in a little plug that we just published – there's a review that's been accepted in Environmental Research that looks at a group of polymorphisms that are known to affect metabolism, PMT, GSTO and AS3MT and [ ] in all of those the only one that we found in association with higher levels of MMA was the AS3MT polymorphism that actually changes the protein structure. So none of the intronic ones seem to have an association with higher levels of MMA but we did not go so far as to actually quantify the risk from that increase.

And with that, that was Karin's comments so now I will turn it over to the rest of the panel.

Are there any comments?

Craig Steinmaus: Well, I had one comment on one thing that you said John was that okay, so we have that a SNIP affects %MMA and then we have that %MMA affects risk. Do we really need to connect those for risk assessment? In other words do we really need to show that, okay, this SNIP affects this health outcome or can't we just use those two separate parts? Isn't that data strong enough?

John Cowden: I'm in a delicate situation here as the co-chemical manager for the arsenic assessment, so I'm going to say that we would be interested in hearing how that data could be used to support risk quantification.

Craig Steinmaus: That's a good answer actually.

John Cowden: Are there any other comments?

Andrea Allan: I just had a quick question for you about the early-life and fetal studies. There's been a thought that exposure during fetal life actually programs the fetus to anticipate a world with that level of arsenic that they're being exposed to and that when they're born, the possibility is that you've got a programming event ready to receive a certain level of arsenic and they may be born into a situation where they have less arsenic or no arsenic at that point and that's what causes it to be more deleterious for that animal.

I was wondering in animal studies particularly, when we have – when we set up these animal models is it better to keep the animal on that level of arsenic that they were exposed to in the fetal period or to reduce it? Because those two things may be different. What do we think would be best for the field?

Craig Steinmaus: Could I bring up some data that we have that we just submitted, which is that from our Chile studies, it is somewhat difficult for us to separate out fetal exposure from childhood exposure, but for, it was interesting, for bladder cancer it seemed that in utero or very early exposure was associated with the highest risk but the lung cancer it seemed it didn't really matter where the exposure came. If it came during the fetal period or if it came even later childhood, 10 years old. Either one of those were associated with similarly high risks. I don't know if that helps with your question or not.

Andrea Allan: Yes, actually it does because I do think that points to the fact that there may be depending on what we're looking at there may be a programming protection or no programming event that's protective.

Danielle Carlin: Okay, John we need to move on to the next one.

John Cowden: Okay. I'd like to introduce Karin Engstrom from Sweden, which I will be her surrogate today.

Her question was, basically to answer what methods or data are needed to identify susceptible individuals or populations? Alternatively, what types of data are needed to consider a mechanistic event – a biomarker of susceptibility?

And Karin was gracious enough to actually send her responses to this so I will read through what she wrote and then I will open up the floor for panelist discussion.

The first thing is that translational research is important. It is important to link cell studies with epi studies consisting of large, well-defined populations.

The second point that she makes is it is important to use primary cells for epigenetic studies not cancer cells. Cancer cells change substantially after malignant transformation as compared to normal cells, in particular if the mechanism of action is through epigenetic changes.

The bottom line is that it is important to combine cellular studies with basically primary cells with epi studies in order to get a broader picture of identifying these populations.

Those were Karin's comments, so at this point I'll go ahead and open up the floor for discussion of these points. Is there anyone on the panel that would like to start off?

Andrea Allan: I have a question. Karin's very focused on cell studies and comparing those to epidemiological studies but transformed cells and cancer cell lines are nice because they're very uniform and very controlled but they are very aberrant. They don't represent what a human being actually does when presented with arsenic. Why the focus do you think on linking cell studies and primary cells and transformed cells to epidemiology?

John Cowden: I can try and answer that and I think that this will largely come from the discussions I had at the meeting with her. Her research is largely focused on these – or at least the research I'm aware of, is largely focused on the polymorphisms and these metabolism pathways and I think they've developed a hypothesized mode of action for how these can actually work in terms of taking in the cells and altering the percentages of MMA that are coming out. What I think she's really interested in, is saying are they seeing these types of affects in epi individuals or in epi studies so that they can correlate what they're seeing in the lab with actually what they're seeing from human exposure.

I'm not sure if that answers the question but I think that was the viewpoint she was getting at.

Andrea Allan: Okay. Thanks.

Are there any comments?

Craig Steinmaus: This is Craig.

John Cowden: Okay, go ahead.

Craig Steinmaus: I just wanted to make a point in that, I work at Cal EPA and I'm a part-time reluctant risk assessor and so I try to view this sort of stuff in terms of risk assessment and setting public health standards and we're setting a public health standard on changes in thyroid hormone, which not for arsenic but another chemical which is not an overt adverse health effect

in a lot of people's minds. And that's a big fight for us. Using biomarkers instead of overt adverse health effects in setting drinking water standards.

I think that most people would agree that changes in thyroid hormonal levels are bad but industry association, that's a big thing they will pick on when you use a biomarker instead of a clear adverse effect in risk assessment or in standard setting. I just wanted to make that comment.

John Cowden: And I'll say in the effort to be transparent and fully disclose that a lot of these questions were drafted with the EPA risk management/risk assessment point of view. Basically, these are the types of questions that we struggle with as well. I think, Craig, your antenna picked up on that, I think, pretty well.

Are there any other comments on this question in particular because we've got about a minute and then we're going to move onto question number four.

Danielle Carlin: I think Michelle has a question?

Michelle Heacock: There's a question that came through. In terms of the polymorphisms that are being seen for instance the ASMT3, how prevalent are the polymorphisms and what effect would they have on the risk assessment? Would you always have to look at those? Would you have to genotype as well as going to Karin's comment, look at that – to link with epidemiological studies and the cell studies?

John Cowden: I can try and – to answer that one. The first part is, that's -- basically the question is how do we know about the AS3MT allele dropped population. I'm not sure that much is known. The value of that type of information, in fact when we were writing our paper we got challenged on this, is why do we even care if we can just measure the amount of MMA in the urine? Isn't that the same thing?

And from a risk assessor's standpoint, one of the things you want to be able to do is estimate the percentage of a population that may be susceptible. Getting this type of large-scale genotype data will allow us to say this percentage of the population is likely to carry this allele and may or may not be more susceptible. For us there's a value in that type of information.

I'm not sure if that answered the question directly but to get that type of information, yes, you would have to do some genotyping from some very specific alleles and the challenge is that as we've seen many of these pathways have many different components and it's maybe not necessarily one in particular gene but several different genotypes that could lead to an exacerbated effect. Getting that mechanistic data to understand that would lead us to that exact – that specific question of estimating the percentage of a population that's susceptible.

And on that note, I hope that answered the question. If not, just tell them to e-mail me and I can talk to them later because we'll move on now to question number four and I'll turn the floor over to Carmen. Carmen, it's all yours.

Carmen Marsit: Great. Thank you. I'm more of a molecular epidemiologist, so I've come at this

question from that direction. I think that will play well with what some of the other discussion that's been happening.

I'm always afraid to use the word mechanism because there's always a reviewer that comes back at me and says you're not looking at mechanism you're looking at associations and that is partly true because often in observational studies, at least, we can't really perturb a system. People are exposed and we can try to understand what's happening but we can't necessarily increase or in some case even decrease their exposures unless we have some kind of good intervention.

I think I've looked at this as you need to think about ways to use the kind of information or feature that we can capture to address these issues. One potential way and this is getting at the first point to try to address where are the susceptible life stage exposures and then how to use that information would be to try to create better integrative biomarkers that provide a more comprehensive understanding.

So for example in our studies, we often use toe nail measures of arsenic as compared to using something like a urinary measure. Urinary measures can change from day-to-day depending on how stable the actual level of exposure is to the person. Where something like a toe nail is actually capturing the exposure over a period of time and provides that more integrated approach.

We can think about that even beyond biomarkers of exposure to think about it as more about biomarkers of affect or intermediate affect. By doing really strong studies to understand exposure at various time points during susceptible periods, say the pregnancy period, and doing repeated measures/tests to look at different biomarkers throughout that period, we then might be able to figure out which is the best marker that integrates over that whole period. That might be more useful for capturing what's going to be important for risk, for health effects later on. If we have really strong biomarkers of exposure across the pregnancy period those might be the most useful to relate to downstream risk later.

People like Dr. Randy Jirtle and his colleagues have suggested, for example, imprinted genes might be these kind of good integrated environmental measures that can really get at some of these issues.

On top of that and this is something the other speakers have been mentioning and I totally agreed with, is that we really need to layer the genomic. When you think about genomic layers or are considering different types of biomarkers and mechanisms, Dr. Allan was suggesting greater importance of gene expression and proteomics, and absolutely I think those things have to be incorporated into our studies, but we do also want to continue to consider changes in epigenetic marks as well like DNA methylation, histone modification, and microRNA.

There're a couple of reasons for that. I think part of it is it's interesting to try and understand the mechanism by which these different layers are interacting together and it may be that some of these marks are going to be more stable for use in the long term. DNA methylation is a very stable mar. We can measure that and we can measure it in various populations and in various tissue samples and be pretty confident that what we're measuring isn't really being effected by

some kind of technical variation.

Things like gene expression or proteomics can be highly affected by the way the sample is collected and what's been -- how it's processed. We have to be really careful about those kinds of things. It's important in those steps but it's also important I think that we really want to try to better understand what these marks are doing.

Something like DNA methylation, there's the [canonical] idea that if there's DNA methylation and [promoter] of the genes, expression is reduced so we should go in and try to remove that methyl mark in the promoter and gene should go up and then that would say it's functional. If that doesn't happen, then it's not functional.

Well, it might be that that methylation in that region is not actually affecting the gene that we are placing it next to, that we are saying that it must be affecting. It's sort of a reductionist view that way. Instead it may be that it's affecting things more distally. The chromatin is three dimensional and so I think we have to start considering that some of these epigenetic marks may not necessarily be playing a role right where we think that they are.

That might be calling for studies like these sort of epigenetic QTL kind of analyses where we'd start again, linking demethylation and chromatin changes and gene expression on a more global scale to try to better understand how these different layers are working together.

And finally, I think it's important that we really encourage transdisciplinary collaborations to really inform all of these things together. Animal models are great because you can start perturbing the system. You can change the exposure and look at what's happening downstream from that but I think we have to be careful about translating those findings directly to humans.

Part of it is an animal is the other nice thing that you can actually study very specific tissues and very specific targets. In human studies we don't necessarily have those options. We tend to be more limited to tissues that are accessible to us, things like blood. In babies, we can look at placentas or pieces of their cord.

It would be great if in these animal models some of the parallel tissues were examined so if we do see effects can we -- are there affects that we're also seeing in blood in the animal that we might be able to move into human studies and translate better? Or look in placentas and ask what's happening there in the animals? Again, considering though that these parallel -- we have to be careful about these parallels.

In some cases there may be challenges in looking at those parallels. A mouse placenta is very different than a human placenta. Imprinted genes for example, vary widely between species. The actual genes that are imprinted and the types of genes and the number of genes -- it varies widely and so those are the kind of things that have to be considered. I think this speaks to the need for good communication and real collaboration between the disciplines.

I will not be trying to perform any animal experiments. I have no background in that and I know I would do a really bad job if I tried. I would rather work with people who really do know what

they're doing and then consider how can we take that kind of – those findings and move them into our human studies. That would be a much more useful way to work through these kinds of efforts.

Hopefully there's discussion.

John Cowden: Well, thanks Carmen. I'll open the floor for the panelists. I have some questions but I'll let them go first since I feel like I've talked a lot today.

Are there any questions from the panel?

Andrea Allan: Carmen, I hope I wasn't sounding like I was poo-pooing DNA methylation because I certainly wasn't. I think it's maybe quite useful for biomarker work. I was trying to answer the question about identifying susceptible pathways, so in that case, we're actually looking for things that are perturbable by arsenic in a more immediate fashion.

Carmen Marsit: I totally agree with you and no, I'm not offended at all. (laughing) I think, again, it's about identifying perturbable and short term and long term and, and (multiple speakers)

Andrea Allan: Right, right. There are two different goals there in a way. You need something more stable and something that you can hang onto with the DNA methylation. I think your work is really brilliant so I think it's quite helpful that in my answer about the RNA and microRNA looks is because they're perturbable and I think we could then link that to a mechanistic affect.

Carmen Marsit: Absolutely, absolutely.

Craig Steinmaus: Hi Carmen. I have a quick question, I think. I think you said DNA methylation is stable but my question is, how stable? For example if you had somebody that was exposed 20 years ago and that altered their DNA methylation and then their exposure stopped over 20 years, would you expect them to have the same DNA methylation pattern 20 years later?

Carmen Marsit: You know, I think that's actually a great question, Craig, and we don't know that. I actually think that we have pretty poor data longitudinally on the same person. There's a lot of longitudinal data that's come out, things like about methylation and aging and those kinds of areas that have all been done in cross-sectional studies. So you're looking at different people, at different ages then trying to link that together.

I don't think we know about that – if you have any exposure during one period and you wait 20 years will you still see that. I would like to believe that the exposures that you have during these really susceptible periods like in utero development probably are fairly long lasting because they're happening during a period where these marks are getting set.

I think more my comment on stability was more technical stability. We don't have to be as concerned with DNA methylation that the way a sample is handled is going to lead to the degradation of those marks, that they're actually quite stable. We can study 20 year old samples

that have been in a freezer and in fact you can study 20 or 30 year old [Guthrie] cards that have been sitting on a shelf somewhere and that methylation is likely stable in that sample.

Craig Steinmaus: I see. Thank you.

John Cowden: So Carmen, I'm going to ask a question that has the answer in a minute or less. Feel free to pass.

I'm interested in exactly what Craig was talking about. Let's say that you have in utero exposure that causes epigenetic changes. Would that mean that if you looked for those changes later on, how would you tell the difference between someone who's just been exposed in adulthood versus someone who's exposed in utero based on that epigenetic mark?

The second question is, in utero you still have a lot of cell division, cell growth to do and my understanding is these marks are heritable across the cell divisions so that you could theoretically have a much larger portion of your cell tissue with these repressed or activated genes in response to arsenic. Would that lead to necessarily different types of health effects than someone who has just been exposed when they're 40 years old or something?

Carmen Marsit: Okay so I'll try in a minute. I think actually in answer to your first question might be what you alluded to in your second. Because the effects in utero, for example, may effect early progenitors or early divisions of cells that could then get propagated more, it may be more of an -- like an amplitude measure that might tell us about what's a more recent effect compared to something that may have happened a long time ago.

I also think that gets at some of the challenges of doing transgenerational work in human studies in that you have this layering of these effects. It might have been the exposure that my grandmother had that is now affecting my health but there was also all the exposure that my mother had. I think teasing that kind of stuff a part will be the challenge.

Again, I don't think we have good data towards your second point but again, I think it's going to be about good proportion of effective tells and how that could then lead to those kind of downstream outcomes.

John Cowden: Thank you. That, I think, is really fascinating stuff but in order to stay on schedule so Danielle doesn't hit me, we're going to move onto question number five, which is Molly Kile. So Molly, I'll turn the floor over to you.

Molly Kile: Okay, can you hear me?

John Cowden: We can hear you.

Molly Kile: Okay great. As an environmental epidemiologist, I have a fairly similar approach to susceptibility as described earlier by Craig and Carmen where the bulk of my research really has been investigating the effects of arsenic on epigenetic mechanisms with a real focus on DNA methylation because that's just the most practical. Most of my studies are done in chronically

exposed people in Bangladesh. So we have that baggage that comes with me when I try to interpret these questions.

What we have observed so far is that relatively low levels of arsenic exposure that occurred in utero is associated with altered DNA methylation. We've done studies in paired maternal and infant cord blood samples, which demonstrate that that life stage factor really does influence the magnitude and the susceptibility of arsenic exposure.

Namely that the cord blood had more DNA methylation at the same arsenic exposure than the mother. I think that gets at the last question that Carmen was answering about the layering of effects.

We've also observed that all exposures in DNA methylation in adults who have been chronically exposed to arsenic and have developed skin lesions so we do also show that these methylation marks are – continue to be modified in adult exposures and seem to be related to the development of chronic disease outcomes.

So it's with this background that I wanted to really address what I think are factors that would influence susceptibility to arsenic on epigenetic mechanisms and I was again, really thinking about this with DNA methylation in mind.

All of this really starts with the intersection at life stage so we know that arsenic crosses into the placental compartment. Mike Waalkes groups at NIH really was the first one that showed that prenatal arsenic exposure increased severity of – or increased the probability of cancer later in life as well as the severity of the tumors. That really led the way to a lot of the models that we've been following in human studies.

There's also been a lot of evidence in humans done by Dr. Steinmaus, Craig and Carmen and others which show that these prenatal exposures are linked to an increased risk of chronic disease later in life. So we know that these epigenetic mechanisms that are at play, we just haven't – we really don't know what they are at this point. And again, our studies which was looked at and shown that arsenic in newborns at P16 promoter regions for instance have been modified by early life exposures.

All of these studies really show that the fetus has a unique susceptibility to arsenic induced epigenetic dysregulation and that the life stage during which the exposure occurs would affect both the magnitude of the impact and also the probability of the impact. But there's also other life stages that can confer unique susceptibility, namely periods where rapid cell turnover is happened such as in early childhood, in adolescence, possibly in women at menopause, things where we know there's a lot of biological activity and those rapid cell turnovers might be able to be influenced by toxic [infects] such a arsenic. But we know very little about whether or not those other life stages have a unique susceptibility.

So that's the first point I wanted to make, which is I think there's still a lot of work to be done trying to understand how arsenic influences susceptibility to epigenetic mechanisms at different life stages besides just the fetal compartment.

Second, we know that arsenic metabolism intersects with one-carbon metabolism. This means that folate, B vitamins, Methionine, choline, all those other one-carbon nutrients could very well influence both arsenic metabolism as well as DNA methylation and possibly both.

There's been some research in this area mostly focusing on folate status and DNA methylation in arsenic which comes out of Columbia University's group. It's produced some really exciting results I think, namely that arsenic altered DNA methylation in blood but that it only really occurred in individuals that were folate replete not folate deficient.

This went against the idea that individuals that were folate deficient would be more susceptible to epigenetic modification, which I think the field was kind of believing at the time. So this really interesting result suggests that dietary factors influence susceptibility to arsenic in regards to that DNA methylation and there's really further work that needs to be done to unravel these relationships.

The reason this would be important is because dietary supplementation is often recommended as an intervention for arsenic exposed populations but clearly we have a lot more to learn about these interactions. I do think that one-carbon metabolism and the nutrients involved with that wouldn't be susceptibility factors for arsenic-induced epigenetic regulation.

On the next slide, the final point that I wanted to make is there's also an intersection between arsenic the redox cycle and susceptibility to epigenetic susceptibility. There's been really some recent data that's come out of the toxicology world that shows that oxidative stress influences epigenetic mechanisms, specifically DNA methylation methyltransferase activities.

It's been well known for a long time that arsenic as well induces oxidative stress and inflammatory processes. This could be another mechanism and/or pathway to disease that really hasn't been explored as -- very much. I think it provides a tantalizing new area of research that would indicate that maybe there's other dietary factors beyond one-carbon metabolism that could be susceptibility factors.

All the antioxidants out there in the world, but there's been very little research on whether antioxidants influence arsenic-related epigenetic dysregulation, although I was really interested to hear Dr. Allan talk about zinc because maybe this could be a factor in here.

In conclusion, the reason I want to know about the factors that influence susceptibility to arsenic epigenetic toxicity is really to develop effective public health interventions and we need more studies in this area that actually look at the entire pathway from exposure to epigenetic change to adverse human health outcomes. This empirical data exists in some animal models, namely Dr. Waalkes work in cancer, but it really is currently lacking in human studies.

I would really like to double-down on what Carmen was just saying – that we really need to work hand-in-hand, epidemiologists with toxicologists to really push this field forward.

I also think it's important area of research in understanding the susceptibility factors both in the

three areas that I mentioned and there's probably others. Craig listed a bunch of factors on his slides. This information would be helpful to inform risk assessment, as well as public health interventions.

After all, public health is pretty practical. Ideally, we work toward establishing evidence that leads to regulatory guidelines for arsenic that would be protective of people whether that exposure is coming from drinking water or diet. Epigenetic toxicity in relation to risk assessment also seems like a brave new world. I think it's really interesting to be thinking about that right now.

This understanding that we have that life stages are the most susceptible to arsenic's epigenetic toxicity is also important when we think about public health and interventions because ideally we'd like to protect those people that are the most vulnerable to any adverse effects. If it is really the fetus that is the most susceptible to arsenic's epigenetic toxicities and that those epigenetic changes do lead to adverse outcomes, that means we really need to protect the fetus, which really means we need to protect women of reproductive age. We need to be thinking holistically about how we actually apply this knowledge to improve public health.

It's also exciting to think that epigenetic mechanisms are perturbable. The potential for dietary factors, be they one-carbon nutrients, antioxidants, or zinc, if these influence susceptibility and we need to understand the potential for these dietary nutrition interventions because this could be another tool for public health that could be used to help mitigate some of the adverse health effects that we see in chronically exposed populations such as what I work with in Bangladesh.

With that, I think I will open it up to questions.

John Cowden: Thanks Molly, that was great. I'll let the other panelists start in. Do you guys have any questions for Molly or discussion points?

Andrea Allan: I was wondering if you know of anybody who's collecting information on exposed populations about lead or anemic states if they have anemia, that's particularly for the one-carbon metabolism story but also hyperhomocystinemia. Do those kinds of health questions get pulled in during the epi studies?

Molly Kile: That's a really good question and a really good comment. They do. The Columbia group with Marie Gamble and Joe Graziano, that group has really been looking at the nutritional factors and those kinds of outcomes. We do collect that information and our cohorts in Bangladesh too. We're analyzing some of that now and more will be coming out hopefully shortly. I think Carmen you do it as well don't you?

Carmen Marsit: Yes, we try to collect it – some of that information, yes. Definitely.

Molly Kile: What's interesting too in Bangladesh anyway, there is a very high anemia rate and the surveys that we've done has shown that it isn't iron deficient anemia. It really does seem to be through probably a one-carbon nutritional deficit. So that would also increase susceptibility, as you're saying. It also might be why Carmen and our data never really lined up perfectly

evenly, even though we're using the same approaches. (laughter) Maybe the underlying nutrition of the population is a bigger player than we think.

John Cowden: Are there any questions from the panel? I have one point that Karin brought up on her slides and that was also the consideration of sex differences. I was wondering if that – if you thought that may play a role in these differential susceptibilities?

Molly Kile: Oh, that's a good question. It definitely plays a role in how you analyze this data because you have to take into account that pesky Y chromosome. We've never seen sex differences when we've looked at DNA methylation changes but we always control for it anyway because people ask this question.

I don't know. Carmen, do you see anything in your epi studies?

Carmen Marsit: Yes, we actually – what's really interesting is we've at least for some specific genes, we have seen, and Andrea this goes toward some of the points that you're making, relationships between methylation and expression that are sex dependent. There's a whole layer there of understanding the function of some of these effects may be very sex dependent. I think it is actually a really interesting layer to consider and to think about. So definitely.

John Cowden: Well, I'm going to pull this discussion to a close. It's actually a really good transition because I wanted to go back to question number one, which we didn't have a chance to have any discussion on.

I can start the discussion by saying that one of the recommendations from Karin was to look at known differences. So for instance, males are known to be more susceptible than females and maybe examining the mechanisms between males and see the differences between males and females. That might be a way to get at some of the novel pathways that may lead to differential susceptibility.

And with that, I will open the floor up to the other panelists to see if they have any questions about the identification of novel mechanisms for arsenic.

Andrea Allen: Let me just address the male versus female question. We just started doing that in our studies and including in utero and assigning the placental sex. We're hoping to see some publishable differences there but there do seem to be critical sex differences in outcomes for sure.

But as far as mechanisms, protein changes, microRNA changes, we're still digesting a lot of that data in our exposure model. It may be that male and female fetuses have a different dose response relationship as well. So this is going to take a little bit of doing but I do think that we should be paying attention to sex differences because I do think that will be critical.

Eric Ditzel: I also think it's important that we're cognizant of looking at how, for instance, some of our more recent work we found increased (unclear) disease in males whereas we haven't seen that type of pathology showing up in our females. We're trying to do a comparison to figure out

why that is but it's also important that when looking at those types of differences that we don't just focus on where we can actually see effects. Understanding why there's differences in effects is something that's critical. So I always say we should have our journal of negative results.

Molly Kile: I also think it's interesting to think about sex because it's pretty well-observed that men and women have very different arsenic metabolism profiles. This also might be an upstream factor that's trickling down onto epigenetics.

Men tend to be poorer methylators so they're not excreting as much of the DMA so they're probably accumulating a little more of a toxic dose from the intermediate metabolite MMA, which has a higher toxicity. So maybe that could also be explaining some of that sex-related difference, which is also how you group all of this together to look at mediation and modification in these pathways.

Danielle Carlin: John? Michelle has a couple of questions that have come in.

John Cowden: Okay.

+++q-and-a

Michelle Heacock: There's one here that touched on what was already said but it just has a different layer to it.

Is diet gene exposure or diet exposure interaction an important area to consider in terms of susceptibility to health effects such as cardiovascular effects? I would even layer onto that, is there any evidence of early life exposures to arsenic where later environmental exposures are worsened?

Do I need to repeat that? (laughter) (multiple speakers)

Craig Steinmaus: Early arsenic exposure and later life exposures, I think I already mentioned our data from Chile where we saw that evidence of – it wasn't statistically significant but there were substantially higher or relative risks for synergy.

Most of our people were exposed in early life to arsenic and most of the other exposures, the asbestos, the silica, and the smoking came after that, came in adulthood. To answer your questions, yes, we did find evidence of early life arsenic exposure and then subsequent exposures where there's a synergistic, not an additive effect, but a synergistic affect. We did see that.

Michelle Heacock: Did anybody else want to comment on that?

Andrea Allan: I was just going to say in our animal studies, although we didn't do a second toxin exposure, we did find our prenatal exposed arsenic animals to be more sensitive to stress in adulthood and that they showed more signs of stress-induced depression than controls. So, stress is another factor that might increase the susceptibility when an animal is exposed to arsenic. They may be more responsive to all kinds of stressors.

John Cowden: Carmen, I think you had a comment? Then we'll go back to Michelle for the last question.

Carmen Marsit: Yes. The other thing that I was going to say was that I think there's a couple of instances in the literature, I know there's some stuff out of here, looking at the relationship between smoking and arsenic and their interaction on bladder cancer risk in adults or lung cancer risks. I definitely think in the cancer literature there's been some instances of that and that's a great thing to think about when we're considering in utero exposures and the cardiovascular risks later. It's all those – the interactive --between the social interactions and stress as well as dietary interactions of these things too.

John Cowden: All right Michelle? Hit us with the next question.

Michelle Heacock: John, I'll actually let you finish up. The other questions we had were more directed to specific experimental details. If there's any more discussion that can occur, maybe we can save those details and take them off-line.

John Cowden: All right. I'll finish up then. Thank you guys, again for putting in the time to put together these answers and these questions – to these questions. I thought this discussion was actually fun and I hope you guys found it fun too.

Thanks again to the organizers. I'm going to turn the floor over to Danielle for her closing comments.

Danielle Carlin: Thanks John for moderating today's session and no I would never hit you, so just for the record.

I would like to conclude this webinar with just a few closing remarks.

First our next webinar is scheduled for May 22, 2014 at 11: 30 Eastern time. I hope you will all join us and it is entitled Global Environmental Cycling and Bioavailability of Arsenic.

I highly encourage you to register for this webinar. Again, you can do so by going to that link listed at the bottom or you can also go to our NIEHS Arsenic Workshop website for more information and also to find links to register for all of our webinars. You can also find additional information and biographies of all of our speakers at that website as well.

We received several questions about posting these slides. We will notify you if and when they are to be placed on the website. We're working towards that. Also, again, if you have any questions or comments that were not addressed in our session today, we will send these to our speakers and their responses will be captured in the final report of the workshop.

I'd also just like to express my acknowledgements and thank yous to the following, including again, Dr. John Cowden. I would certainly like to thank the panelists. I think you all have done an excellent job today. My colleagues here that are sitting in the room with me, especially Dr. Michelle Heacock, she has fielded all of the questions that have come in. We have Justin Crane

here, Maureen Avakian in the room with me and again, thank you for your help with the webinar logistics and then finally Marisa Naujokas, she is on the line and she's helping us out with the workshop publication.

Finally this will conclude our webinar and we hope to hear from you all on May 22nd, so I think we're going to end now.