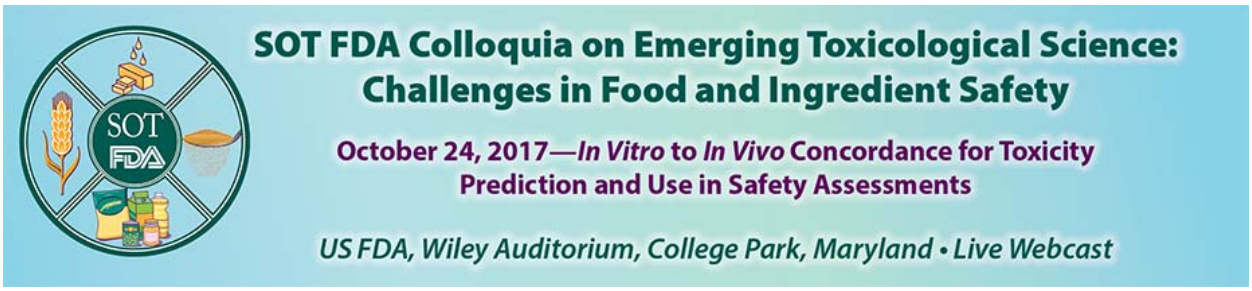

Real Time Captioning

Schedule

8:30 AM–8:35 AM	**Welcome from FDA** Steven Musser, PhD, Deputy Director of Scientific Operations for CFSAN, CFSAN, US FDA, College Park, MD
8:35 AM–8:50 AM	**Welcome from SOT and Speaker Introductions** Bryan Delaney, PhD, DABT, ATS DuPont Pioneer, Johnston, IA
8:50 AM–9:30 AM	**'Omic Biomarkers for Assessing Cellular Toxicity: Integration of *In Vivo* and *In Vitro* Data—Why It Is Important** Bruce Fowler, PhD, ATS, Toxicology and Risk Assessment Consulting Services, LLC, Rockville, MD
9:30 AM–10:10 AM	**Establishing an Integrated *Ex Vivo* Female Reproductive Tract in the Microfluidic Platform: Screening of Reproductive Toxic Chemicals** Shuo Xiao, PhD, University of South Carolina, Columbia, SC
10:10 AM–10:30 AM	**Break**
10:30 AM–11:10 AM	**Investigation of an *In Vitro* Method for Protein Hazard Characterization** Bryan Delaney, PhD, DABT, ATS, DuPont Pioneer, Johnston, IA
11:10 AM–11:50 AM	**Analysis of *In Vitro* to *In Vivo* Concordance Studies for Food Safety Assessment in Humans** Miriam E Mossoba, PhD, U.S. FDA, Laurel, MD
11:50 AM–12:50 PM	**Roundtable Discussion** Moderator: Paddy Wiesenfeld, PhD, US FDA, Laurel, MD All speakers

Welcome from FDA and Overview
Steven Musser, PhD, Deputy Director of Scientific Operations for CFSAN, CFSAN, US FDA, College Park, MD

It gives me great pleasure to introduce and start off the fourth annual SOT FDA colloquia. Today we are welcoming the fourth year and 13 in a series of SOT FDA colloquia on emerging topics a logical science, challenges in food and ingredients safety. I thought we had quite a few challenges in this area. It is nice to have a colloquium like this we can be discussing these items.

I would like to mention this is an agreement. These colloquia are arranged between the Society of Toxicology and the Food and Drug Administration. These colloquia are put on as a product of the memorandum of understanding between the two organizations. They provide a forum to engage in the newest methodologies and inform the work of the FDA Center for Food Safety and Applied Nutrition employees by utilizing leading experts in toxicology's from around the world. I believe this is an important aspect to help our staff and employees and scientists understand the newest and latest and state-of-the-art in *in vitro* technologies, and the concordance of those *in vitro* technologies with *in vivo* experiments. I think the nice thing about this is the number of people that are here in even though there aren't many folks in the auditorium, I understand there are over 500 people registered online watching via WebEx, so we welcome those folks who are watching. This meeting is open to the public and I should note that while these colloquia are intended to inform CFSAN scientists, they are not for offering recommendations to the agency on any regulatory issues. They are intended to discuss the latest toxicologic and regulatory science.

Why concordance? It is important we discuss concordance because we need to move away as much as we can from animal models. The only way to do that is if we understand the *in vitro* technologies used are accurate and reflect what might be seen in the human model. One example is a glycolic acid concordance study recently concluded. Most of the toxicology research is done in the center and they completed the study looking at this impurity on the synthesis of -- preparations. In this study been established all the *in vitro* tests used did test used did manage accurately with the *in vivo* operations.

It's important to point out we have quite a few people from around the FDA. We have program offices in the center that help and provide information and requests and experiments that need to be done by people in the laboratory which is done at OARSA and we are happy to have people from the program offices and OARSA, and other folks from around FDA which might include the National Center for Toxicologic Research in Arkansas. With that I don't want to take too much longer but there are a few administrative items for the people here. The restrooms are in the auditorium upstairs just before you come in. There is a public cafeteria at the front of this building. There are cards handed out to those would like to submit questions for the panel discussion later in the morning. However, in the interest of time please ask your questions earlier in the program if possible.

The invited speakers will review the state-of-the-art in applied *in vitro* technology to safety assessments. This is a good program. I looked at the speakers and we are fortunate to have been speaking to us this morning and it is my pleasure to introduce Bryan Delaney from the Society of Toxicology to get everything started.

Chair: Bryan Delaney, PhD, DABT, ATS, DuPont Pioneer, Johnston, IA

Good morning everybody and thanks for coming. We have 500 participants via WebEx. Thank you for joining this morning. I'm thrilled to be here. I am a toxicologist and work at DuPont Pioneer in Johnson, Pennsylvania.

A little bit about the colloquium series. The partnership between SOT and the U.S. FDA Center for Food in Safety and Applied Nutrition provides for the exchange of high-quality, cutting-edge, and future oriented toxicological science.

All the information presented is for FDA employees and the public. All the slides will be made available and open access soon. This is also not a public forum for discussion of regulatory issues. It is all about science.

The Society of Toxicology mission is to create a healthier and safer world by advancing the science and increasing the impact of toxicology. Priorities are to strengthen the relevance and impact of toxicology, develop and support toxicologists to capitalize the future opportunities and expand the outreach and impact globally. Again, I'm not sure how many people in the audience would consider themselves toxicologists, but this is the mission of the society itself.

This is the fourth year and there is quarterly colloquium, so we have one of these about every three months. We have one coming in December entitled “Neurobiology of Food Additives.” I'm anxious to learn about that topic. In February, the colloquium will be on Risk Assessment of Mixtures.” In May, we plan to host a colloquium on “Biotechnology of Modern Agriculture.” We include as much aspects as we can. We would be welcoming to consider additional topics for additional colloquia. Specifically related to toxicology in food safety, but that is a broad term and we are always looking for new symposia. We typically think of the people in the room being the audience, we understand there are more than 500 participating in the webcast. Many countries, I think 25 countries. This is probably where you're going to find the slides for resources, www.toxicology.org.

Also, an opportunity to discuss the Society of toxicology in the opportunity for late breaking data. I would encourage as many people to attend the meeting in San Antonio, Texas. It is a nice venue to attend in March. Please take a few minutes to visit the Society of Toxicology web site to learn more about the Annual Meeting and all the other resources that the Society provides. I would like to recognize the rest of the SOT FDA Committee who are listed on this slide. I am the chair, but I want to acknowledge my co-chair, Paddy Wiesenfeld is sitting in the second row and we are here as

attendance in looking forward to the busy agenda today and a lot of information. We will be starting with Dr. Bruce Fowler talking about ’omic and biomarkers and then Professor Xiao and then we would take a quick break a little after 10:00. We will then move on to work from DuPont Pioneer with a presentation entitled “Future Methods for Approaching Hazard Characterization.” We will finish up with *in vitro* to *in vivo* Concordance Studies,” and follow up with a roundtable discussion.

To keep us on time I'm going to go ahead and step back and let Dr. Fowler gave his presentation.

’Omic Biomarkers for Assessing Cellular Toxicity: Integration of In Vivo and In Vitro Data—Why It Is Important
Bruce Fowler, PhD, ATS, Toxicology and Risk Assessment Consulting Services, LLC, Rockville, MD

I'm happy to be here and share some thoughts with you on approaches we have taken over the years. I hate to say how many years to looking at mechanisms by which toxic chemicals, and I would deal exclusively with inorganics. We will start with arsenic which is of interest to FDA about arsenic in food for items like rice. Also look at the issues surrounding risk assessment from exposure and chemicals that also may be with it.

What I'm going to try to cover broadly the need for test systems, which I'm sure everybody watching this knows. We need test systems that are fast, accurate and cost-effective. The demand for answers to chemicals new and those coming online every year is always present, so we can't wait around for 10 or 20 years. There just isn't time. The "omic" -based tests are basically a set of mechanism-based approaches. One of the areas of increasing interest has to do with mechanism remote affection risk-based assessments. These tests, genomics and proto-makes fit in this well. The issue of sensitive subpopulations, I'm going to spend quite a bit of time on this because this is increasingly an issue about the fact one size does not fit all about chemical exposures. There are individuals within a population who are more sensitive than others and we need to consider how we protect them.

In order for these "omic" biomarkers to be accepted for risk assessment, they must be validated, and this is where ancillary information and modifying factors comes into it, so I will spend some time on that.

I'm going to talk a lot about starting with *in vivo* data, which is where historically toxicology has been. And then talk about *in vivo* data and the use of computational methods to extrapolate between these two and how we get these two in concordance and how you convince yourself the answer you get is relevant or germane to what might happen *in vivo*. Like I said, ultimately, I believe in my opinion this is what toxicology is -- if we can understand that we can better determine who might be at special risk for chemical sensitivity.

Here is a diagram of what I just said. We know within the general population there are number of people who are resistant or tolerant. But then there is a sensitive group, and this follows about the joke about the person who drowned of the people whose average depth was 2 inches. No matter what level you set there will be some people who will be especially sensitive. Bee stings is an example.

Ideally what we would like to do with these biomarkers is develop tests that could pick up toxicity before the development of clinical disease. It is much better from the point of view of public health to pick up something before a group of individuals are exposed. Then you must figure out why. We had the issue of mixtures. This also is a clear and present issue for toxicologists. The fact is there are thousands of chemicals in commercial use and new ones every day. We really don't know a lot about the number of these, so we must have a way of assessing those but also assessing those alone or in combination. Again, this is mechanistic linkage between the biomarkers and other parameters up toxicity, and I will show an example of the issue of consistency across species. Basically, we are all biological organisms. There are a number of these systems that are sensitive to chemicals, which exist whether we are talking about a human or a hamster or a chicken. This is how they survive on this planet. They need these systems to exist. It would be handy to validate these biomarkers across species, so you can move quickly between them.

This is Fowler's idea of how such tests could be done or a way of looking at them. Fundamentally we have *in vitro* studies, (which is agreed upon is the place where we should be focusing our efforts.) At some point in the biological scheme of things, we need to move to intact organs or intact animals and then ultimately up to the population at risk. There are factors that will influence us in many ways. We have diet and ender, genetic inheritance in these things we are increasingly aware of that have an impact on who might be at special risk for toxicity. Now I'm going to move into these biomarkers, and I'm sure most of you in this room are aware of these definitions. Basically, we are looking at genes, protein responses and metabolite system responses, which are the three main ones. We are learning a lot about these. The trick is to move these to the level of risk assessment

There are modifying factors for these biomarkers. Things like age and gender, which is one I will talk about in particular. Nutritional status, people with healthy diets tended to do better and genetic susceptibility. There are compensatory mechanisms, inducible enzyme systems and stress proteins, and antioxidant systems. We and other species have developed ways of coping with chemical insult. We are not defenseless is what I am saying. As I will show you, these defense mechanisms are finite. They can't go on and on. Eventually if you raise the dose or increase the duration or exposure, or if you have a genetically inherited or defective system, you become susceptible.

What you are looking at is a carpet of kidney cells. These were hamsters that were put in culture, and these are black areas in the middle of the green that are holes in the carpet where the cells have died and lifted off. Normally, this culture started with cells looking like this. As they become sensitive, they start to round up and lift off. This

culture was stained with a dye that was used to measure with hydrogen peroxide. These cells are exhibiting signs of oxidative stress. One other question, and they were obviously exposed at the same time at the same level of arsenic 3, but why should it be some of the cells died and went away, others are dying and lifting off and others are just sitting there?

I'm going to talk quite a bit about this organelle, the mitochondria. As most of us know from taking biochemistry or even high school biology, the main source of energy for the cells, but it has many other functions it carries on. It has enzymes work chemo biosynthesis and carbohydrate metabolism. These functions are located at different parts of mitochondrial compartments. There is some on the outer and inner membranes and in the matrix. This makes perfect sense if you have time to get into the biochemistry. The point is by having knowledge of these systems and the fact they are integrated we can use this to our advantage to come up with mechanistic linkage is.

This is a P31 spectrum of ATP. This is *in vivo* and from a surface coil on a rat. These three peaks are the three phosphorous peaks of the ATP. The animal was given an IV injection of arsenic 3 at this time point. What you can see is these peaks go down over time, over the next two hours. Interestingly these peaks which are inorganic phosphorus and the number of species go up. The phosphorus must go someplace so what it starts doing is attaching itself to other molecules inside the cell. In other words, we are changing the metabolism and changing the metabolic regulatory systems. This is one kind of effect. This is a semi-slide? that attempts to cover some of what is going on at one time. One of the things arsenic will do is induce epitaphs. It causes many transcriptional factors. I'm going to start with the *in vivo* study. The first set of slides, I'm showing this, so you can see what the experiment looks like. At this time, we are interested in -- and how I will get into the mixture of binary mixtures. Electrophoresis was done in the liver, kidney and urinary proteins. One of the things that happens with inorganic arsenic is it gets methylated. The particles are broken down, in this case -- is released. The arsenic is handled very much like arsenic is it arsenide. What does this have to do with biomarkers? This is the pathway in the mitochondria. It has been known for many years this outlet interferes with a couple of steps in this pathway and inhibits the enzyme. When that happens, we can get a rise in zinc -- there is a? -- that result in ? -- coming out in the urine. Alleges? that the only thing that can do that. Arsenic and mercury can do with. They inhibit the pathway at different places so that is a different metabolic profile of things you can measure in the urine. From our experience, these patterns show does relatedness. -- dose related.

This comes back to the hamsters dosed *in vivo*. We also ran some inorganic arsenic controls and compared them to what we got with in vitro? Or unknowns?-- you can see that no two are like this is important because it means in a mixture situation of indium arsenide there is a separate profile for -- as well. The idea is you have a biological system?-- with a dose exposure and it changes over time, whether at one month or three months, we get a different pattern. I will look at arsenic for a moment, this is below does this is the high dose. You can see the magnitude of the effect is much

greater at 3 milligrams per kilogram. 3 milligrams per kilogram was chosen as being about one quarter of the -?- and that is about where we are in the dose response. This is .3 milligrams per kilogram but still we are getting and affect. What happens? One of the things that has been appreciated over the last 30 years or so is when we or other animals are exposed to chemicals or toxic insult, this induces changes in gene expression patterns or protein expression patterns. This is a two D gel map. These are basically proteins synthesized, and this is the pattern for nontreated control l. This is what you would expect. This is referred to as housekeeping genes. At ten days at the low dose of arsenic would get a very dramatic change in this pattern. If we raise the dose, we get a different pattern.

If we look at the patterns and compare -- at ten days and 30-days, there is a striking thing that happens. Basically, the indium arsenide at 30 days is showing a suppression of the expression patterns that are observed. The reason that is important is this, these are urinary profiles stained with silver stain which is a sensitive stain for staining proteins and gels. You can see the strongest pattern occurred with the indium arsenide. We believe what we are seeing is a suppressive effect from the indium component of this binary mixture; the arsenic is turning on through oxidative stress. These cells can respond, or the animals can respond. This is *in vivo* treatment with ex vivo incubation. The capacity to do this is finite. The indium, which is a toxic element in its own right, can suppress that effect. I will get to how this happens, but back to the notion of concordance. These are *in vitro* studies where these are hamster kidney cells incubated with arsenic or indium arsenide. We are looking at two different markers of cellular toxicity. One of which is leakage of ? AR?-- into the media. We are measuring -- in the one hand the open circles, and this has been a test around for a long time to assess the healthy cells in cultures. The indium arsenide raises? the lactic acid?-- level so it is more toxic depending on the dose. This is LMR blue. We were interested in how well the assay correlates with the lack lactic androgynies?. In this case the LMR blue goes down rather than up. You are having a suppressive effect. There are a number of images in play, but the idea is we have a way of looking at the help of the cells using *in vitro* methods. This is sort of the summary of what I just told you. The thing I find professionally interesting is we did Western studies looking for a number of the main stress quarantined families and we looked in the urine and we didn't find any. This means the protein we sought is not cell death. If it were, these things would be picked up in the urine. These proteins are regarded as chaperones and remove other damaged shall proteins -- damaged cell proteins. The hypothesis is they can take the damaged proteins to the cell membrane and delve them out. That is the reason for those patterns. We have a way of going after a potential mechanism.

The problem is too much information, and this is where computational methods commit to play. This is a digitized image analysis map of gallium arsenide. You can use this technology -- the expression of proteins in these cell cultures relative to controls. We were comparing -- the program assigns numbers to the spots and expression patterns to the controls. You can see we are getting in males suppression of many of the proteins whereas if we use gallium arsenide we use -- the spots are increased quantitatively. It says they are eliciting different responses when run head-to-head --

we get a different expression pattern. The females seem to be much more responsive than the males. That is important and what we were interested is the female semiconductor workers -- a large portion of the workforce are female, so you would like to know whether they are at greater or lesser risk or how they are responding.

Those are animal data. The question before the house is what we can do to directly compare these animal data or see if these patterns exist for humans. We have access to a library of human kidney cells. The cells were age matched by both genders. We brought up the cells and exposed them to Gallium? or indium or arsenic at different times. The thing I would call to your attention to is we again see the same pattern. These are the control expression patterns untreated in saline and then we get the pattern for arsenic and then one for gallium and one for indium. If we go to the mixtures, we get different patterns again. In this case, the indium arsenide actually produced reduction in the expression of these proteins. There are differences between the females and the males. Here is the male and the female and again the females are responding into a much greater extent than the males at this dose level. There is a reversal at 100 micromolar. Nonetheless, you can grind through these kinds of studies very quickly and cheaply and gather information about the populations at risk.

This is a hypothesis is much as anything, but it is a composite of many studies. Basically, the idea is the arsenic component will decrease respiration in decrease the potential and increased degrees of hydrogen peroxide and -- oxidation of other proteins in the cells. If we have indium present, what we observe from electron micro studies is basically the indium would damage the end of plasma it -- and cause the shedding of ribosomes and it that way it acts like [Indiscernible]. Ultimately you can generate apoptosis and you can have cell culture and cell death. The idea is we can use the basic tools of modern biology and cell biology and molecular biology to inform and address the question of the people exposed to these substances alone or as mixtures and who would be the most sensitive and who to focus resources on.

I'm going to finish up with a summary of this. Keep in mind I'm using gallium and indium as examples to go along with arsenic, but it could be many other substances. You can do things with lead or cadmium that might be of particular interest to the FDA or regulatory agencies in general. With regard to trying to sort populations at special risk. These biomarkers like anything else that are talks talk so logical pillows? must be validated and checked for them to be credible and for people to trust the information or give them insights to where they should be looking for effects. Then the integration of data from *in vivo* to *in vitro* studies in using approaches for risk assessment and identifying them?-- is where we are now, and this is where we are going to go. These are my collaborators that did all of this work. These are post-doctorate fellows and students who contributed also. And that is it.

Delaney: We have time for one or two quick questions, and I'm encouraging to follow-up to submit questions for later for the panel discussion.

Audience Question: I have a quick question regarding the hamster studies. I noticed the doses used are pretty high compared to the human injection or exposure. I wonder if there have been any studies using a lower dose? On similar pathways or studies?

Fowler: Yes and no. These "omic" tests are as we say a moving target. Over time the tests become more sophisticated and different test evolve. Not these exact same data. What we have done looking at arsenic in drinking water is to look at these same protein markers using antibodies at much lower doses and closer to ambient. You are right on the edge of two tech show in. It just varies. Bear in mind you are using these rodents, particularly with something like arsenic, the rodents have an apartment?compartment? that combines arsenic that we don't have and that is mainly there for. A fair amount of arsenic will go into the further fur? of the animals if you're using normal kinds of rodents species so you need to be aware of that. It is hard to get the doses to line up because there is another external compartment the animal can put the chemical into.

If you ever observed the [Overlapping/Multiple Speakers] ?

Fowler: I have but not in this context. What she is asking is there is there a U-shaped dose response curve that can occur where you get a greater affect the lower dose and then it goes down and comes back again want to get higher. Not for these substances. Part of this in my opinion when that happens is we are going to get back to these compensatory systems and -it?- might be one if you get up high enough which if it doesn't occur at the lower dose levels and if that isn't triggered, the chemical can have a more direct effect on a sensitive sub cellular system. Did that answer your question? Sure.

Delaney: Thank you. What I'm going to do now because I didn't do before is to read Bruce Fowler's bio. And then I will introduce our next speaker. Bruce has had a long career as a toxicologist is currently the owner of Toxicology and Risk Assessment Consulting Services. Over the years he has served on SOT council and president of the *in vitro* Specialty Selection. Includes service in the Department of Health and Human Services at the NIH and in NIEHS and CDC. He served as a research scientist to develop a reputation regarding toxicology of lead, cadmium, mercury and other?-- appointments such as the World Health Organization, international agency for research on Cancer in the National Research Council which is supported a public health. Recommendations for these elements.

He has served on the Swedish Medical Research Council using professorship and contributions in the field of toxicology. He has received many honors over the years. Again, we thank you for your presentation and being here this

[Break]

Delaney: It's 9:30 and we will continue with our next speaker who is Professor Shuo Xiao who is the newest member of the Department of Environmental Sciences in University of South Carolina, Arnold School of Public Health since January of this year.

Dr. Xiao's research focuses on reproductive health and toxicology with multiple *in vivo* and *in vitro* models and investigates how chemicals adversely affect fertility in young female cancer patients and how environmental chemicals affect female reproductive function and how to provide fertility protective options for women at the risk of reproductive dysfunction and infertility. His second interest is using microfluidic technology to establish an integrated reproductive tract for pharmaceutical drugs, environmental screening and testing and he is originally from China and he studied preventative medicine there. He has as a Bachelor degree and a Master degree in toxicology at Peking University and the School of Public Health in Beijing and the he dug deeper into toxicology with a doctoral degree from The University of Georgia. While performing research on uterine biology and embryo implantation in the Department of Pharmacology and Pharmacology. He focused on female toxicology with professor Teresa Woodruff in Northwestern University? can my interest in medical and toxicology. With that I turn the microphone over to Dr. Xiao.

Establishing an Integrated *Ex Vivo* Female Reproductive Tract in the Microfluidic Platform: Screening of Reproductive Toxic Chemicals

Shuo Xiao, PhD, University of South Carolina, Columbia, SC

Thank you, Bryan. Good morning everybody. I would like to thank the organizing committee for inviting me to give this talk and happy to share the research data with the toxicologist and other people who are interested in female toxicology. Today, I will talk about our research about how to microfluidics technology to make the female reproductive tract and how we use this technology to do the female toxicological research.

For all the data I present today there is no conflict of interest. Like the other organ systems, the reproductive system is also important for our human health and in the female, the female reproductive is not only the host of pregnancy, fertilization and hormone secretion but also very important for the general human health. For example, they maintain the bone health and also the health of the cardiovascular system. This cartoon indicates the general structure of the female reproductive system so here is the ovary, the fallopian tube, the uterus, cervix and the vagina.

Follicle is the basic functional unit of the ovary. It is activated from the primordial stage to the primary stage and then goes to secondary, parentral, and antral stages for maturation. When the follicle reaches maturation, the pituitary gland will secret the luteinizing hormone that forms a LH surge to induce ovulation and the follicle will undergo the ovulation process by releasing a mature egg into the fallopian tube. The follicle itself will differentiate into corpus luteum and secrete progesterone for later early pregnant events, and if the egg is fertilized it will develop into a different stage of embryos and transport from the fallopian tube to the uterus and then implant into the uterine wall, establish the placenta, and finally develop into a fetus. So, the female reproductive system is highly regulated by the hormones. For example, the follicle maturation and differentiation process are highly regulated by the pituitary hormone including both follicle stimulating hormone and luteinizing hormone and the follicles will

secrets estrogen and progesterone to regulate the uterine endometrium proliferation and differentiation in order to maintain the 28-day menstrual cycle. Because this system is highly regulated by the hormone they are also potential targets for chemicals and clinical drugs. For example, the most well know chemicals are the endocrine disruptors. Right now for the female reproductive toxicology, the gold standard is the *in vivo* animal testing. We are using the chemical to treat the female animal and then we take the reproductive organ away for the morphology and histology to see if there is any pathological changes in the female reproductive system. We can also check a vaginal smear and determine whether the ovarian function is damaged. We can also the ovulation and perform the fertility study and check the pregnancy, litter size and perform multiple generational study to check the developmental toxicity. However *in vivo* animal models are time-consuming, costly and harmful to animals. For the downstream reproductive organs including the uterus, fallopian tube and cervix, there are no good *in vitro* models to do toxicology studies. For the ovaries, a lot of ovarian follicles are used for in vitro cultures to perform toxicity testing, however, the two-dimensional follicle culture cannot represent the *In vino* physiochemical microenvironment and the three-dimensional tissue architecture.

My talk today includes two parts. At first, I would like to introduce how we use the microfluidic system to establish the female 28-day menstrual cycle and then we can integrate the whole reproductive tract on the chip. The second part is about how we use this organ on a chip technology to perform the female reproductive toxicology research.

In 2011 the NIH established a new program which was focused on the organ on a chip technology by using the microfluidic technology. Different research groups are focusing on different organ systems. Some of them are focusing on the brain system and some other people are focusing on the blood vessel, the lung system and cardiovascular system. In our group in Northwestern University, we were focusing on the female reproductive system.

This is because the early testing of the chemical toxicity in females is much more difficult than in males? the male because there are the menstrual cycle and hormone changes. In addition to the existing menstrual cycle and the hormone change there is also a lack of awareness and the importance of gender as a biological variable and ethical issue when people are performing the clinical study because the women may have potential or current pregnancy. Some drugs have been withdrawn from the market because they have more toxicity in females than in males. So, in 2015, the NH released a new policy about including both genders or sex as a biological variable and all NIH funded research need to include both male and female. If you are only using male, you *must* justify why you are not using female.

The hypothesis of our project is that whether we can use the microfluidic technology to mimic the 28-day menstrual cycle hormone control to make the ovary on a chip, so we can perform the female reproductive toxicology *in vitro*. On the other hand, whether we can connect the different female reproductive organs together to make a whole female

reproductive tract on a chip. In this project, we are collaborating with the bioengineering people from the Drapper laboratory and designed this is microfluidic system. It includes three interfaces. Here is the tissue culture modules and here is the fluidic interface and the actuator interface. For these materials we are using to build the system, they can be sterilized, are non-toxic to the cells or follicles and do not bind hormones. This is important because we are doing the female reproduction, so the hormone is very important for the results.

The picture on the right indicates one enlarged cultured unit. Here is a media donor model, here is the tissue culture module and here is the past media reservoir. The pumping pattern of the microfluidic system is computer program controlled. We can design certain computer programs and the culture media at a certain flow rate can flow from the media donor module to the tissue culture module and then to the past media collection module. Then you can collect the conditioned media whenever you want for the analysis without disturbing the current running cultures.

For the microfluidic system we designed, the other advantages are that it's easy to handle and load the tissue. As I just mentioned you can also collect the most recent past media without disturbing or distracting the running culture. This is an advantage for the microfluidic system because normally when we do the traditional cell culture, we have to change the media every two or three days and then you are kind of changing the culture environment and the secreted hormone or other factors will go up and down. For the microfluidic system, the cultured tissues are experiencing a stable secreted factors or the hormones.

In our laboratory we are using the alginate encapsulation method to perform the three-dimensional follicle culture and the alginate encapsulation could maintain the three-dimensional architecture of the follicles and support the follicle development from the preantral stage to the antral stage. When the follicle reaches maturation and we perform the hormone treatment, the follicle can undergo the ovulation process just like *in vivo* and release the MII oocytes which can be fertilized and the develop into a different stage of the embryo. If we transfer this state back to the recipient mice they can be pregnant and deliver live pups. So, based on this model we want to see whether we can apply this 3D *in vitro* follicle growth process to the microfluidic system for a long-term culture and mimic the flow of ovarian cycle including follicular phase, ovulation, and luteal phase. To mimic the human 28-day menstrual cycle hormone control we culture either the individual follicles or the ovarian tissues for 28 days. From day 1 through day 14 we use follicle stimulating hormone to mimic the follicular phase and the follicle could reach maturation when we give a high amount of the hCG to induce the ovulation on day 14. Then we use a growth media without FSH from day 14 to day 28 to mimic the luteal phase. During follicle phase, follicle could grow from secondary stage to antral state for maturation in the microfluidic culture system. On day 14, when follicles were stimulated by LH or hCG, follicles underwent ovulation in vitro that is similar to the *in vivo* ovulation process and produce MII oocytes which have the potential to be fertilized and develop to the embryo. After the LH surge of ovulation,

the follicle also differentiated into the corpus luteum which his pretty similar to the luteinization.

This is the hormone secretion profile over the 28-day culture. We can see that for both estrogen and progesterone, they can mimic the 28-day menstrual cycle. For estradiol, it is gradually increased and peaks on day 14 when the follicle reached maturation. After the in vitro ovulation, the estradiol level was decreased and the follicle secreted a large amount of progesterone that peaks around 48 hours after *in vitro* ovulation and then gradually decreased. This hormone expression patterns mimic a 28-day menstrual cycle without the pregnancy. If we keep treating the follicle with hCG, follicles can keep secreting a pretty steady level of progesterone which can mimic the hormone secretion pattern during early pregnancy.

For the data I mentioned above, we have successfully made the ovary on a chip. We know that different female reproductive organs are not working by themselves but coordinating and interacting with each other to support the whole female reproductive function including the pregnancy, fertilization and the 28-day menstrual cycle hormone secretion. Thus, for the next step, we want to see whether we can translate the ovary chip to the whole reproductive tract chip.

Then we collaborated with our friends from the Drapper lab and invented this new microfluidic system which can integrate and interconnect different organs together *in vitro*. Because we are doing the female reproductive toxicology, we include the fallopian tube, the ovary, the uterus, the uterine cervix. In order to introduce the metabolism, we also culture the liver tissue in the microfluidic system.

This is the microfluidic system with real tissue loaded and we call it EVATAR. Here is the universal media that can support the tissue growth, here is the liver, here is the ovary or individual ovarian follicles. Here is the fallopian tube tissue and the uterine endometrial tissue and the cervix tissue. You can collect the media to do the ELISA to measure the level of hormones or other secreted factors. Another advantage for this system is that it could pump certain percentage of media or conditioned media back to the liver tissue in order to introduce the recirculation so we can mimic the recirculation in our human body. And for the system we only use the mouse tissue in the ovary module, for all the other tissue we use human tissue because we have limited ovarian tissue.

Because I already mentioned how we successfully make the ovary chip so I won't talk too much about the ovary and we will focus on the downstream tissue data. Here is a fallopian tube tissue and currently our result indicates that the microfluidic culture could support the human fallopian tube viability and the cilia is beating for 28 days and the beating activity and cilia length is controlled by the hormone secreted from the ovaries. For example, on day 14 when there is higher levels of the estrogen, there is more active cilia beating and more active expression level of OVGP1, a fallopian tube marker.

For the uterus part, we use another new technology that is called the decellularization. This is because we cannot get all the tissue ready at the same time. We use 1% SDS to remove all the cellular components and only keep the extra cellular matrix so we can make the uterine scaffold. when we have the other tissues ready, we can isolate the primary uterine endometrial cells and reseed the primary cells in to the uterine scaffold which is called recellularization. However, these results cannot tell us whether the cells are smart enough to form the uterine glands or form the luminal epithelium layers and stroma.

And for another downstream tissue uterine cervix, the result indicates the uterine cervix tissue respond to the hormone secreted from the ovary. We have also demonstrated that the three-dimensional culture model could maintain the 3D structures of the cervix. When there is higher levels of estrogen, there are thicker layers of the uterine cervix epithelium and higher levels of cell proliferation.

Since we want to introduce the metabolism, we also introduce the liver in this microfluidic system and the data indicates we can support the tissue of viability for all 28 days. The liver tissue is secreting albumin for 28 days. But right now we cannot demonstrate whether the liver tissue can metabolize the chemical or whether the hormone change could change the metabolism activity of the liver tissue and this is our future work.

Another new discovery for this study is that we found if we only culture the ovary in the microfluidic system, we can get a very high hormone expression level, but after we introduce the downstream tissue including the uterus, fallopian tube and uterine cervix and liver, the estrogen and progesterone are significantly decreased. The peak level is only 200 pg/ml and this is actually more relevant to the human serum estrogen level. So these data indicate that after we introduced downstream tissue, these downstream tissue can consume the ovarian secreted hormone, or they can change the ovarian hormone secretion pattern.

The second part of my talk is that we want to see whether we can use this female reproductive tract chip to do the female reproductive toxicology study.

So, we use microfluidic system to test and screen the female reproductive toxic chemicals, and all data is based on the ovary because we don't want to make the data too complicated, so I choose to use the DOX which is a chemo chemical for different cancer diseases including breast cancer, liver cancer and leukemia. The DOX is also highly recommended by WHO. As we know, DOX can kill cancer cells and also kill normal cells like the liver and the heart and cause liver damage and heart failure. Some studies have already demonstrated that DOX can kill the reproduction and cause infertility for young female cancer patients. Our data indicates that after we introduced DOX, there is significantly decrease hormone secretion including both estrogen or progesterone. These data indicates if we treat a young female cancer patient with DOX, they may have infertility.

If I take the follicles out form the microfluidic system anc check the follicle survival, we also found that doxorubicin has dose dependent toxicity on the follicle growth, follicle survival and the hormone secretion. All follicle were died on day 6 when follicles are treated at 200nM of dix. This indicates if the patients receive the doxorubicin at 200nM, they will get infertility right away and the menopause will be 10 years or even 20 years earlier. And I want to point out here that the doxorubicin we used is around 200 nM which is very relevant or even lower compared to the human serum level.

Another new discovery for the study is that: we found at 200 nM we can kill all the tissue with culture on day six but at lower levels of 20 nM the follicle growth and survival are not significantly different from the control group. However, there is significantly increased percentage of the oocytes with abnormal spindle morphology and the chromosome alignment. This is even more dangerous because it is more difficult to be clinically recognized. When the young female cancer patient receives a low level of the doxorubicin they will feel okay and won't have stop bleeding, but it can increase the risk of infertility, miscarriage and even birth defects. We think the patients or doctors should try to avoid to cryopreserve the embryo and avoid the pregnancy during this window. >> For the take-home message today, we are using the microfluidic technology to make the ovary chip and the whole female reproductive tract chip. Another advantage of our study is that we can introduce the gender or sex to the co-culture tissues If we want to have certain chemicals whether they have liver toxicity or have the heart or kidney toxicity, we can do it *in vitro* and connect the follicle with them together and then introduce a 28-day menstrual cycle hormone control and tell the gender difference and tell whether the hormone change can change the toxicity or efficacy of certain chemicals or clinical drugs. And another take-home message is that DOX has ovarian toxicity and at the low doses is more dangerous for the patient, so they should avoid this window for cryopreserve and the donation and pregnancy.

If you are interested in a more detailed information you can check out our papers and email me and I am happy to answer any questions. And at last but not least I would like to thank all my collaborators because this was a collaborative study and without everyone's great contribution we could not make this happen. I would like to thank my lab members at the University of South Carolina and all the bioengineering people in Drapper lab and the people in Northwestern and I also want to thank the financial support from NIH and my home school. Thank you and I'm happy to take any questions.

Delaney: Two questions online. The first is your system is very elegant. Does it reduce the number of animals needed for a given study? I am assuming that's compared to traditional reproduction] study.

Xiao: So currently I think this system is ready for the *in vitro* study and can replace some animal studies especially if we want to study the ovarian toxicity of certain chemicals and if we want to test whether a chemical influences ovulation and affects the oocyte quality and whether they can damage the fertility.

Because I think you mentioned there was one cell type that you still needed mouse ovaries for because you didn't have the source of human ovary tissue.

Yes, for the ovary we are using mouse tissue because we have limited human tissue and the human follicle culture is much more difficult than the mouse follicle culture. For the mouse follicle culture, we can use some bioengineering method and totally mimic the human hormone secretion. They are similar not only for the secretion of estrogen and progesterone but also for some other peptide hormones like AMH and inhibin A and B, which can mimic the human follicle maturation and differentiation in human. We can also make this cycle shorter and we don't have to do it for 28 days. We can make it for 7 days or eight days to make it quicker.

Delaney: The second question coming in from online is how long can these tissues stay alive under microfluidic conditions?

For the ovary tissue because we must let them grow, mature and then differentiate, so after we complete one cycle for the follicle culture, we have to use the new tissues. We also did some preliminary studies and see whether we can get two cycles in the microfluidic culture.

Okay, thank you. Any questions from the audience?

Thank you. [Applause]

Investigation of an In Vitro Method for Protein Hazard Characterization

Bryan Delaney, PhD, DABT, ATS, DuPont Pioneer, Johnston, IA

I hope everyone is back and refreshed from our break time, had some coffee and ready to go to the next phase which is the next part here. I will go ahead and do this since there is no microphone changed involved and I am Bryan Delaney and I am a toxicologist with DuPont almost 15 years and before that I was with Cargill and before that I was with [Indiscernible]. Bachelor degree in chemistry from University of Nebraska and the last 20 years has been rough for me on Saturday mornings. I got my PhD right down the *street* at the College of Virginia in Richmond. My postdoc was University of Wisconsin and I am a fellow of the Academy of Toxicological Sciences and in on the board of toxicology. Also, editor in Journal Food and Chemical Toxicology if anyone is familiar with that publication. Also authored probably about 50 to 60 papers and spend a lot of time in the last 15 years working with agriculture biotechnology and over the course I've learned a lot about what biotechnology is as applied to agriculture and some of the tenants we do for safety assessments. We will talk about today some *in vitro* methods and one in particular that we have been investigating for a number of years. We will talk about that for protein hazard characterization.

Accomplished back for the conflict of interest statement I have no conflict of interest in the funding for all studies was provided by DuPont Pioneer. The studies conducted at Massachusetts General Hospital, Harvard Medical School and were conducted as contract research funded by DuPont Pioneer. We expose this in the publications itself and we will have a limited amount of data we will cover but an extensive bibliography of all the publications which are available with open access, so we can have a look at them if you want more information about this specifically.

Let me talk about biotechnology. It's not always the case but in many cases, it is that the crop phenotype is a product of expression of a protein. From a non-native source probably, the ones that you are most familiar with would be insect resistance from proteins from this source. When my people say BT corn some people think it is biotechnology, but it is not. We also see herbicide tolerance which is also in a form of EPS PS from Agrobacterium. This is also from glycoside. Finally, disease resistance from expression of viral coat proteins. You can see the phenotype we are looking to develop is largely dependent on expression of a protein from a non-native source. Always variations from this and plenty of things not protein based but many are and that's what we will talk about. The protein itself and the safety assessment of the protein.

Almost 10 years ago I could participate on a work group for international food group Bio Informational Council where we developed a weight-of-evidence approach which is multi-tiered and it was expression in crops that we developed the system where it's the weight-of-evidence approach where we borrowed it from allergy people using a similar weight-of-evidence approach but maybe not concerning the same data. You take into consideration the history of safety for the protein. In other words, if you get the protein you expect in the crop and express in a ubiquitous environmental bacterium you are not as concerned about it, but you might be more concerned if the protein came from anthrax or some pathogenic bacteria.

You understand the history of safety is an important consideration. We also look at bioinformatics where that is the sequence comparison and we compare amino acid approach with sequence of known protein toxin to see if they are similar or not. Then consider the mode of action and specificity. In the case of prion proteins, we know that in the target species a lot of times boring insects and I don't mean boring like but a board that they drill and what it is they have an alkaline gut so what happens when the use proteins as they tend to crystallize in an alkaline condition and form pores and combine two receptors as a target insect so the more you know about the specificity of action probably the higher level of confidence you can have. We also consider resistance to digestion *in vitro* and this reflects a purified protein usually from a bacterial expression system and expressed in a bacterium purified. You can put it *in vitro* with digestive enzymes and see if it stays intact or falls apart. In nearly every case you find the proteins fall apart within seconds after being exposed to digestive enzymes which means likely that would happen [Indiscernible].

We consider them expression 11 -- expression level and dietary intake because we want to have something that gives an idea of what type of exposure you will have from my expression is the protein. Generally speaking this is a tier 1 hazard identification and I could amplify identification because the way we put this recommendation together is if you didn't see evidence of adverse effects from the hazard identification then there wouldn't be any need to go and do further hazard characterization studies. The way this works out because of regulatory requirements, it doesn't matter. It's not every agency that requires every study, but enough agencies do require a few toxicology studies for proteins regardless of whether they are indicated or not from a tiered approach that we proposed.

The problem then comes when we look at some of the products that are in development right now. If you look back at the list of proteins that I identified before like the insect resistance or herbicide tolerance proteins, they don't represent that much of a technical challenge to purify but if you think about acute toxicology study it's really designed for chemicals. For chemicals produced at industrial scale to do an acute toxicology study isn't a problem. Indicates and proteins that aren't hard to isolate and can still be difficult if you must do isolation and characterization to show the *in vitro* version of the protein you have is equivalent to the ones expressed in the lab. And that we slip into the next generation of plans a pull together another work groups several years ago where it was originally proposed that the group would work on safety assessment of membrane proteins. We had a group of people together from different companies in a couple of academic folks and turned out that membrane proteins are extremely difficult to isolate in any quantity at all much less in a quantity to preserve or conduct an acute toxicology study. That we got a bunch of people together and looked at signaling proteins and transcription factors and glycosylated proteins and resistance proteins also. To change the name of the group for historical purposes from membrane proteins to difficult proteins which everyone hated because it sounded like proteins that had a bad attitude. We changed the name from that to intractable proteins which I think is good because it indicates it's not impossible but very difficult to obtain.

I use a reference here, but all will be referenced at the end of the presentation as well so if you don't take notes they are publicly available in open access. I am reemphasizing what I said before and if you look at hazard identification process it doesn't take that much protein to identify whether you have a hazardous thing worth characterizing. Hazard characterization requires gram quantities of proteins to do acute toxicology studies and considering the fact we probably wouldn't expect them to be required to do them and the FDA to my knowledge doesn't require them but nevertheless we have what will be a regulatory requirement. Knowing we have proteins that are externally difficult to isolate but we must have information on acute toxicology, what do we do?

What it allowed me to do was to go back and look at the nature of hazardous proteins and if you go out in nature you look for toxic Protegent you can find plenty of them out there but many of them you don't eat them, but you must be stoned, injected orbit from poisonous insects or fish and jellyfish which are all capable of producing toxins that are

quite prudent. Also sneaks. The problem is when you look at the information for proteins that are toxic there aren't that many of them. You must focus on one's we really do know are toxic and one for example we looked at is undercooked kidney beans. The undercooked kidney beans contain phytohemagglutinin E and it's a case where if you eat undercooked kidney beans and have enough of this in them it will mimic the clinical extent of food poisoning. Far enough away from lunch I can use words like vomiting and diarrhea, but it will happen and in fact definite clinical observation been reproduced in humans and animals alike.

So, we go back and look at what can we learn about chemicals like phytohemagglutinin and we learn they damage the intestines of the ?apple LIFFE helium. -?- [Indiscernible]. I consider it to be a target worth looking at which is what would a protein do if present and has this. I thought about that and said we need to think about investigating many people who decide at the end of the day in this presentation that we test *in vitro* with the least as good as an animal study and something you can replace and smaller quantities of protein. Keeping in mind we can get 10 milligrams of protein and what can we do with that? Me know we will never get a full gram or multiple grams and pack.

Obviously, we want to reduce the use of animals in the laboratory. In fact, you try not to do any animal studies at all. And for purposes of getting greater access to this type of methodology we want to use inexpensive reagents and methodology. While it doesn't say it on the slide I can tell you also we had a dose fact definite no intellectual property. The idea is if it works for us it to work for others as well. These are the overall goals we had before venturing into *in vitro* testing. When we thought about the intestine epithelium is the cell lining and the ones we investigated were T 84 and Caco-2 and HCT eight all derived from human colon cancer. And they are used for a variety of purposes including drug bioavailability. If you look for publications on intestinal epithelial cell line monolayers there are thousands. It is very well-established and not even investigating new territory or new methodology but looking to see if it can be helpful in differentiating between hazardous and nonhazardous proteins. So, we know the cells when you put them on a Transwell insert and we have a cartoon on the next slide to show you what it looks like that they differentiate in the monolayer. They have been used for investigating drug bioavailability.

This is a cartoon version of what they really look like but this is the Transwell insert and this looks like an upside on top at and it's right on top of a tissue culture like this and has mesh on the bottom. You see with the cells if you let it mature depending on culture conditions from one week to 3 weeks until they form a mature monolayer. They will form a thin individual cell monolayer across and representative of what you see *in vivo* with humans. So, we tried to take proteins we knew were hazardous and those that we knew were not hazardous and put them on the apical side and looked-for evidence of cytotoxicity. In other words, the proteins kill the cells. We look at LDH release and MTT reduction more for mitochondrial representation but also representative of toxicity and look at whether it affected the monolayer integrity. We wanted to use something sensitive to small obstructions which is transepithelial elect

real -- electrical resistance. It's like a tuning fork that goes out here and you measure the electrical resistance between the wells. You get pretty high levels and you would be surprised. We look for something that would damage the cells more than something as simple as TEER so we looked at -- inulin and we start out with tritium and we went to [Indiscernible] because the lab we were working with got a rate -- got away from radioactivity. And then the monolayer we look for transport from this cell to the [Indiscernible].

So, you think this is sensitive form biosynthesis but this is up to about 3000 molecular weight in this is about 70,000. >> So we have a little bit of time to talk about the data and I will focus on one study more than others. We have a number of publications and I will start with the group from [Indiscernible] investigation which is a group I discussed and we start with a list of hazardous proteins and you can look at the list and some bad ones in here like scrap the life and C. difficile toxin A and B and we have mated a pair of males -- messed up Erin -- motuporin. We pick something innocuous which is PSA and then we also have these substances and I think the jury still out on whether these are considered innocuous or not but nevertheless when we started the study we thought it was. >> This is where I must refer everyone to the actual publication to look about the methodology but what I summarized, and my coworkers like to call it the heat map. You look at indicators for the toxic proteins in the top half and innocuous proteins on the bottom. You see three letters here and the green N means nothing happen and a red Y mean something did happen in each column is a different cell. These second and third refer to cytotoxicity indicators and LDH and MTT and monolayer integrity was measured over here with these subsets. I draw your attention to instead of focusing on all data we are looking for 100% concordance because I knew we wouldn't get that but when we did the study for 24 hours there was an overwhelming large number of Y and even better, yet we didn't find any evidence of adverse effect when we look at common dietary proteins.

I will tell you since we completed this group up concept study we go out in the next publication which should be undergoing internal review when I can share with you we extend the timeline beyond 24-hour period, we see more red Y on the top but not the bottom. It's suggests to us under the conditions we look at the proteins that are considered hazardous are more likely to cause adverse effect than proteins that are nonhazardous and we found the most sensitive indicator is transepithelial electrical resistance. This is the work we did with a professor from Massachusetts General Hospital and Harvard Medical School, Bryan Hurley. The paper I have a picture up here and is available online.

The second thing is we want to look at like we do with the multicomponent hazard assessment process. We wanted to look at digestive enzymes and in other words if you had a hazardous protein that was sensitive to digestive enzymes and how would it perform in the study?

What we did is we took some proteins we knew were not sensitive to degradation like these here on the left and one we knew should be susceptible to degradation and the

presence of gastric fluid and tested for innocuous proteins to see how they work. What we did is we have kept in and gastric fluid and suspended them and sequentially exposed them to similar gastric fluid followed by pancreatic and stopped the reaction to put that mixture in with the intestine epithelial cell and monolayers.

What we found is this is a similar work that her own toxicology lab did to measure 24 hours and 48 hours the cytotoxicity measured by neutral writeup taken monolayer integrity and the tight injunction integrity and TEER and looked at light microscopy.

I will summarize some of the data I picked out for emphasis. What we found is this cause adverse effects of monolayers not particularly cytotoxic but maybe not even variability which is a surprise but we found that it's largely resistant to degradation and the presence of gastric or sequential fluid. Regardless of how you expose it seems to affect monolayer integrity at subsequent time points but this is extremely potent cytotoxic at early time points exposed to gastric fluid for brief period uptime completely loses that affected not cytotoxic and doesn't affect monolayers and if anything increases [Indiscernible].

This is the observation that you have been always told and I was always told that you can't take polypeptide drugs orally because your stomach breaks them down. It's hard to find data that supports that. Similar textbooks my publications that show it proved to be more difficult than I thought. Really what this shows and this is a light microscopy and you can see it's interesting that the cell monolayers look like white microscopy you see regardless of whether they are intact or exposed to gastric fluid or simulated intestinal fluid but all look pretty good. If you compare that with not necessarily the prettiest pictures but look radically different when exposed to what we call adverse effects or these hazardous proteins. If you start to look at this for example, these look almost exactly like the ones that are nonhazardous proteins.

What we are seeing is no effects are exposed to digestive enzymes or not and some hazardous pro times proteins completely degraded and monolayer integrity but those resisted degradation and were damaged in the monolayers after exposed so looking back obvious things that we would have expected to see but now we have data that supports that hypothesis. After visiting some colleagues out here a couple of years ago a question came up that you are working with human cell lines so that will be a homogenous population of cells which are all intestinal epithelial cells but the human gut is not like that I made up a lot of cells so the question came what if you use primary cells?

We went out to investigate some human [Indiscernible] epithelial barriers are what we call them. We didn't start with these studies because cell lines aren't that much money and most can get for free for writing to someone else at a PCC for a small fee and the primary cell lines are about $1500 a plate. We spent a lot of money but could only afford two proteins. We were very careful in picking the proteins we did. We picked PSA of the innocuous protein and *C. diff* toxin A as a toxic protein because we knew it would perform in cell lines but thought these primary cells could be looked at as far as

monolayer integrity for toxicity and we found and I point to reference that Bryan Hurley his goes right -- goes right to left and we see the resistance we follow here in all monolayers beginning with the study and 24 hours later you see when you add BFA doesn't have any effect. It's dropping a little bit but just because the cells were hungry. If you put tocsin A it decreases monolayer integrity substantially even at the smallest dose we tested and for reference this is 2100 which is when we completely blast the cells apart.

Very low concentration and exactly what we saw with the variable affected in the monolayer of the cell line. You see also the indicator of high junctions and you see at small doses of toxin A cause big increases in the flux going up to an almost reaching the same level you see with the trait detergent. With then looked at HRP NUC in the media you see no change in the flux but we see significant damage to monolayers in *C. difficile* toxin A and not quite the level you see it with TX but pretty significant. Importantly we see the viability which is important and exactly what we saw with the PHA and some of the other studies with the cell lines which is we don't have any effect on viability. Especially by LDH release. Might be a slight uptake here but didn't reach statistical significance as opposed to over here that you see with Triton which has a release of LDH from the monolayers. What we see is the damage monolayer integrity is comparable with those seen in cell lines and might have been some slight difference but clearly no big difference and largely the same effect in the same dose range. We also saw as we did with the cell lines that BSA did not damage monolayers at any of the tested concentrations.

Finally building to intractable proteins the time to obtain intractable proteins is difficult because there aren't that many in the catalog and trying to keep it a high-level purchase. And we can identify a number of different ones in different categories. You see at the top we have bacteria represented by the transmembrane protein which is a human her pad aside growth factor receptor and is a signaling protein and we also have a signaling glycoprotein and activating transcription factor and we you see we tested not high concentration because primarily hard to isolate and not very soluble. You have limited opportunities to work with them and what we wanted to do was to see how they performed in the cell lines and this is a heat map and more specifics available the publication. I point your attention that to the range retested to hear which is higher than the range of toxicity we saw with toxin A which caused the significant changes in a lot of these different variables. These doses of these individual hazardous and intractable proteins we didn't see evidence of changing monolayer integrity. You could go be tempted to use higher concentrations than we used or additional proteins. There is always a desire for most compelling but within range of what we could do with the funds we had available, this is as far as we got to be.

What we found is looking back this is the answer but intractable proteins in general would not necessarily be considered hazardous in the results we have from test support that. None of the proteins we tested altered membrane integrity except for *C. difficile* toxin A which we thought it would. So, coming up to some of the conclusions. The human epithelial cell and monolayers appear to respond differently to hazardous

and nonhazardous proteins and building on more information on that as we speak right now. We need to figure out a way to incorporate possible digestive enzymes components to be do so in the hazard assessment and we keep focusing on that because the first year is the hazard identification so in other words something in the first year lead you to believe you have additional safety data and repeated dose or whatever study you want to do. We look at this for hazard characterization.

You would only probably need to do these studies if there is indication you need to do an acute toxicology study. We figured out a way to incorporate the component and the results we find that cell lines coronary -- correlate with them primary cell monolayers that we could test. We believe this might be useful for intractable proteins and primarily driven by the fact you need a lot less protein to do the work. I have listed here a list of publications for the presentation itself. And I believe these are all available online so if you have an interest in following you can go ahead and look and note also we have a number of additional publications in preparation that they are looking to investigate to see if we can find additional variables to look at or what we need to do and look at more proteins are variables we haven't talked about today.

We will keep working on that to keep your eyes open and finally I'd like to thank all the people who participated in this. Starting here the bottom with the DuPont Haskell global centers for health and environment we have Jason sitting over there and Stephanie and Lauren and Greg who all are working together on the studies for many years. And it looks a little different now from some of these decisions but we have done work for them in the past. Moving over here to Harvard Medical School we worked with Bryan Hurley and his staff listed here. They have done a nice job for us and conducted the study's unsupported publications as well. And finally, the DuPont Pioneer, Cindy Zimmerman manages day today and Mark Harper who started off with the company and this project many years ago and has since bought the company and then Ray Layton who supported the project and testing for many years and he is also moved on to a different thing now. I believe that's all I have for you today.

[Applause]

Thank you.

Audience Question: I was wondering if some of the intractable [Indiscernible ? proteins?] may not be stable.

Delaney: We have not. It's something you normally would do with a study to make sure your testing something stable. We have limited access to these proceeds -- proteins and their very expensive. We're still looking for the comp set -- concept. We did not. >> There was another symposium on the -- my friend said when it comes to oral toxicity stuff we are dealing with the elephant in the room is the gut [Indiscernible]. I went home and came up with six more. This is the one that's tied to you. What role does microbiome have in all of this?

There are opportunities to investigate microbiome. I don't mean to sound defensive. We think about it in terms of what we are replacing. [Indiscernible]. We are looking at is what we've done a sexology report in the past we've isolated enough that we've isolated [Indiscernible] and give it to the animals. You don't look at the microbiome in the study. That becomes something you'd be interested in looking at it would be eight interests but more experimental than it would be regulatory. There may be a point in the future to do that. When we are looking at *in vitro* study does the damage that monolayers or does not? In the future there could be a possibility we could investigate the gut microbiome. Calling an elephant, the B a vast opportunity.

[Indiscernible - mic is too far from speaker] Now we have *in vitro* systems that don't have a microbiome. What difference does that make? I think we must address it. It may not make any difference. I would have a hypothesis that the [Indiscernible] of different proteins are so hard to come down in terms of those symmetry. What we're looking at is the study and limit those . We are trying to replace it and whether comes to additional experimental information, I feel that's up for discussion. It's becoming a more important area. Back in graduate school we did not know much about them or why they were there and the changes would have on the diet. We only knew if we had the wrong ones because they made you sick. There's another publication that just came out a month ago. What it does is attract the development of the toxicology test from where it was first introduced back in 1927 two how it's it is it -- transitioned. It's a continued toxicology studies that involve. If you look at it you can see the history of it.

Whether it evolves into getting some that out of the study looking at microbiome that may be the next thing. Right now, it's not requires we have not taken it into consideration. One more question.

Question: [Indiscernible - audio cutting in and out] Sorry about that. I was curious about the control you use them to what extent those represent the universe of orally hazardous proteins.

Delaney: The question is about which type of protein. The answer is I don't know if those proteins are [Indiscernible]. If you eat the bacteria it produces [Indiscernible] and cause adverse effects. Within the world of orally proteins there isn't [Indiscernible]. They are hard to find. There is not much known about putting toxins. The ones that are known income from bacteria -- in most cases the bacteria need to be present with the toxin to produce the adverse effect otherwise the protein itself -- given by itself would be hazard. For the logistical problems, one of the problems in investigating -- learning more about protein toxin would be to go out and buy proteins and test them. If you go by the proteins, you will be registered in that patriot act. They will be wondering why you must have it. We have logistical issues. Some of the proteins I propose say we don't need that in our lab. There are logistical purposes that is not on paper but that's what it is. Thank you.

I will thank everyone for listening. I will introduce our next speaker. I will introduce Miriam Mossoba. She is a research biologist at the FDA, Division of Toxicology andNeurotoxicology, and in vitro Toxicology Branch . Within the Center for Food Safety and Applied Nutrition. She is a background in molecular, cellular and [Indiscernible]. She completed her training at the NIH. She's a member of five American scientist societies. She is also a [Indiscernible] toxicology *in vitro*.

Analysis of *In Vitro* to *In Vivo* Concordance Studies for Food Safety Assessment in Humans

Miriam E Mossoba, PhD, US FDA, Laurel, MD

Thank you. Thank you for that introduction. I am thankful to be here. I would like to thank the FDA for the chance to speak to you today. This is my contact information if you have any questions. You can should meet an email to answer any questions. I do not have any conflicts to declare.

Lots of conflicts but nothing to declare. Will start with a simple definition of concordance. Concordance is a state in which things are great do not conflict with each other. For the talk today, when I talk about concordance I will be talking about the agreement between *in vitro* modeling as well as *in vivo* human outcome. Understanding the level of concordance between *in vitro* and *in vivo* outcomes will help establish the value of *in vitro* models for many purposes. I've listed a few here. Understanding modes of action, from Connecticut features, prediction modeling of a DME and potential toxicity and risk assessment which is more regulatory. If you survey the literature for the last five years the publications that use *in vitro* cellular models you will find the actual number of publications for each of the different listed Oregon systems here are strikingly like the number of publications for in the last five years for *in vivo* model. Yet there's papers that connect the dots and establish bona fide concordance.

What are some reasonable expectations relating to assessing concordance between *in vitro* and *in vivo* outcomes? There are many different considerations. I would like to think about them for discussion at the end. What types of models are being used? What aspects of *in vivo* system is being modeled *in vitro*? Is the concordance between them being assessed qualitatively quantitative? And finally, can concordance assessment be extrapolated to both healthy and disease models? Most publications use healthy models. That's something we should talk about. When you look at *in vitro* cellular modeling, it is a jungle. There are many different categories and everyone has their favorite model. If you step back and look at the big picture, you can classify *in vitro* modeling according to the following schema. Your immortalized cells cop primary cells, induced [Indiscernible] stem cells and thin sections of Oregon explants. These cell types can be cultured in 2-D, 3-D, static microenvironments and micro-fluid environments. In the *in vivo* modeling side there are a bunch of different models. [Indiscernible], rodents, insects and nonhuman primates. Sometimes they are taken from the wild, sometimes they are inbred . Many researchers have not out and in strains. Finally, a lot of animal another type of animals' art transplants. The process of

comparing outcomes from *in vitro* to *in vivo* systems is quite diverse among the research community. There is guidance on the use of *in vitro* methods. OECD official testing methods exist that gets updated regularly. Tox21 from the NIH which also performs the [Indiscernible]. There are also published the purchase to establish concordance that are not related to other official testing. Due to the large variety of *in vitro* and *in vivo* models available, there is a wide variety of rubrics and statistical tests that can be reasonably used to decide on the extent of agreement between systems.

One example is a study at the division of toxicology using tactile? colic acid also called TGA. It's an impurity produced during the sins that this of several carbohydrates preparations used in food products. The products like ice cream, cake baking mixes and different syrups. All of these have different levels of VGA. Studies conducted between 2009 and 2011 identified TGA is a minor metabolite of by fouling by call. And one of two active agents responsible for human renal toxicity associated with DG 80. Mass poisoning of products adulterated with TGA -- DAG have resulted in renal toxicity and TGA is a metabolic byproduct of Dee Gee?. There's also a case study documented accidental ingestion of TGA solution that should direct evidence of renal and other Texas cities. When we perform our concordance study we began with a study to identify the target Oregon. This is followed up with a *in vivo* 28-day oral repeated dose toxicology study. In parallel we performed *in vitro* liver and renal biomarkers analyses and finally we compared our *in vivo in vitro* findings to human exposure data to look for concordance. In the absence of comprehensive safety data on TGA and *in vivo* safety study in rats were performed and complemented with *in vivo* -- *in vitro* cellular testing to understand the concordance between these systems.

On the left we compared diglycolic acid to one positive control that was *in vivo* was quite toxic to. We included an innocuous control [Indiscernible]. It's great to is a establish model of [Indiscernible]. We evaluate the toxicities. We evaluated oxidative stress by the oxidative issue level. We performed the measurements of the kidney injury biomarker. For our *in vivo* study we performed an animal study in which routes were in a different dose of diglycolic acid. It was for 20 days except for the highest dose on the far rate group 8. 300 mg per gram of body weight. The data from the *in vitro* human cell study were compared to data from the *in vivo* rat study as well as human case report outcome. Directly exposing HK 2 cells into the G-8 *in vitro* for 24 hours resulted in HK 2 cells exhibited with deuce ATP production significantly. That shown on the top left graph. The reactive oxygen species production was greatly increased shown in the graph below it. The top right graph shows there was a decrease in MMP and that reflects the membrane integrity is affected.

Finally, the graph shows Kim 1 expression suspended positive control. All animals on the study were dosed with daily oral doses for 28 days except for the high dose group of 300 mgs per kilogram of body weight which could not survive past five days. Shown on the far left you can see clearly where the controlled kidneys have normal or renal cortex and renal with tubule in color the pale color of their renal cortex in the dark color of the renal Mridula (medulla?) is striking in the 300 of the gram per kilogram body weight. The analyses show that the top two images show 400 and 100 times

magnification of controlled kidneys. The [Indiscernible] is completely intact. We were interested in understanding the genetics of the happening and we performed a second study just containing the controlled group with no diglycolic acid.

We looked at the levels of Kim 1 overtime. The significant -- [Indiscernible] took place at day three. The HK 2 *in vitro* data were concordant with both the *in vivo* rat model data as well as the reported human exposure data. To summarize *in vitro* findings, HK 2 cells were directly exposed to TGA for 24 hours in the trope showed clear evidence of decreased cellular and mitochondrial health and increase oxidative stress. Treated cells also upgraded the biomarker Kim 1 in a dose dependent manner. For our *in vivo* findings treated rats demonstrated interest high dose renal effects. For the human exposure data, the above observations are consistent with the effects reported for an unintentional human exposure case report.

When considering establishing the process of concordance, I think there are two main variables that need to come to light. Whether you're dealing with *in vitro* cellular data, animal model data, human outcome data or methods to compare data across different systems it boils down to the availability and reliability of the data and methods. Some cases of concordance are fairly clear-cut and others are not. Disparities exist among approaches by toxicologist in trying to compare *in vitro* to *in vivo* data. These variables are present for both food and drug toxicity studies. The availability and reliability of data from *in vitro*, *in vivo*, human is important along with the different approaches to compare data across systems. For the availability and reliability of toxicity data from *in vitro* cellular system depends on many factors. I will go through a few over the next three slides.

First accurate dosing in a direct exposure model. *in vitro* dosing is dependent on many things including the solubility of the food or food ingredients. For example, lipids are often modified with albumin in or first dissolved in appropriate solvents prior to use in cell culture. What does that do for the reliability of the data? Also, the range of doses used *in vitro* may not reflect an *in vivo* situation. Even when *in vivo* blood levels are used as a guide, Organ specific concentrations may differ greatly. Think about the higher local concentrations that can be in proximal tubules of the kidneys or lower local concentrations in the brain due to the BBB.

Another factor affecting the accuracy of dosing is a definition for acute versus chronic dosing and they are often arbitrary. Some variations in general cell culture methods and growth conditions exist from lab to lab. Primary cells as well as cell lines used by different researchers are subject to different media especially FBS and occasional contamination by Mycoplasma or even other cell types. That comes out to the reliability of the data. Cell lines that are over passaged undergo genetic drift. Over time, polymorphic and tell it a change for the place. Culture conditions may alter the differentiation state and epigenetic status. Source of the cell lines may not be taken into consideration when modeling *in vivo* outcome. Gender is a variable in toxicology outcome. Age, species of selling source as well.

Finally, co-culturing cell type introduces new variables. Adding bacteria to intestinal cell models can change the outcome and in some can improve the *in vitro* model depending on the question. I want to mention there's a big push in the literature for converting 2-D cell models to 3-D representations of solid organs. Their newer technologies that impose stretch forces on cells that are emerging. Sometime a necrotic core can form inside a cellular spheroid and that can confound.

Finally, for considering *in vitro* outcome data, micro-fluid systems versus static *in vitro* cellular culture. A few points about that. Current micro-fluid systems still rely on one or two cell types to represent entire organs but has potential to accurately mimic Oregon function. Oregon on a checkmate permit multiple organs to be connected to mimic the effects of toxic outcomes affecting downstream organs. High throughout designs is not readily available decreasing the satanic power of typical *in vitro*. For *in vivo* outcome considerations the availability and reliability of toxicity data from *in vivo* models depends on a number of factors that I hope you'll take into consideration when reading the literature and having the discussion at the end of this talk. The fidelity of the selected model to represent human outcome can have a huge effect on the reliability of the data. Inbred strains of rodent a popular *in vivo* model and using more than one strain could improve its concordance human outcomes. Accounting for potential discrepancy in vulnerability to toxicity between genders, female rats are more likely to show toxicity than male rats. The ability of disease models to display disease phenotypes is critical. If there's increased vulnerability to human -- of human to toxins should be reflected from the disease state model. There are a lot of concerts cellular signaling pathways among animals and other *in vivo* models that can offer a mechanistic window into understanding toxicity. Despite lacking the same anatomy as human’s example tear ducts, liver non-mammalian *in vivo* models often have conserved cell signaling pathways.

Finally, *in vivo* models with humanized organs can yield a unique way to study human toxicity in an experimental study. When *in vivo* models are transplanted with human blood or solid organs, exposure to toxins can be assayed in a psychological environment. Turning to human outcome considerations. The availability and reliability of toxicity data in human subjects depends on many important factors. Clinical trial data collected right industry, academia or government are not always published into the public domain. It's not as commonly available for food and ingredients as for pharmaceutical drugs. That puts an ability for the data for research to perform the *in vivo* to *in vitro* concordance analyses. Also, accidental ingestion or food poisoning events are available through online databases but the scale of the event vary widely sparse occurrences versus mass poisoning. Both unfortunate. Sometimes events are population specific. That needs to be taken into consideration whether it's a genetic predisposition for that population or not. Also, people are not thinking about the toxicity data that is available from human outcome could be due to unusual circumstances such as the mislabeling or miss identity of a specific foods that cause the toxic event in the first place. Many fish on the market at the grocery store are unstable and that's something people don't realize.

When deciding on whether data generated from an *in vitro* model are concordant with *in vivo* outcomes, it's important to note that portions of *in vitro* models can yield data that are concordant with *in vivo* outcomes. Transporter protein expression on possible to bills may yield data on toxin entry into cells that is concordant with *in vivo* situations, but brush border enzyme expression may be absent.

Mechanistic information may only be necessary to establish mechanistic concordance as opposed to full toxicological data. Relying on *in vivo* outcomes from nematode, insect or animal models to establish concordance with *in vitro* systems could be undermined by the concordance of the *in vivo* model with human outcomes. Toxicity observed in humans can sometimes be measured using *in vitro* cellular models but not necessarily *in vivo* systems. The ability of potential toxins to be processed *in vivo* through a DME pathways could undermine the relevance of any *in vitro* data. Ingested foods can bypass certain organs thereby reducing their actual exposure *in vivo*. The loss of toxicity may not be correctly captured in an *in vivo* model of exposure. When discussing concordance defining what functional or mechanical aspect of an *in vitro* model is being used to assess its *in vivo* counterpart is critical. Functional characteristics should help guide model selection. Functional characters are important.

There multiple endpoint analysis that could help avoid artefactual data and better-established levels of concordance such as our GGA model. *In vitro* to *in vivo* cordons for toxicity prediction and use in safety assessment is still in development. As toxicologist continue to collect concordance data, how to improve *in vitro* modeling for better concordance will become clearer. I*n vitro* modeling and methods continue to improve over time as official guidance on how to determine that quality of their end points increases. As the quality of *in vitro* modeling improves, concordance with *in vivo* outcomes will also improve. Collecting data from multiple *in vitro* or *in vivo* models will likely offer more leverage to establish concordance with greater confidence. The diversity in endpoint and genetic assay *in vitro* and *in vivo* will create new opportunities to generate more complete comparison of selected systems. Efforts for -- from researchers to scrutinize *in vitro* and *in vivo* data will also lead to better understanding. To overcoming discordance will happen as time goes with *in vitro*.

Advance technologies are emerging to help improve the quality of *in vitro* cellular models and methods. Increasing the number of organs are being developed as organ on a chip system which can be interconnected. Stem cell differentiation protocols are being developed to increase the number of cell types of various organs to have a more comprehensive collection of *in vitro* models that will present both male and female organs. Genetic tools that can help model disease of interest are no longer prohibitively expensive. *In vitro* cellular methods can yield rapid results on mechanistic aspects of toxicology testy -- testing especially as better models are being developed *in vivo* approaches allows for a systemic understanding of toxicity.

Perhaps running experiments in parallel comparing results will provide information on how to improve *in vitro* cellular models. Integrating the two approaches will therefore create better *in vitro* models and improved concordance for food safety assessments.

I've listed a long list of references for your consideration in case you are interested in this further. I acknowledge the SOT FDA colloquium. I hope that this talk will stimulate some great talk. I hope to answer any questions now or later with the panel.

Thank you so much.

[Applause].

Question: I was wondering you may have said this, did you evaluate -- this is -- did you evaluate the urine from these mice?

Mossoba: Yes, that's how I got the Kim 1 measurements .

You evaluate it your data from the highest dose group so if the lower dose group did you still see Kim 1 performing fairly well ?

Yes, but not as robustly as the mice? Study was done in rats so I am a little confused here? group.

Thank you. Thank you.

[Applause].

Delaney: We have a roundtable discussion to discuss some topics.

Here we go. A few more slides and will put the panel together. Paddy Wiesenfeld will be our moderator.

The next upcoming colloquium is in December. I encourage everyone here to attend and invite people to attend. It's a great opportunity to learn more. We have risk assessment of mixtures in February and in May biotechnology of modern agriculture. That's my area of interest. I look forward to seeing how that develops. Always looking for new ideas. If there's something that's related to food and toxicology, it would be a consideration. The colloquial materials, recording, slides and text are at www.toxicology.org. You can go back and watch different videos and get transcripts as well. Also thank you for participating. We appreciate your input from the people in the room and online. We appreciate you attending. We will send you a link and a survey so please fill it out.

With that I propose we should go to the panel. We are scheduled to be here for an hour. I would like the panel to join me a. And take some questions.

Wiesenfeld: I won't keep you here if there are no questions. I want you to feel you can engage in this conversation. I think you can tell by the diversity of the talks that were presented, you can see the challenges that we have in order to address concordance between *in vitro*, *in vivo* whether it's an animal or in humans. It's a challenging field and

I think we would love to have your input. If we've missed a key element how can we make it more concise, make it more selective, more sensitive and be able to address these issues of *in vitro* to *in vivo* concordance please submit suggestions.

I want to thank those members of the SOT- FDA Organizing Colloquium Committee. They were very helpful in getting this meeting organized and delivered. I can't thank them enough. I want to especially want to thank Allen Rudman, Jeffrey Yourick, and Bryan Delaney. They've been on the committee for many years and they know what's been going on. I thank all the wonderful speakers we have had today. They were really excellent. They were very clear on so many different areas of expertise and shared them with you today. I hope it's been informative and challenging for you as well. I now open the microphones for you in the audience and for you online to please, come up and ask a question. If you don't, I'm liable to.

Audience Question: Thank you all for the great lectures today. My question is related to *in vitro*/in vivo. Discuss the population of changes that are going on especially in the U.S. How is extrapolating the results to minor populations and the changes in the general [Indiscernible] population. How does that change now and the future of toxicology facing these populations of changes?

Response: I think you're presenting an interesting concept. [Indiscernible - background noise] We will be able to test human cells from that have different genetic tests.

I'm looking at things to check that box so it's fortunate for what I've been looking at. It's a simple study. The social aspect beyond the testing guidelines for the study. It's also indicated the studies -- I think people think of them being how to do a well designed [Indiscernible] study. If you go back and look at the paper that was published a few weeks ago the first time the toxicology studies were conducted were? straight forward?[Indiscernible]. When you progress from 1927 till 2017, the tests have increased in accuracies.

One more thing to consider is at the CDC which shares with the Office of Public health genomics which when they do the [Indiscernible] study they collect genetic information on the subject . They have them by race, sex, age and genetic background. Among the things they measured and I know this because I happen to be there when they published the study, they looked at a number of drug metabolizing enzymes and other enzymes that would be of importance to toxicology. They still have the cells archived so they can do more but don't have the resources to do it. That information is available to a limited extent but there could be more. I'm suggesting that data mining may be another way to go at this because this is public available information. This study was published in 2010 out of the National Center for Health Statistics. Public health genomics.

Question: In one of the slides [Indiscernible - background noise]. [No audio]

That's a great question. The question was -- [Indiscernible - background noise] him -- the doctor made note of the fact there are different organ systems that I showed in which there could be potential concordance and he asked whether there are any analyses whether -- if people know how much concordance is present particularly for the newer systems which had the highest number of publications in both *in vitro* and *in vivo* testing. Where we would we start for something like that? Did I get that right?

Will there be commonality on the different organs or similarities when trying to evaluate?

[Indiscernible - mic is too far from speaker]

Response: Comparing *in vitro* to *in vivo* as I mentioned can take on different flavors. It depends on what questions is being asked. Is it more of a full toxicological systemic type of evaluation of *in vitro* to *in vivo* concordance. There is a possibility of literature establishing concordance? There's a huge data gap in the literature to be filled in my opinion. Rules are arbitrary at this point whether different organs will require different rules, most likely, because that's the nature of the beast. Every organ has its own nuances you cannot say I will say 80% concordance equals levels of this biomarker compared to levels of this or that biomarker. Those biomarkers don't exist for other organs. I think it would be a fair statement to say it would be difficult and lots of different rules will have to be established. We will likely rely on OECD and TOX21 and other organizations that are involved in this. Anyone else have anything to add?

Response 2: I completely agree with that. Basically, if you look at the level of the cells in those organ systems they are composed of many specialized cells which vary in the level of things of responsiveness? [Indiscernible]. Capacity to have inducible systems that can be used to protect the cells from toxicity. Again, we cannot do a one size fits all. You will have to, as she said, look at this from the point of view from the question you want to get answered. To have straight line toxicity data that would give you let's say a RFP or something like that, that's maybe not so easy to do realistically but what you can do is to ask questions about relative sensitivity, individuals if you pull the cells out look at the individual based on genetics, gender.

The other things as I pointed out or try to point out, we are worrying about more and more which is who is at risk from an exposure for me to pick -- dose level.

Question: [Indiscernible - mic is too far from speaker]

Response: You want me to comment on that? Ideally -- to repeat the question you are asking me -- you are pointing out that most of the data we show today would give someone inside in rank ordering as opposed to a quantitative ordering that could be used to develop the regulation. I think that's where we are right now. I have option is him that because of the tools that are being brought on line the use of modern tools of biology and computational toxicology, those tools are being applied and improving. We are getting better, I believe. How long this will take to come into everyday practice, I

don't know. It seems to be moving faster and faster or are moving slower and slower. I'm not sure which one it is. The idea is there's much to be done. The toxicology community is challenged in a number of ways including not only the new chemicals that are being brought in to use, the types of chemicals. We haven't talked about nano materials. Those kinds of things are challenges for toxicologists. On the other hand, we have these wonderful new tools. How it starts is if we can get a range finding, rank ordering, that's where we start. As the tools get sharper and we understand more the interactive factors that will influence which way the dose response might move, we get smarter. Then we can get there. I believe it's possible. It's hard work as Miriam pointed out.

This is playing hard but it can be done in my opinion and it will be done because it must.

Response: I have a couple of thoughts. I hate to answer the question with it depends but it kind of depends. If you're looking for quantitative values and things like that, it seems more difficult when you start getting too precise values. If you take a step back into some of the regulatory they don't require quantitative identifiers and [Indiscernible]. The dose has nothing to do with those [Indiscernible]. Depending on which variable you're trying to investigate, I think it could be a desire to do [Indiscernible]. In my case not so much.

Question: I wanted to thank the organizers of this colloquium -- my name is Dave Hatan. Not many years ago I was part of this organization. I retired in 2014. Admittedly I have not stayed on top of the state of toxicology but not too many years ago I was directly and indirectly involved to the OECD test guideline program. There was a recognition of this difficult transition time. I think among most of the people, then there was a burst of enthusiasm especially in certain of the large corporate bodies and in some of our government laboratories that we could substitute somehow *in vitro* testing for animal testing. I was concerned for considerable time that people seem to be leaping ahead about the meaning, significance, interpretation of toxicological data. Perhaps in hopes of going directly from *in vitro* testing to some kind of a risk assessment. I think you showed how difficult that actually is. There are several if not many interim steps and research programs that must be applied so that at some point in the future weekend melt the information from the *in vitro* system and *in vivo* systems and make the whole toxicological determination of the whole system better and stronger and perhaps more mechanistically oriented. Thank you again for what you're trying to do. I realize how difficult this is. I think some of this could be addressed if both the drug and the food industry would at the same time when they're doing animal testing on new products, a new substance, if they would also look at some of the more promising of the *in vitro* methods and try to get dose related affect between the *in vitro* data and the *in vivo* data. What you are doing is a necessary step but I think this other step is also essential If we want to substitute *in vitro* for *in vivo* that must be done carefully and it must be done step-by-step.

Response: Thank you.

Question: This one is for the panel. Dr. Delaney mentioned the cost of the experiment. It's a concern for every toxicological study. I'm interested in the panel's thought regarding study cost and whether the technology under development realistically has the potential to be more cost-effective than more traditional city paradigm considering that even if the costs were higher the data quality may be superior.

Response: It important consideration from us because a lot of biotechnology is being developed in countries that would be described as developing economies. The countries that would benefit most -- the actual studies we've been doing -- I would say inexpensive -- the people who gave us the money would not say it's inexpensive because it's research and research is expensive. I hope to get something available like the human cell line. For us the cost is more up front. The study itself would cost tens of thousands of dollars opposed to a toxicology study that will cost $5000. The test itself can cost hundreds of thousands and millions of dollars to produce. If we can't afford it would seems safe to say that other will not afforded. I think the question is more applicable to the more sophisticated studies. Our study is not that hard to do. Your looks more difficult.

One suite demonstrates working well the next step we would make it [Indiscernible] and more efficient.

Response 2: The issue of cost particularly for developing economies is a real thing. On the other hand, I've become a recent convert to computational toxicology or computational modeling and the ability to mind existing data sets which are not frequently looked at but you can do it, this is a quick way -- it depends on the level of answer you want. If you want a precise answer per risk assessment purposes or someone wants to develop something in our country that we don't know whether how risky it may be there are ways to find out. AME's is one such place to look at. There are others. Databases come out of a SGR or the NTP. You can go in it is publicly available if you have a computer and Internet access. You can learn a lot is what I'm trying to say.

Response 3: I think I would like to add that sometimes cost is a factor that can be high in a way that we choose it to. For example, we tend to perform particular toxicology that we feel to do because the field is doing but not necessarily the cheapest. However, there can be a plus meant assay that are cheaper that we tend to shy away from because we don't want to veer off too far. In some cases, cost is a compound. There are also in-house methods that can be performed but again the cost becomes high. Cost is a big issue and that will prohibit a lot of studies. It comes down to the availability of data. If you can perform to do the study how can you generate the data? That's an important question.

Question: I don't know if this is a question. Sometimes I remember we should keep our eyes on the prize. What is the prize? The prize is our legislative societal purpose. That is defined conditions and levels of substances that are safe for humans. The

problem with that method is because the absence of harm. That's the goal of all of this. The way we can get there is through very good dosimetry. I want to repeat that it's very easy to find things toxic. Everything is toxic at some level. What we must find out is where the levels of conditions are not toxic. If we have 100,000 chemicals out there we're not going to destroy all of them or eliminate them. We need to know what levels we can live with and that are safe.

Response: When dealing with chemicals I think it's more realistic you be concerned and be able to define the risk values and things like that. For the field I'm working our prize is checking the box in many cases. We have regulatory requirements saying you must do the study and it's expected to do it a certain way every time. To your point about human exposures being the prize, if you look at the concentration of [compound?Indiscernible] in the amount of crop you must be exposed to expose the same out of putting is no weight related. In some cases, you need millions of kilograms. Is a related in this case to exposure not so much -- the chemical is a different situation. I will leave it there and say I understand where you are coming from.

Response 2: That's part of the clue. Even though the animal studies don't work for proteins or food -- you must come to the conclusion that it's safe. You do this using -- maybe we should look at how you do that to get there because that's the goal.

Delaney: I agree. I was having a discussion with my younger son and this is a curveball. He explained to me that every time I fasten my seatbelt I increase the odds that I will die of cancer. Technically he is right. It's not the right question. I get where he's coming from. He's a smart, eight-year-old, good kid, but ultimately the question is about the dose and I'm thinking about the [Indiscernible] those are the variables you want get out of the animal study and if you're using *in vitro* system to replace those, I think it's a great question.

Response: I keep thinking about the lawyers lament about [Indiscernible] that we all have 100 arms because we keep saying, because on the other hand. We are talking about several kinds of things. What we are about is we are trying to move the ball down the field conceptually to see what we can do. That's with the tools we have in hand now and the tools we may need. We can measure chemicals. We can get access to the data relatively inexpensive. The question before the house is, is it safe, yes or no and if it is, at what level is it safe? That's really where toxicology must come through. We are getting better at it I believe. We are considering some of these other factors that we've talked about. These ancillary data that would help inform the decision. We are getting there. That's the best I can say. We still have some ways to go. One other thing I want to say is the issue of urgency, the issue of time. What happened with the Gulf oil spill and some of the cleanup in Texas that's going on when the sites got pried open by the last hurricane that want to Houston, these are public health situations where we cannot wait around to do an animal study even if you could design an animal study that would look at the hundred chemicals in a mixture into it by Friday. This is where the tools that we must bring to bear have to be agile. They must

be fast. The Corporation of can't -- computational toxicology tools are the ones that can move that fast. We don't have time to do weaker studies. I will stop there.

Question: Another question from online that touches on several issues that have been brought up. I would like to know more about the approaches they used to convert *in vitro* concentration to the *in vivo* dosing. It gets back to the symmetry and things like that. Did they use a protein similar to that *in vitro*/*in vivo* extrapolation work that the [Indiscernible] computational toxicology group is using or different approaches? How are you selecting you're *in vitro* dosing?

Response: That's a great question. I don't think there's any one answer. When selecting *in vitro* dosing the *in vitro* data has as much meaning as you give it with respect to your controls. If you have a test compound and you deemed non-toxic, what is that relative to? When choosing doses, it's important to cover a wide range especially depending on the organ in interest that's being modeled. I realize that? [Indiscernible] can come into play and limit things etc. but the factors *in vitro* dosing should always be performed relative to the correct control of interest. That would be helpful for understanding the nature of the toxicity of the compound of interest but it's not comprehensive necessarily. Many people try to look at blood levels and that's not always necessarily a good idea. Sometimes blood levels are not even available and blood levels don't reflect what tissue levels exist compounds. That's a good question. I don't know whether there's an exact answer. Anyone else have anything to add?

Response 2: The comment about the blood. Our toxicologist always consider dosage. For my study I would [want to know Indiscernible] wether it is metabolized activated or not[Indiscernible]. That's a question I asked myself. I would start from the blood level but always ask another question whether those chemicals can reach the blood-brain barrier?[Indiscernible] systems. Maybe that makes them different. Another question is, there is metabolism [Indiscernible] so whether I can choose a chemical concentration to make more relevant to *in vivo* .

Response 3: The only thing I would add regarding the stipulations is I don't think we talked about this before. It's the health of the *in vitro* cultures themselves in other words to make sure in using the data from *in vitro* studies that there is an assessment made on how healthy are the cells? Are you dealing with cells that are in good condition or are they going out? If that is the case -- if you get a dose high enough you will get that. You like to believe you are moving your studies with cultures in which are in good condition to start out. That sometimes is not addressed.

Response 4: I don't have anything additional to add to others than to say if you are reflected upon the entire presentation -- there's a desire to use *in vivo* /in vitro data with animal studies. We are doing methods and efforts to try to get more scientific information produced numbers that are relevant to safety. The question comes up like it did on the phone. These methods will be good, will they be too expensive and exclude everyone else? There may be instances of cases that *in vitro* data will not cut

it and you must do animal studies. In some cases, it may not be possible to get that without animal studies. Each one is on a case-by-case basis.

[Indiscernible - mic is too far from speaker]

Question: My question is for Miriam Mossoba. I ask you did you use the systems with multiplex systems to evaluate Kim 1, that biomarker? Did you look at any other biomarkers in toxicity like [Indiscernible] in your TGA experiment?

Mossoba: I looked at the whole battery of biomarkers that were relevant – this mike is not working very well .

Question: [Indiscernible - background noise] As far as the other biomarkers, how well do they perform and how has the field -- have they become more accepting of these biomarkers to use in early detections of renal toxicity?

Mossoba: I did not publish that data. If you are interested in learning more, I would be happy to communicate with you through email.

Question: This is what you were talking about Dr. Delaney, I'm wondering what the stop point is for the use of *in vitro* studies versus *in vivo* studies? The study you are doing with the filters and that cell line, if you found a protein substance that clearly caused issues in your *in vitro* studies, would you still want to continue in the *in vivo* study or higher-level studies to move on with that pathway or would that be the endpoint? That's the idea if you're trying to use *in vitro* studies. When does that *in vitro* study that that's a stopping point?

Delaney: I've had that question many times internally. The question was along the lines if you have a new protein in your excited about it. I would need questions for it to be suitable to continue. It's not necessarily over. Just because you don't see 100% concordance with *in vitro* and *in vivo*. If you could use that data to explain why you didn't do a mouse study, you cannot. Would not be supportive. The way the world works is that -- in the world when things are clear or dark you know exactly what to do. If it's toxic and use [Indiscernible] integrity then you would think it's hazardous. There still one component of the overall hazard assessment. The way I have explained it internally cut let's say we have five proteins and you must decide to carry forward three of them. If those three are the ones that tested clean in the *in vitro* assay and you don't take the two that have areas of gray and not look as clear, right now it would be considered supplemental data. I don't think -- it's not yet considered a substance for an acute mouse study but eventually we would want to see if it would be ready.

Your question is a good one. Things with intractable proteins they tend not to be soluble so what do the protein chemist like to do? They like to put him in Triton. We still have issues to work out so we don't get a bite [Indiscernible].

Question: As far as moving -- can you clarify something for me. I'm not clear with the cells being used for the ovaries, was that tissue for an animal or person or was that primary cells?

Xiao: They were primary tissue.

Question: They include a large majority of the cell types or do you know the balance of the [Indiscernible] cell?

Xiao: As far as that goes where they push you into first and primary cells because it supposed to be more representative. The problem tends to be a was in liver for long time. Liver loses its polarity and polarized [Indiscernible] very rapidly. You see the same type of [Indiscernible] versus -- intestinal cells have the same issue. I imagine you didn't want to cross that.

I did not show the data here. If you were to look at the lecture given by Dr. Hurley from Boston he showed the cells do not lose orientation. If you look at the electro [Indiscernible] -- I know *in vivo* they have other cell types down there. As far as it goes we did not look for orientation or things like that. We looked at the monolayer intact and they did and responded the way the cell lines did. It supports that while you could use the primary cells rather than cell lines there's not an obvious advantage of doing so unless you have a whole lot of money to spend. It's very expensive.

I think we should also remember the concordance does not have to be perfect.

You must know what is not toxic and what is safe.

You have the ovaries, you have the liver, and the pituitary gland. That is where it comes from. I wanted to comment on that.

I see, so right now because in the system, [Inaudible] right now, because of our limitation and the model is not perfect, we artificially added that onto the system. I believe, for example, if we look at what it will be like in five years, maybe we will have a model we can use. That will make our system more perfect and more relevant to a real human.

Question: Is there a test that shows accessible coordinates, and what would be your next step how would you use that?

Response: Basically, if I get some data from an individual, first I would want to confirm [Inaudible] a conflict.

Question: Let's assume it is confirmed and you have the concordance. What you do with it? You propose to use that for regulatory decisions? Do you propose to do that -- can use that as a tier 1 test that you would recommend to everyone?

Response: On my side, I would definitely recommend FDA/EPA, and others to use that system to replace the animal. That is my personal opinion.

Question: That is why I am getting confused. For that one effect, unless you have a whole battery eventually, the whole battery of tests that would represent a very structured -- restructured animal and you are being -- saying we can do -- I am not sure where you go with this information once we get it.

Response: I think right now I would say you would use them to help internal business decisions. If you have five things and one of them is clearly good and one of them is clearly a good. It is an indication of what performance you could do in the study. I would say if it becomes a regulatory study, we must consider with the cute study is and what it does. We look at -- there are not many parables out there. We could do liver-- liver enzymes, but we do not because it is not required in the study. What we're seeing now is it is reasonable to say [Inaudible] what I say -- when I say belongs in tier 1, not necessarily because we are looking at talks -- study we get most of this information you need about this protein, any individual protein, with a Chivas ability and exposure. Those are primary components that should tell you whether they should be a concern. That is the world where I live. And forcefully, the world where regulators live, they want studies anyways. They do not want this study, but others do. When it comes through, what we are seeing is in the case were proteins are going to be impossible or extremely difficult or extremely expensive to conduct a study on, but you decide you want acute toxicity data, and you ran it for your study. That is where we are, so is a ready for regulatory reflection? That is not my call. I do not think anybody would say it is yet.

Question: I can understand the point of whether there is a difficulty -- not material, but for most of applications, how would this be used?

Response: It has been used for drug and food, in fact, that is where I will take credit for using proteins for this person, but I was not the first one to test this for nutrients and food additives. Not so much for quantitative purposes, as much as is it available? Is it predictive of what you would expect to see in the animal? Does this entirely eliminate these studies? No.

Question: You are not really advocating a regulatory role for these tests?

Response: I do not know if we are ready for that yet. I know we are talking about if it could be or what it would take to get it there, right now I would say we have not gotten approval to use it by a regulatory agency. Do we have an interest? Yes, we do. That is why we are setting the time and effort for the science.

Questioner: Thank you.

Response: I wanted to at the sometimes when we find concordance and we find concordance toxicity it does not mean you need to take any action. For example, it is

highly toxic at the highest dosage tested, however, does that mean we need to take any regulatory action? It depends, what are the levels in food and human exposure? Those questions go hand in hand. So, yes, it can be one day replaced as they introduced without question, but sometimes the question is do we need to take any action? I think that is an important consideration to think about as well.

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