FOOD AND DRUG ADMINISTRATION

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DRUG-INDUCED LIVER INJURY CONFERENCE XV

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THURSDAY
MARCH 19, 2015

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The Conference met in the University of Maryland Marriott Conference Center, Chesapeake Ballroom, 3501 University Boulevard East, Hyattsville, Maryland, at 8:00 a.m., John Senior, Paul Watkins, Mark Avigan, and Lana Pauls, Organizers, presiding.

PRESENT

JOHN SENIOR, Organizer
PAUL WATKINS, Organizer; Moderator, Session IV
MARK AVIGAN, Organizer; Moderator, Session III
LANA PAULS, Organizer
ALBERT CZAJA, Moderator, Session III
GYONGYI SZABO, Moderator, Session IV
JACK UETRECHT, Speaker, Session III
EINAR BJORNSSON, Speaker, Session III
DAVID BERMAN, Speaker, Session III
ARIE REGEV, Speaker, Session III
PAUL HAYASHI, Speaker, Session IV
TOM URBAN, Speaker, Session IV
MERRIE MOSEDALE, Speaker, Session IV
DAN ANTOINE, Speaker, Session IV
BRETT HOWELL, Speaker, Session IV
MINJUN CHEN, Speaker, Session IV
ALEXANDER GERBES, Speaker, Session IV
ANREAS BENESIC, Speaker, Session IV

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Session III 19 March 2015 (8:00 a.m.)

Moderators: Mark Avigan, Albert Czaja

Dr. CZAJA: Good morning and welcome to this session of the conference. This session is entitled "Autoimmune Hepatitis or DILI -- One or Both?"

My name is Albert Czaja and I am Professor Emeritus of Medicine at the Mayo Clinical in Rochester, Minnesota. And I will be co-moderating this session with Dr. Mark Avigan, who is the Associate Director for Critical Path Initiatives at the FDA Center for Drug Evaluation and Research.

Our goals this morning are to describe the forms of immune-mediated liver damage that are clinically manifested as drug-induced autoimmune-like hepatitis or and classic or idiopathic autoimmune hepatitis. And we hope that this discussion will actually lead to vigorous interchange that will allow us to explore

everyone's opinion about the nature of these different diseases and the best approach to diagnosing them and ultimately managing them. Now, with that foreword, I shall begin the session by introducing our first speaker, who is Dr. Jack Uetrecht, Professor of Pharmacy and Medicine at the University of Toronto. And Dr. Uetrecht will present a topic entitled “Navigating Immunologic Responses to Drugs and Biologics to Predict Clinical Outcomes.” Dr. Uetrecht, welcome.

Uetrecht photo, biosketch, abstract

JU#1: Thank you very much. I don't know how many of these meetings I have been to but they are always very enjoyable. And John is just the energizing bunny to keep this going the way he does.

I didn't choose this title but I think it is not inappropriate. So, in other areas, there hasn't been much question that idiosyncratic drug reactions are immune-mediated. But in the area of hepatology, that was not the case. I think more

and more people have decided that these things really maybe immune-mediated. And certainly, they have the same characteristics as other types of idiosyncratic reactions, in terms of delay and onset, et cetera.

JU#2: So, there are several pieces of evidence that I am going to point out. I can't point to all four screens at one time, so I apologize. But some of the evidence that these things are immune mediated are at first just the characteristics. I mean this is the sort of typical type of characteristic for immune-mediated reaction. The delay and onset, often a rapid onset on re-challenge, et cetera.

There is often the presence of eosinophils, fever, rash, et cetera, that suggest an immune response but even if those features aren't there, it does not mean that these reactions are not immune-mediated. Often we see the presence of anti-drug antibodies. That doesn't prove that it is an immune-medicated reaction. These could be

an epi phenomenon but, again, it is consistent with the hypothesis that these reactions are immune-mediated. And unless you know what the reacting metabolite is and can make the appropriate antigen, you can't test for antidrug antibodies. And so the number of drugs for which this has been shown is relatively limited. More recently, there have been HLA associations. And again, that is pretty strong evidence that the reactions involved are immune-mediated. And finally, there are positive lymphocyte transformation tests. So, in this case, you take cells from the patient who has had an idiosyncratic reaction, incubate with the drug involved, and if they proliferate, that means that the lymphocytes have recognized the drug. And that is, I think, very strong evidence that the reaction is immune-mediated. I used to not understand why this reaction would be positive because, in most cases, we think it is a reacting metabolite of the drug and not the parent drug that

is responsible. So, why is the immune system recognizing the parent drug?

JU#3: What we have seen is that once you get strong immune response, you get epitope spreading, so that often, the immune system recognizes the parent drug, as well as drug-modified protein. So, even though I think these things are immune-mediated, I would be the first to admit that we do not have conclusive evidence, in most cases. It is just this pattern that looks like an immune reaction. So, how do we really test the hypothesis that reactions are immune-mediated? What we really want to do is test patients but we want to know what happens before the patient gets sick. What are the events leading up to this immune response? And of course, we don't know who is going to have an idiosyncratic reaction. So, that is very difficult to do.

As in other areas of medical research, animal models are very important but we always have to make the link between the animal model and humans. We

are really interested in humans, not animals, and unless the characteristics of the animal model faithfully reproduce what happens in humans, they are really not very useful.

Unfortunately, although animals have idiosyncratic reactions, they are just as idiosyncratic in animals as they are in humans. And unless you have a pretty high incidence, it is not going to be very useful. And if these reactions are immune-mediated, you would think that we could just stimulate the immune system and that would allow us to develop -- easily allow us to develop animal models. I don't know how many, and I mentioned this last year, how many graduate student years of mine and other people, I am sure, have been wasted trying to develop animal models by stimulating the immune system in various ways and it never worked. And this, to a large degree, mimics what we see in humans, that patients with preexisting liver disease and inflammatory conditions like inflammatory bowel disease are not

at significantly increased risk. And so, stimulating the immune system, somehow the immune system seems to be able to differentiate the drug from other inflammatory stimuli.

JU#4: A classic drug that was not believed to be immune-mediated is isoniazid. And part of this was based on classic studies done almost four decades ago with isoniazid. And it was shown very clearly that in rats, when you gave a really high dose of the drug, you got acute toxicity that was mediated by a metabolite of acetylhydrazine. But it is the wrong model in the wrong species because that is not the sort of toxicity that we see in humans. It is always delayed in onset. And when we looked at the metabolism, in fact, in the upper right-hand corner, you see so that we developed an antibody that recognizes with isoniazid bound to protein and in four different mice you see covalent binding to a range of different proteins. On the left you see the same immunoblots from control animals that weren't treated. So, you can see that

the antibody is quite specific for recognizing isoniazid-modified proteins. It’s bioactivation of the parent drug, not acetylhydrazine in these mice, that is leading to the covalent binding.

If you compare mice and rats, there is a little bit of covalent binding of the parent drug in rats but much less than in mice. And if you look at human microsomes, you see covalent binding of the bioactivation of the parent drug. So, we more like mice than we are to rates.

JU#5: And in collaboration with Will Lee, we took sera from quite a few patients that had isoniazid-induced liver failure and we see a pattern, a different pattern in different patients of antibodies that either recognize isoniazid or autoantibodies that recognize one or more of the P450s that form the reacting metabolites.

Again, this isn't proof that it is immune-mediated but certainly consistent with that hypothesis. And we needed to know what the reactive metabolite was, in order to be able to test

this hypothesis. But still, when we treat mice with a reasonable dose of isoniazid that would give comparable to therapeutic concentrations in humans, we don't see any toxicity. So, we don't have an animal model.

JU#6: And so why is it so difficult to develop animal models of idiosyncratic drug reactions? Well, they may have the wrong MHC repertoire or T cell receptor repertoire. But if you remember that immunoblock that I showed you with covalent binding of isoniazid, it looks like a coomassie blue stain. It is binding to any protein that has a lysine on it. And each one of these proteins is processed to several peptides. So, there ought to be some MHC T cell receptor complex that would recognize one of those peptides. Another possibility is you don't have sufficient activation of antigen presenting cells. But again, we tried to do that and at least the ways that we tried to do it didn't work. We have also tried to increase the formation of reactive

metabolite, to deplete glutathione, to do all sorts of things and none of those methods work.

And it appears as if, especially in the liver, the default immune response is immune tolerance. That is the key, I think. So, of course you are familiar with the fact that if you give a whole bunch of people isoniazid, in most cases, nothing happens. So, if you consider Homer normal, that is the result.

JU#7: In a study that I will show you in a minute, up to 20 percent of the patients will have a bump in ALT but you can continue to treat with isoniazid, the ALT comes back to normal, nothing happens. That is adaptation. And only the rare patient, less than one in a thousand, develops liver failure. Now, if the injury is mediated by the immune system, this adaptation must be immune tolerance. And a good example, I think, of that, Paul mentioned this yesterday with lumiracoxib, it is associated with a specific HLA genotype that is pretty good evidence that it is immune-mediated.

And it is the same HLA association for the mild toxicity as it is for the severe toxicity. So, again, if that reaction is immune-mediated, that adaptation must involve immune tolerance.

So, although it is difficult to do prospective studies in humans, we did it with isoniazid because the incidence of mild injuries, actually pretty high, up to 20 percent. And what we found is that in those patients that had a mild increase in ALT and the ALT just went from what is it, 18 to 93, I think only one of the six patients that had an increase was over 100 and they continued on treatment and it goes back to normal.

JU#8: In those patients that had an increase in ALT, you see an increase in Th17 cells. That is in the upper right-hand corner, this is one example but all six of them had an increase -- what did I say -- all those that had an increase in ALT had an increase in Th17 cells, which are proinflammatory cells but they also had increase in T cells producing IL-10, which is an

immunosuppressive cytokine. So, even in these mild injuries, we are seeing a risk immune response. With isoniazid, we don't see any liver injury in mice at a reasonable dose of the drug.

JU#9: But with another drug that causes both liver injury and agranulocytosis, amodiaquine, here is a metabolic scheme showing the formation of the reactive metabolite. We do, in mice, see mild injury. So, there is an increase in ALT. We continue treatment with the drug, and then you get adaptation. Again, we believe this is immune tolerance. So, if it is immune tolerance, one possible way to overcome that immune tolerance is to immunize. We know what the reactive metabolite is. We can bind this molecule to protein. The immunized mice with amodiaquine-modified hepatic proteins, along with adjuvant, and then we wait a couple weeks and then we treat with oral amodiaquine. We should now get a much stronger immune response.

JU#10: And it may be hard for you to see but the bars that are elevated are the ones that were not immunized. We get an increase in ALT. But in those that were immunized, that immunization, instead of making a liver injury worse, it was actually protective. It was a paradoxical response.

JU#11: And if you look in the liver of these animals, you see an increase in myeloid-derived suppressor cells and T regulatory cells. So, this immunization actually induced immune tolerance, even though we used adjuvant to the drug-modified proteins.

JU#12: So, another strategy, if the dominant response is immune tolerance, maybe if we block immune tolerance, we could get more injury. And as you probably know, there are a lot of drugs being developed now to block immune tolerance for the treatment of cancer. And it is a very promising area of research. And two of those molecules are PD-1 and CTLA-4.

JU#13: And this is a complicated slide but you see in wild-type animals, again with amodiaquine, there is an increase in ALT but, despite treatment, the ALT goes back to normal.

JU#14: If we co-treat with anti-CTLA-4, we get a stronger immune response and more injury, but it still goes back to normal, despite continuing treatment.

JU#15: On the right side, these are PD-1 knockouts. Again, we get a stronger immune response and injury but it resolves, despite continued treatment.

But if we co-treat these animals with anti-CTLA-4, now -- and the scale is different here, now it doesn't resolve and we get histopathology of piecemeal necrosis that looks just like what happens in humans with severe liver injury. Now, despite the fact -- and the ALTs are not that high but, as you know, clinically, I would much rather have a high ALT from ischemic liver injury than a sustained liver injury over a long

period of time. And we do see an increase in bilirubin in these animals, along with the histopathology but we don't get overt liver failure.

JU#16: There is decreased function but not overt liver failure. And we also see, and again, this, I am sure, is difficult to see but in the wild type animals, there is an increase in T cells that express PD-1, that express CTLA-4, et cetera. In the PD-1 knockouts, there is an increase in Treg. So, even though we are getting a strong immune response and liver injury, there is still -- the immune system is trying to down regulate that immune response. In the lower quadrant here, you see also an increase in cytotoxic T cells. These are CD8 T cells that express granzyme B and perforin. And so this suggests that injury may be mediated by cytotoxic T cells. And there is evidence clinically that some of the most severe liver injury is mediated by cytotoxic T cells.

JU#17: So, what we did is deplete CD8 T cells and sure enough, it totally protects these animals from liver injury.

JU#18: So, how about other drugs? And Arie was very enthusiastic when I presented some of this data last year with a different way of trying to block immune tolerance. We weren't seeing injury with isoniazid, so I was a little hesitant at that point. But when we used the same system with isoniazid and I say here it increases liver injury, that is actually a misstatement because without using PD-1 knockouts and anti-CTLA-4, we don't see any liver injury but in that model, we do see liver injury.

The same thing happens with nevirapine. We don't see any liver injury in wild type animals but, in this model, we see livery injury with nevirapine. So, it looks like blocking immune tolerance is exposing the potential of a drug to cause immune liver injury. And there is another drug that I can't tell you about because of the

confidentiality agreement but a drug that is used to treat cancer by modulating immune response, we are seeing the same picture. Now, there are a lot of different cells and molecules involved in immune tolerance.

And Lance Pohl has a paper that has been accepted in *Hepatology*, where he looked at it from a different perspective. Lance did work with halothane some three decades ago, that actually convinced me that these events were immune mediated. And Lance, for three decades, has been trying to develop animal models without success. But finally, he succeeded. Unfortunately, he had a stroke and has had to close down his lab. But instead of going after immune tolerance with PD-1 and CTLA-4, he depleted myeloid-derived suppressor cells and he gets liver injury with halothane that looks very similar to what happens in humans. There are multiple mechanisms, redundant mechanisms for immune tolerance and any one of these can have an effect. The other interesting point is that some

of the most severe liver injury, I think, is mediated by CD8 T cells and we showed that we could block that in the amodiaquine model, in his model, it looks more like halothane. He sees eosinophilia and if he blocks CD8 T cells, it doesn't protect but if he blocks CD4 T cells, it does protect. These drugs are causing immune responses that damage the liver but the immune response can be different with different drugs and even the same drug in different people.

JU#19: And how about biologicals? It is not surprising that drugs like interferon alpha would cause autoimmune hepatitis. It is stimulating the immune system. What is more surprising is that drugs that are supposed to be immunosuppressive like infliximab also can cause autoimmune hepatitis. TNF alpha is doing more -- it is more complicated than just that this is an immunosuppressive drug. And not only can some of these drugs used to treat cancer cause liver injury but they can interact with other drugs. So, for

example, if you co-treat with ipilimumab, and I am not that familiar with that drug, but the drug can cause an increase in ALT but you combine with anti-CTLA-4 and it markedly increases the risk of severe liver injury. So, as we develop these drugs, we are going to see drug interactions with other drugs because it uncovers the potential of the drug to cause liver injury.

JU#20: And I will go through this quickly because it is not liver and I need to go through it quickly. We developed an animal model for nevirapine-induced skin rash. Now, it is a lot easier to induce an immune response in the skin than it is in the liver because the liver, the default immune response is, again, immune tolerance.

JU#21: And again, we have found that in rats we get a skin rash that looks very much like what happens in humans and this table lists the different characteristics; it is very similar between rats and humans.

JU#22: And we were able to show that there is a reactive sulfate formed in the skin that is responsible for this skin rash.

JU#23: And then the next question is, because we could prevent the covalent binding and the rash with a topical sulfotransferase inhibitor, the next question is how does covalent binding of this reactor metabolite that we showed clearly is responsible for the rash, how does it induce this immune response that leads to the skin rash?

JU#24: And it was known that chemically reactive agents applied to the skin -- poison ivy, or dinitrochlorobenzene -- cause contact hypersensitivity. And it is known from that literature that animals that are deficient in the inflammasome apparatus are resistant. And although we were getting a reactive metabolite formed in the skin from a precursor that came from the liver, otherwise it should be a similar mechanisms to contact hypersensitivity.

JU#25: So, maybe activation of inflammasomes is an important early step in the induction of an immune response. And this is just a pictorial of the inflammasome. It is a complex structure. What is important is that procaspase gets activated to caspase 1 and that converts pro-IL-1 beta to active IL-1 beta. And if something increases the level of IL-1 beta, and you can block it with a caspase 1 inhibitor, that means it must have come from an inflammasome.

JU#26: So, we looked at pairs of drugs that caused idiosyncratic reactions, one of which is much safer than the other. So, we compared telaprevir with boceprevir. Telaprevir had a black box warning because of severe skin rash, boceprevir doesn't. Dimethyl fumarate is a drug being developed for the treatment or has been developed for the treatment of multiple sclerosis, is associated with contact hypersensitivity and a bunch of adverse reactions.

Ethacrynic acid is an old drug. It is also a microacceptor. If you are a chemist, you know what that means. If you are not, you probably don't. But these drugs are chemically reactive but yet ethacrynic acid, although it is known to covalently bind to protein, forms a glutathione adduct, I went through the literature and I couldn't find one report of an idiosyncratic reaction to ethacrynic acid. I don't know why.

JU#27: So, when we looked in in vitro assay of the ability of these drugs to activate inflammasomes, so this is a dose response curve, telaprevir activated inflammasomes. We could block it with an caspase inhibitor. Boceprevir didn't significantly activate inflammasomes. A different scale here, dimethyl fumerate really activated inflammasomes and ethacrynic acid, not a bit, even though it covalently binds to protein.

JU#28: One thing that I have been interested in for a long time is clozapine and olanzapine. Clozapine causes agranulocytosis, as mentioned

yesterday, can also cause liver injury. In most patients treated with the drug, there is an increase in IL-6, neutrophilia. It clearly causes an immune response. Olanzapine doesn't do any of those things and I thought the difference was dose. The structures are very similar, as shown below, and both form a reacting metabolite. The dose of clozapine is more than an order of magnitude greater than olanzapine. So, I thought that was the major distinction between the two.

JU#29: But in terms of inflammasome activation, at the same concentration, clozapine activates inflammasomes and olanzapine doesn't. So, there is some other difference than dose between these two drugs. I don't know what it is but it clearly shows up with inflammasome activation.

JU#30: Amodiaquine, the drug that we used for the liver injury model, it also activates inflammasomes. So, this may be a biomarker for the ability of a drug to cause an idiosyncratic

reaction. Now, with drugs that are intrinsically reactive, that is easy to test. Even with clozapine, there is enough mild peroxidase in these THP-1 cells, we get bioactivation and covalent binding. I didn't show you the data but we did covalent binding of clozapine to the THP-1 cells. But if the drug requires P450 bioactivation, these cells don't have a significant amount of P450.

My best guess, and it really is a guess, is that maybe the hepatocytes make a reactive metabolite. It is known that hepatocytes release exosomes, or microvesicles, or whatever you want to call them. These would be taken up by antigen presenting cells, Kupffer cells, and other antigen presenting cells and proactivate them. And so we have started studies looking for this. Unfortunately, in the way that we isolate them, it is just killing the THP-1 cells. So, I think we have to go back and not use a simple way to isolate them but use a more complicated way.

Am I running out of time? Yes, okay.

JU#33: So, what are risk factors in humans? Genetic factors are, obviously, important. T cell receptors are formed by random recombination events. So, even identical twins have different T cell receptor repertoires. I talked about activation in the immune system and, again, clinically, in the ways that you might expect preexisting liver disease, et cetera, that doesn't seem to be important. Deficiency in immune tolerance, the patients that have idiosyncratic reaction do not have the degree of immune tolerance deficiency that these animal models do. So, I think we are uncovering something but I don't think that is a major issue in humans, although polymorphisms in IL-10 can affect the type of immune response you get and the mortality of DILI. It doesn't seem to affect the risk.

One point I would like to make is I think the immune system is a product of everything. It is like the brain. It is a product of everything it

has ever been exposed to and so different people are going to respond differently.

JU#34: We'll pass over that one.

JU#35: So, I think valid animal models are important. There is compelling evidence, I think that most idiosyncratic reactions, including idiosyncratic DILI is immune-mediated, genetic factors play a role but there are other factors that are important. I think, again, environment, you know it is nurture-nature issue again. I think environmental factors important but we don't know exactly what they are. They are not the obvious environmental factors. I think prior exposure to different pathogens set how our immune response responds.

And finally, the most severe reactions are ones that persist after you stop the drug. And if you know what the mechanism is, whether with some of the most severe, it is due to cytotoxic T cells or with other ones that have a more immunoallergic type. I think we have an opportunity window to

treat these patients, so that they don't develop overt liver failure, so they don't die or require a liver transplant. And if we could treat them better, I think it would be much less a serious problem. In other fields of idiosyncratic reactions, attempts are made to do this but, for some reason, although patients are often treated with steroids, there has been no good trials to see what works in treating these patients.

JU#36: And finally, I want to thank the people that actually do the work, not me, and I thank you for your attention. And I'm sorry I went long.

Czaja photo, biosketch, abstract

AJC#1: My task is to discussion idiopathic autoimmune hepatitis, which, by definition, is defined as a disease of unknown cause. But I think as I proceed through this presentation, you will begin to identify themes that resonate quite nicely with what Dr. Uetrecht has already mentioned.

AJC#2: My goals are actually to describe the advances that are transitioning autoimmune hepatitis from and idiopathic disease to an explainable disease.

And I will also indicate that this transition is far from complete, as new knowledge actually brings new questions about the nature of this entity.

AJC#3: Idiopathic autoimmune hepatitis is an inflammatory liver disease, which, by definition, is of unknown cause. Now, it is characterized by the presence of autoantibodies, hyper gamma blobulinemia, especially high levels of serum in globulinemia levels and, by the presence of interface hepatitis on microscopic examination.

AJC#4: Now, codified diagnostic criteria for definite autoimmune hepatitis requires the absence of viral markers. And there must be no or low likelihood of alcohol-related or drug-induced disease. Additionally, the immune manifestations must be substantial, as reflected in serum

autoantibody and gamma globulinemia levels and there must be no evidence of homeostasis, either biochemically, clinically, or histologically.

Now, liver disease is of similar immune manifestations but with known causes must be designated by their etiologic agent and, therefore, they must be classified separately from idiopathic autoimmune hepatitis, mainly because their treatments and their outcomes are different.

AJC#6: Now, two types of autoimmune hepatitis have been described, based, primarily on their serological markers. Type 1 autoimmune hepatitis is characterized by the presence of antinuclear antibodies or smooth muscle antibodies. And Type 1 autoimmune hepatitis affects all age ranges and it is the most common form of this disease worldwide.

AJC#7: Type 2 autoimmune hepatitis is characterized by antibodies to liver, kidney, microsome type 1. It affects mainly European children. And in fact, it is relatively uncommon in the United States both in children and in white North American adults with this disease.

Interestingly, both types of genetic predispositions but they actually differ in regard to their susceptibility alleles.

AJC#8: Now the susceptibility alleles that have been implicated in Type 1 autoimmune hepatitis are DRB1*0301 and 0401 in white, Northern European and North American patients.

DRB1*0404 and 0405 have been associated with an increased occurrence of Type 1 autoimmune hepatitis in Mexicans, Japanese and mainland Chinese.

And HLA DRB1*1301 is the primary susceptibility allele in Argentina, Brazil, and Venezuela, especially in very young children.

The susceptibility alleles that have been implicated in Type 2 autoimmune hepatitis are DRB1*07 in British, German, and South American patients and DRB1*03 and DB1*02 in Spanish patients. A report in the DQB1*0201 is in strong linkage to this equilibrium with DRB1*07 and DRB1*03. Therefore, it has been proposed as the

principal genetic determinant of Type 2 autoimmune hepatitis. The diversity of these susceptibility alleles that have been associated with autoimmune hepatitis really suggest that individuals are selected to develop this disease by their genetic predisposition to respond to certain sensitizing antigens and that, in fact, because of these different susceptibility alleles, different sensitivity antigens are likely to generate the same clinical disease.

AJC#9: Susceptibility alleles do encode the antigen binding groove of Class II molecules of the major histocompatibility complex. And the antigen binding groove, as depicted on this slide, actually can determine the nature of the antigen that is accommodated. Various amino-acid sequences coded by the susceptibility alleles indicate that the occurrence of type 1 autoimmune hepatitis in white North America and Northern European patients is strongly associated with a sixth immunoacid sequence, included as LLEQ K R at positions 67

through 72 of the DR beta polypeptide chain of the Class II MHC molecule.

AJC#10: Now, the strongest association with Type 1 autoimmune hepatitis in this population is actually the presence of a positively charged lysine at the DR beta 71 position.

AJC#11: If we look at the susceptibility alleles that have already been described in North Americans, Northern Europeans, and Asians, these susceptibility alleles all include a sixth amino acid sequence between positions DR beta and 72 that are the same or similar to the ones that I have just mentioned. The only exception is the substitution of a positively charged arginine encoded as an R for a positively charged lysine coded as a K at the DR beta 71 position. These findings suggest that patients with these susceptibility alleles may in fact respond to the same or similar sensitizing antigens.

In contrast, DRB1*1301, which I have just mentioned as the predominant susceptibility allele

in South American patients, especially children, that susceptibility allele encodes a different six amino acid sequence in this DR beta 67 or 71 position, especially different in that it encodes a negatively charged glutamic acid encoded as an E in the DR beta 71 position.

Clearly, these different susceptibility alleles for the same disease in different ethnic populations and in different age groups suggests that the analyses of these susceptibility alleles and the engine binding groups that they encode might well provide some valuable clues about the nature of the sensitizing that actually causes this disease.

AJC#12: It is also important to note that multiple genetic polymorphisms have been described in idiopathic autoimmune hepatitis but their role is clearly unclear. Recently, a polymorphism for the SH2B3 gene has been described in a cohort of patients with Type 1 autoimmune hepatitis from

Northern Europe. This analysis was done by genome-wide association studies.

The variant of SH2B3 may well affect immune reactivity by altering the activation of T cells affecting cytokine production and modifying the adaptive immune response.

Another variant, a variant of the CARD10 gene, has also been implicated in Type 1 autoimmune hepatitis in the same genome-wide association studies. And this variant might well affect pro-inflammatory signaling pathways. The important message here is that multiple polymorphisms have already been described in idiopathic autoimmune hepatitis and that many of these polymorphisms are not disease-specific. In fact, many do occur in multiple immune-mediated non-liver-related diseases and, in fact, they probably contribute to modulating the vigor of the inflammatory response but are not clearly essentially for the development of the disease.

AJC#13: Now the cytochrome oxygenase CYP2D6 is now recognized as the principal target autoantigen of Type 2 autoimmune hepatitis. Antibodies to liver kidney microsome in certain Type 1 inhibit the activity of this enzyme in vitro. Liver-infiltrating cytotoxic CD8 cells are sensitized specifically to CYP2D6 in patients with Type 2 autoimmune hepatitis. And human CYP2D5 administered by immunization or by infection with an adenovirus vector actually induces experimental autoimmune hepatitis in mice.

AJC#14: CYP2D6 has five epitopes, which are recognized by antibodies at LKM1 and the dominant sequence spans the positions 193 and 212 on the recombinant CYP2D6 molecule. This sequence is recognized by antibodies to LKM1 in 93 percent of the British patients with Type 2 autoimmune hepatitis. Importantly, homologies exist between the epitopes associated with CYPD26 and amino acid sequences within hepatitis C virus, cytomegalovirus and herpes simplex virus type 1.

Now, these homologies suggest that repeated or protracted infection or exposure with viral antigens that closely resemble self-antigens can overcome self-tolerance.

The prominent target autoantigen of Type 1 autoimmune hepatitis, which is the most common form worldwide is still unknown.

AJC#15: Animal studies have indicated that molecular mimicry is an important mechanism for losing self-tolerance in autoimmune hepatitis. This mimicry between human and mouse CYP2D6 can actually loss of humoral and cellular tolerance to mouse CYP2D6 in experimental autoimmune hepatitis and actually induces the disease in these animals.

Epitope spread is also an important mechanism for sustaining or exacerbating this disease and animal studies have indicated that reactivity to CYP2D6 early in the course of the disease is directed against closely homologous epitopes to the mouse CYP2D6 but that reactivity later in the course of experimental autoimmune hepatitis begins

to be directed at neighboring epitopes and remotely homologous epitopes.

AJC#16: Now, interesting to this group and to me is the fact that the principal autoantigens that have been implicated in the various clinical syndromes associated with autoimmune hepatitis have all been drug metabolizing enzymes associated with the P450 system.

Type 2 autoimmune hepatitis, the autoimmune hepatitis has been associated with autoimmune polyglandular syndrome Type 1. The autoimmune-like hepatitis that has been induced by tienilic acid all have been associated with drug metabolizing enzymes in the P450 system. So that clearly, the P450 system is pivotal to the emergence this form of liver disease.

AJC#17: The cell mediators of idiopathic autoimmune hepatitis are components of the innate and adaptive immune systems. The cells that are at the center of this very complex interactive

network are the regulatory T cells and the natural killer T cells.

AJC#18: The regulatory T cells have broad immunosuppressive effects that have been really a hot focus of attention in idiopathic autoimmune hepatitis. These cells are natural thymic-derived cells but they can also be induced from naive conventional T lymphocytes by antigen exposure, by stimulation with transforming growth factor beta. The important thing is that the deficiencies in the number and function of these cells have been described in idiopathic autoimmune hepatitis but in fact these results have been recently challenged and that the exact role of the regulatory T cell in idiopathic autoimmune hepatitis is controversial.

AJC#19: The early studies described that a reduced number of the regulatory T cells in the peripheral circulation of patients with autoimmune hepatitis compared to normal healthy controls, regardless of the degree of inflammatory activity.

These early studies also demonstrated that the addition of regulatory T cells to preparations of CD8 cells failed to significantly suppress the activity of the effector CD8 cells.

AJC#20: So, these studies really generated great interest in the regulatory T cells as a possible mechanism that could be a target population that could be manipulated and improved through various pharmacologic and cellular interventions. But the fact is that recent studies using more restrictive and rigorous definitions for regulatory T cells have actually contested these findings.

AJC#21: These studies demonstrated that the number of peripheral regulatory T cells in patients with autoimmune hepatitis actually were similar to those of healthy normal individuals. And furthermore, the addition of regulatory T cells from patients with autoimmune hepatitis to preparations of effector T cells reduced the

proliferative activity of the effector T cell population similar to normal controls.

AJC#22: The critical determinant of the activity of autoimmune hepatitis may relate to the relative balance between the activities of the regulatory T cells and the effector T cells, rather than to the absolute number or function of individual cell populations.

AJC#23: The natural killer T cells are really emerging as the key regulators of immune reactivity in this disease. The natural killer T cells have dual personalities. They can respond very rapidly to cites of tissue injury within the liver and behave like an innate immune response and they can be sensitized to specific antigens and behave as an adaptive immune response. They have surface markers both of natural killer cells and conventional T cells and they have stimulatory and inhibitory actions that are, in fact, dependent on the nature of the sensitizing antigen, who like the lipids, actually sensitize these cells through CD1

molecules that are class 1 molecules of the major histocompatibility complex. And the nature of the lipid antigen, whether it be a ceramide or a sulfatide can actually determine the predominant action of the NK T cell population. So, the NK T cells are actually emerging as an exciting area that might lead to therapeutic manipulations by designing antigens that would elicit disease-specific functions.

AJC#24: The migration of inflammatory and immune cells to sites of tissue injury within the liver is actually orchestrated by a variety of chemokines. But the chemokines CXCL9 and CXCL10 have been increased in autoimmune hepatitis and their levels have actually been closely associated with disease activity. The cytokine exotaxin-3 has also been increased in immune-mediated liver diseases compared to viral-related liver diseases. And in fact, this finding suggests that eosinophils are preferentially recruited to sites of tissue liver injury that are immune-mediated. The

chemokines are currently being evaluated primarily as indices of disease activity and indices of treatment response.

AJC#25: Lastly, I would like to mention apoptosis, since apoptosis is the principal mechanism of how to cite loss in autoimmune hepatitis. A receptor mediated extrinsic apoptotic pathway predominates in this disease and it mainly results in the activation of caspase-3 and 7, which result in the fragmentation of the nucleus. It is also important to note, however, that an intrinsic apoptotic pathway associated with mitochondrial dysfunction induced by reactive oxygen species also contributes to the apoptosis, mainly through activation of caspase, through the development of an apoptosome and then activation of caspase-9.

The apoptosis of hepatocytes has an important consequence, the release of apoptotic bodies, which can serve as allele antigens, activating the lymphocytes that can actually expand the

inflammatory autoreactive and fibrotic responses in its self-amplification loop.

AJC#26: I would like to close by emphasizing that idiopathic autoimmune hepatitis is an important model by which to begin to understand immune-mediated liver injury. It is also a disease which can be distinguished from most forms of autoimmune diseases that have known causes, mainly by its self-perpetuating nature, its strong genetic predisposition, and its spontaneous occurrence.

It is also possible that deficiencies in the modulation of certain immune cell responses may distinguish the disease, as may propensities for life-long fluctuations in disease activity and progression to cirrhosis.

AJC#27: The key questions that I see as being unanswered as yet are: Does autoimmune hepatitis have a cause or does it emerge spontaneously? Can triggering exogenous antigens actually be discovered and validated? What is latent

autoimmune hepatitis and does it exist? And can autoimmune hepatitis be predicted and the risk mitigated or obviated?

I think these are questions that offer great challenges that must be addressed by future investigation.

AJC#28: In conclusion, I hope I have indicated that autoimmune hepatitis actually reflects multiple imbalances in a complex homeostatic network that involves cellular and molecular interventions; that genetic factor strongly influence antigen selection and immune reactivity; that the cytochrome monooxidase CYP2D6 is the target autoantigen of Type 2 autoimmune hepatitis but, in fact, the principal autoantigen of the dominant form of the disease, Type 1 autoimmune hepatitis, is still unknown; that deficiencies in the number and function of regulatory T cells have been described, they have been exciting, but they are now controversial; and in fact, natural killer

T cells seem to be emerging as the key regulators of this disease.

Certainly autoimmune hepatitis has moved beyond the idiopathic stage but, clearly, its transition to a fully explained disease is far from complete.

AJ29: Thank you very much. (Applause)

Our next speaker is Dr. Einar Bjornsson. Dr. Bjornsson is the Chief of Gastroenterology and Hepatology, as well as Professor of Medicine at the National University of Iceland in Reykjavik, and he is now spending a sabbatical at the National Institute of Health. Dr. Bjornsson will discuss autoimmune DILI, its recognition and management. Dr. Bjornsson.

Bjornsson photo, biosketch, abstract

EB#1: I would like to start by thanking John Senior and the organizers for inviting me. Thank you very much. I appreciate this very interesting meeting.

I just would like to mention, before I go into this drug-induced autoimmune hepatitis, the features that Jack Uetrecht mentioned before of the immunoallergic reactions. When I was working in Sweden, where I spent almost 20 years, we analyzed reports that came to the Swedish Adverse Drug Reactionary Committee from physicians in Sweden.

EB#2: And cases of disulfiram and others, this is a very well-documented hepatotoxic drug. And we found among these patients that were reported, eight died. This is in accordance with Hy's rule, about 10 percent mortality.

EB#3: To our surprise, we found two different phenotypes histologically. This phenotype with immunoallergic features with hepatic and peripheral eosinophilia. You can see in the liver lobe that there are numerous eosinophils, which is an inflammatory infiltrate. These patients all had a very favorable outcome. They all survived.

EB#4: Whereas, with a centrilobular dropout of necrosis, this feature not surprisingly lead to

a very bad outcome with death from liver failure or transplantation.

And we looked at report from different registers around the world and it turned out to be true that, for example, in the Spanish hepatitis registry, patients who died very, very rarely had any immunoallergic features. It is interesting.

EB#5: We also looked at all the drugs that are very well documented, and we found the same thing. There was a lot of difference between those who had immunoallergic features and those who did not, in terms of severity of liver disease and prognosis. So, this was truthful for all these drugs.

EB#6: So, all the time you present something that is new, people become skeptical, for good reason.

EB#7: So, I was very happy to see that this could be reproduced in another cohort and this was a study from India, where tuberculosis in India is a big health problem and will still haven't come up with all the drugs that do not include isoniazid.

And a lot of children in India die from isoniazid-induced liver injury. And he looked at patients, actually children, with drug-induced liver injury and he found that those with hypersensitivity have much better outcome. Those who had hypersensitivity features have no mortality, whereas, this was present in almost 50 percent of those without these features. I would just like to mention this because this is an immunoallergic feature.

EB#8: So, coming back to this autoimmune hepatitis, Dr. Czaja has mentioned, this can be defined as an adverse immune response to proteins within the liver, initiated by a drug. And this is similarly clinically and biochemically and also histological to idiopathic autoimmune hepatitis.

As was shown and mentioned before by Dr. Czaja, tienilic acid was a prototype in the '80s or '70s for this type of reaction. This has been removed from the market, I think. And that the reactive metabolites created through hepatic

metabolism of some drugs have been shown to bind to cellular proteins such as cytochrome P450. And this can be recognized by the immune system as neoantigens.

EB#9: There are some drugs that are particularly associated with this type of liver injury: nitrofurantoin, still in wide use; minocycline, alpha-methyl dopa, and hydralazine. More recently, TNF-alpha antagonists and statins have been implicated in this type of liver injury. So, this has been caused by drugs. There are limited data comparing these patients with other patients with autoimmune hepatitis.

EB#10: So, when I spent time at the Mayo Clinic a few years ago, I looked for these cases in the Mayo Clinic diagnosed medical intakes and we searched for the text in the medical records. Not anywhere in the world, and not even at this fine clinic, can we trust the diagnoses that doctors make. Isn't that right? (Laughter.)

So, this is the way to look for diagnosis. Look for it in the text and then screen to see if this terminology is present in the text, we can look for this case and this can be a differential diagnosis. It can be a history or family history and so on. So then we can come up with a number of good cases.

And in this part, we excluded overlap syndromes with PBC and PSC and decompensated liver cirrhosis.

EB#11: So, among 261 patients with well-characterized autoimmune hepatitis, we were able to find 24 drug-induced autoimmune hepatitis, mostly due to nitrofurantoin and minocycline in this series.

EB#12: Interestingly, a very similar proportion of those with drug-induced autoimmune hepatitis and idiopathic had antinuclear antibodies and smooth muscle antibodies. There was no difference. And interestingly, the histological grade and stage were similar in these

two groups, but none of the drug-induced autoimmune hepatitis had cirrhosis at the baseline; whereas, this was present in 20 percent of the matched autoimmune hepatitis cases.

EB#13: We looked at liver imaging because they found that this was abnormal in the nitrofurantoin patients. This was normal in all the minocycline cases. We saw that liver atrophy and confluent fibrosis centrally was characteristic for the nitrofurantoin-induced autoimmune hepatitis. See atrophy of the liver and here is the confluent fibrosis.

EB#14: we looked also at the corticosteroid responsiveness. This was very similar but the only difference we could identify was when the immunosuppressive drugs were discontinued. When this was tried, physicians -- there is a difference between the doctors how eager they are to change anything. And if they wanted to discontinue this immunosuppression, when this was tried, this was successful in all these cases and no relapses.

Whereas, during this follow-up in the autoimmune hepatitis group, 65 percent had a relapse.

EB#15: So, we, from this series conclude a significant proportion, between nine and ten percent of patients with autoimmune hepatitis have drug-induced autoimmune hepatitis. And these groups had similar clinical and histological patterns. But at least, according to our data, they do not seem to require long-term immunosuppressive therapy. So, I think that the DILIN network is now working on a further analysis of their cases with drug-induced autoimmune hepatitis. This may involve minocycline, hydralazine, and alpha methyl dopa. And I think an abstractor from this work will be presented at the ESIL meeting.

EB#16: As Jack mentioned before, TNF-alpha antagonists have been found to be associated with drug-induced liver injury. There are numerous case reports but the largest series, until recently, included 6 patients from the U.S. in the

DILI network. And these 6 patients are presented with additional 28 cases from the literature in a paper published in 2013.

Little is known about the absolute risk of liver injury with these drugs. And, in Iceland, this is a small country, but we have advantages that we can cover the whole country. We can trace all these patients and look for them where they hide. And they cannot leave the island unless we test them.

EB#17: So, we found in a recent paper that an absolute risk of DILI associated with infliximab was one out of 148 treated patients. This was over a two-year period in a prospective study. And we because we have the Director of Medicine who doesn't have a medicine registry, all prescriptions, both within hospital and outside hospital are registered, so we could match these patients with the registry. We come up with these figures.

EB#18: So, we wanted to look both before this two-year prospective study and after for a five-year period to look for if this is true also for the paired outside the study in a population-based study.

So, we tried to identify all patients with suspected drug-induced liver injury treated with TNF-alpha antagonists in Iceland and we analyzed the clinical characteristic and features of autoimmunity.

EB#19: So we could, during this five-year period, come up with 11 patients. And much are females and a total of nine patients have been treated with infliximab. And I just think that this reflects the use of these drugs. Infliximab was the first TNF-alpha antagonist and most widely used still. Only two of these patients have inflammatory bowel disease; whereas, mostly had rheumatological conditions.

EB#20: And during this period, over 1,076 patients had been started on infliximab. We could

even find a higher proportion patients develop DILI. One of 120 patients treated with infliximab developed this kind of liver injury.

EB#21: So, just more than a third had jaundice, and the particular phenotype was hepatocellular with very high ALT and AST and features of autoimmune hepatitis or autoimmunity.

EB#22: What we wanted to do that nobody had done before was to match these patients with controls on TNF-alpha antagonist not to develop disease, not develop this reaction. And we matched these patients by age and gender, as well as the indication for which the drug was given. I think this is very important because these patients, mostly those with rheumatological conditions, have immune-dysregulation. So, it is important to match or think about the immune features before or at baseline. And we didn't find any difference between these groups except for the presence of methotrexate. This is a widely used drug in rheumatology. And also we looked at the

ANA positivity prior to TNF-alpha therapy. There was no difference in those who have been tested.

And it has also been taken into consideration that some of these drugs induced ANA, although, in some of these patients, they don't necessarily develop autoimmune hepatitis. But among those who developed liver injury, a significantly less proportion of patients were on methotrexate, whereas in the controls, this was more frequent. So, in this context it seems to protect against this type of liver injury.

EB#23: We have liver biopsies on approximately half, mostly hepatitis.

EB#24: And you can see a patient, 40-year-old woman who developed dense inflammatory infiltrate yet, you see apoptopic cell here and these features might look like autoimmune hepatitis. What do you say, Albert?

DR. CZAJA: Yes.

And these are the figures that she presented with, and for a two-month period her ALT doesn't seem to

go down. And there was a problem with the biopsy. She had elevated APTT and we have to look for and explain that. So, we didn't do the biopsy until two months after the presentation. And the biopsy was, as I showed before. And she had positive ANA, immunoglobal, et cetera. She started steroids and became rapidly improved, clinically and biochemically. She is now off immunosuppression and for a follow-up of two years, she hasn't had a relapse.

EB#26: This is another type of reaction, which also showed ANA. This patient was symptomatic presented approximately with ALT 800. And as you see here, when you follow the patient, she spontaneously goes down and no immunosuppression was required.

EB#27: So, half of these patients were treated with steroids and this could be discontinued in all where we tried but in one patient, he is still on treatment. And that is a decision of the responsible physician to do so.

EB#28: We found infliximab was more often associated with DILI than other TNF-alpha antagonists and autoimmune features are frequently in these patients and required steroids in approximately half of these patients. But despite this, the overall prognosis is favorable. So, the vast majority do not need steroid, long-term. And what was important was that when we tried other TNF alpha antagonists, it was always safe.

EB#29: So, I am just turning a little bit about, turning my attention to this association between drug-induced liver injury and autoimmune hepatitis. IN a long-term follow-up of patients who have concomitant jaundice leading to hospitalization, autoimmune hepatitis developed in several of these patients during a mean of six years.

EB#30: And it has also been shown that ANA can be detected after DILI and later on during follow-up.

EB#31: Interestingly, in the Spanish hepatotoxicity registry, nine out of 700 patients or 1.2 percent had evidence of two drug-induced related episodes caused by different drugs. And an interesting finding was that four out of these nine cases developed drug-induced autoimmune hepatitis in the second episode. This clearly exceeds the chance of association of this liver injury phenotype. So, we don't know why this happens.

In most cases drug-induced autoimmune hepatitis have developed injury associated with drug intake and autoimmune features.

EB#32: And the question is if it is adequate for diagnosis to have the drug intake and an elevation of autoantibodies. Probably not, because some drugs can lead to develop of autoantibodies. Maybe it is important to also take into consideration the history, if this preceded the symptoms of liver injury.

EB#33: And we often need to do a liver biopsy, particularly those with a persistent liver injury. And when this was done in a subgroup analysis of the use of liver biopsy and distinguishing autoimmune hepatitis and drug-induced liver injury, we found that the severity of inflammation and fibrosis was similar but marked fibrosis was very much -- was only seen in patients with classical autoimmune hepatitis, as I mentioned earlier.

EB#34: For management, we need to identify the role of drug. I am going to skip slides here a little bit because of the time.

EB#35: And I think some patients do not require immunosuppression, as with the second patient I showed you. And of those who do not normalize their liver test, we need steroids. But the question is: how long do we require the immunosuppression?

There has been success with drugs in most cases that have been reported but I could only come up with

three cases where this has not been possible. Of course, you need to follow the patient.

EB#36: I just want to finish with an email I received recently from Turkey. I am a pediatric surgeon. I have a 17-year-old daughter. She has been diagnosed with Type 2 autoimmune hepatitis. I have doubts about the diagnosis, the treatment protocol, and duration of treatment. That was all she had concerns with. So, I read your article "Drug-induced Autoimmune Hepatitis". We need your suggestion and advice.

EB#37: My daughter had no complaints; physical examination was normal. She had a problem with acne vulgaris. And on the fifth of August 2014 she was prescribed Rosaccutane, isotretinoin for acne vulgaris. And these were the liver test prior to treatment with Roaccutane AST 36, ALT 43, slightly above the limit. But after a month, ALT goes up to 140 and -- ALT is 91 and two weeks' later it is 141. And

she has ANA positivity and also anti-LKM. Other causes are excluded.

EB#38: And the histopathology showed portal and periportal plasma, accelerates inflammation, fibrosis 1/6. And this was the suggested treatment: prednisone 60 milligrams daily for -- it started with 60 milligrams daily with tapering and also azathioprine at the same time. This was supposed to go on for two years.

EB#39: And we questioned the diagnosis, diagnosis Type 2 AIH or drug-indiced hepatitis? Was the treatment protocol suitable? How long should the treatment be, et cetera, et cetera?

EB#40: So, I don't think that drug has been associated with drug-induced autoimmune hepatitis but for the first I don't think that a 60 milligram. That is quite a high dose. Maybe 20 or 30. What do you think?

DR. CZAJA: Yes, I think the standard recommendation was, for severe disease, to start on prednisone 60 milligrams daily and decrease it gradually back to 20 milligrams daily for a month. But in mild to moderate disease, as in this particular instance, particularly in a young female, I think a 30 milligram dose is sufficient.

DR. BJORNSSON: Yes, 30, that is what I would have done. And the question is whether there was an association with the drug. I think we cannot exclude that. So, to treat this woman for two years, I think I wouldn't have given azathioprine at the start. I would treat her for two or three months and see what happens, if she had a relapse.

DR. CZAJA: Exactly. I think that azathioprine really doesn't act very quickly and usually you're not looking at an advantage with the addition of azathioprine for probably six to eight weeks. So, if you intend to institute therapy over a short term, a four to six week interval of

treatment is no longer than three months, it is probably reasonable to just treat with prednisone alone and then you will get a clearer understanding of how rapidly this disease is responding. And here, you are really uncertain as to whether this is drug-induced or whether it is autoimmune hepatitis that is spontaneous or latent or preexisting. And in that particular instance, the severity of disease does warrant a treatment intervention. Just discontinuing the drug alone with a disease of this severity is probably -- it is possible to do but it is probably not what most people would do. You are not going to stop the drug and wait six weeks or two or three months to see if things get better. I think you stop the drug and you add something because of the severity of the inflammation. Thirty milligrams of prednisone would be reasonable.

Ninety percent of the time, if this is idiopathic autoimmune hepatitis, there will be a significant reduction in that aminotransferase

level within four to six weeks, actually within two weeks. You can usually make a pretty good judgment as to whether this individual is responding. If the individual doesn't respond quickly, I think you do have to carry out the therapy to a point when the laboratory tests are normal, before you discontinue.

I don't think that you would need a liver biopsy at that time, but that is possible if you wanted to really ascertain complete resolution of all of the manifestations of the disease. But stopping the drug at the time that the disease is in remission by your laboratory and clinical assessment would be appropriate. Then, the key aspect is monitoring the process after that and, if there is relapse, then you are dealing with autoimmune hepatitis, however you want to identify its beginning and not a drug-induced form of immune-mediated disease.

DR. BJORNSSON: So, in conclusion, in a patient with a high clinical suspicion of

drug-induced liver injury with positive autoantibodies, immunosuppression is indicated if aminotransferases remain elevated, despite discontinuing the drug. And discontinuation of immunosuppression, when attempted is usually successful and is really required long-term. Thank you very much.

DR. CZAJA: Thank you. Would the other two speakers come forward to the podium?

DR. SENIOR: May I suggest to Dr. Czaja that we extend at least a ten-minute discussion period and move the coffee break back a bit, while people come to the microphone.

I have a question for Jack and also for you, Al. Jack talked about adaptation. How does this happen? We talked about yesterday how humans communicate with each other by speech and by writing. How do hepatocytes communicate with each other? They can't talk. They don't write, at least not in the kind of writing we use. But they do send exosomes. They pinch off little bits of

their own membrane and there is something inside that goes in and can be taken up by a different cell. What are they saying to each other? What is the message? Are the injured cells saying look out for troglitazone or look out for genetic abnormalities? What are they saying to each other? What are the exosomes telling each other? Dr. Szabo is going to talk this afternoon aboutexosomes. And you mentioned exosomes. I think they are very important. What are they saying to the other cells?

DR. UETRECHT: Well, certainly, they have lots of things in them and different exosomes have different things in them but they have HMGB1, ATP, all sorts of things that can stimulate antigen-presenting cells. But, it's complicated.

DR. CZAJA: I think that just one analogy that I can compare is that there are certain enzymes which are important in the generating of the active metabolites, the drugs that we use to treat autoimmune hepatitis that actually do seem to

induce increased activity of those enzymes through continued use of the drug and that, in fact, actually improves their metabolism and they improve their efficacy, as well as reduce their toxicity. So, a substrate challenge may actually improve enzyme activity and contribute to that response. I don't know really what your answer is but that is one observation that we have had, especially in patients who have thiopurine methyltransferase deficiency and who we are giving azathioprine.

DR. PRATI: Some of the drugs that you have indicated as linked to autoimmune hepatitis, for example, alpha methyl dopa, are also linked to autoimmune hemolytic anemia. Did you look at any combo mechanisms for these conditions?

DR. BJORNSSON: No, I cannot recall but I don't think that any of these patients have also the phenotype of autoimmune hemolytic anemia. I am not aware of that.

DR. PRATI: Did you look at any Coombs thyroid -- Coombs test?

DR. BJORNSSON: No, this was a retrospective study.

DR. PRATI: Thank you.

DR. REGEV: I have a question. I guess both questions are for you, Einar. I'm trying to use a case example just to clarify how you view the differentiation between autoimmune drug-induced and non-drug-induced. You used a fibrosis stage of four as a cutoff between a diagnosis of autoimmune that is idiopathic and autoimmune that is drug-induced. And my question is if the case of infliximab, for example, has a three-month history of treatment on infliximab and then presents with autoimmune-like presentation and a liver biopsy shows a stage 3 fibrosis, how would that be used as an indicator to differentiate between the two?

DR. BJORNSSON: I'm not convinced that you can use a cutoff of three and four. I mean, we have -- it is more complicated than that. We have some

reliability. And also in idiopathic autoimmune hepatitis, it has been described that some people have cirrhosis that can disappear with treatment in a new biopsy, if it is something but I don't know. And I think it is difficult to -- but the only thing is that if you have significant fibrosis in a biopsy, it makes it more likely that this has been a long-standing process. Undiagnosed people are often asymptomatic for a long time.

I would still try, because if they tried to discontinue treatment because I don't think it is danger to stop the immunosuppressant if you monitor the patient closely. Because you know what to expect. The severe thing is that people go undiagnosed. Nobody knows about it. Ninety percent would see severe jaundice and they can come with acute liver failure. But if you follow them very closely with biochemical test prior to symptoms, it is easy to treat them, I think, and get them into remission again.

DR. REGEV: Thank you for that. I am just summarizing. It is more a case-by-case thing, rather than a cutoff of four or three.

DR. BJORNSSON: Yes.

DR. REGEV: And my second question is related. You mentioned the Indian study that actually associated hypersensitivity syndrome is actually a good prognostic sign. There is quite a lot of data that suggests the opposite. And I am curious to hear from other people as well, where is this -- and including the recent DILIN article. They actually nine patients with severe skin manifestations and eosinophilia as part of the presentation and they have four out of the nine. So, they saw that as a bad prognostic sign. So, where is this? Is it a population thing? Is it a data collection thing? Why the differences?

DR. BJORNSSON: I think that skin reactions are something else. That was not included in the reaction. And this is a complicated thing with the different pathways. The eosinophils can be

destructive and it has also been shown to be protective. And it was shown that in patients with ulcerative colitis when they were biopsied during the active phases, versus when they were in remission that the eosinophils were more prominent in the inactive phase. And this has been shown in several studies, suggesting that in some pathways, the eosinophils have a protective role in healing the mucosal injuries. So, I think if you have hypereosinophilic syndrome, you can have a destructive pathway. So, it is very complicated. Maybe Jack can answer this but there are different pathways. Do you want to come up?

DR. UETRECHT: Only to repeat it is complicated. (Laughter.) So in the same cell, there are neutrophils that are tolerogenic. So, I think we are developing pools now that we have never had before to very carefully phenotype cells. I think the way that we have done it in the past has been inadequate to determine the function of these cells.

PARTICIPANT: Yes, speaking of skin reactions, recently there have been a lot of reports about the occurrence of psoriasis in patients with anti-TNF alpha drugs, especially with infliximab. So, I am wondering at the Mayo Clinic or elsewhere whether you can get some of these patients to see if the immune environment in those patients would give you any clues about the occurrence of anti-TNF-induced liver disease as well.

DR. CZAJA: Certainly, I need to look at it.

PARTICIPANT: I haven't seen it.

DR. CZAJA: I think I can't really answer that question for you.

DR. UPENDER: So, that goes to the dermatologist.

PARTICIPANT: Yes, well, I think there may be some rationale to look at some of their immune cells and their immune regulations. The imbalances that you are thinking about are present in those patients as well.

DR. CZAJA: We have time for two more questions.

DR. AVIGAN: So, I had a question about the tolerance mechanism for Jack. So, you were making an argument that one of the steps in the cascade of pathogenesis is the loss of a certain tolerance mechanism to a regulatory cell network. So, that raises the question of are there opportunities to provide therapeutic intervention for resetting, essentially, the network, when you see a relation. And as an analogy, desensitization, which, of course, is something a little bit different.

But why it is confusing is that you have many patients on these drugs, some of these drugs, which would develop autoantibodies but don't develop clinical syndromes. So, dysregulation is not binary. It is more of a kind of a continuum. The question is if can you reset the level of tolerance. Is there, from the way you are thinking about this, to at least eliminate in this cascade of

perturbations the clinical injury step, the injury step? Where is the tolerance broken down?

DR. UETRECHT: I think the immune system is everywhere. So, a lot of what we look at is in the liver but we also look at lymph nodes and spleen. So, you know when antigen-presenting cells are activated, they go to drain lymph nodes to get maximal interaction with T cells, et cetera. So, it isn't -- in terms of location, it isn't one place. I think always there is a balance. So, as I said, I don't think that most patients that develop idiosyncratic DILI have a severe impaired immune tolerance. But it is this balance by depleting or decreasing immune tolerance. We are tipping that balance but there must have been something previous that led to this very strong immune response that tolerance was not sufficient to overcome it. I'm not sure I am answering your question.

DR. AVIGAN: Well, obviously, it is complicated. So, there are lot of cells in the

network and there is a balance. Did anybody have an idea about how to intervene when you had breakdown?

DR. UETRECHT: Well, I think what we need to do, and I am a little disappointed it hasn't been done, is doing more controlled studies. Obviously, if you have a patient who has idiosyncratic DILI, and you stop the drug and give them steroids and they get better, you don't know whether it is because of the steroids or just because you stopped the drug. And until we do controlled studies, not just with steroids but sometimes if we understand the mechanism better and it is going to be different in different people, if we target cytotoxic T cells or whatever, we will have a much better chance of selectively saving that patient, rather than just using the same therapy for everyone. We need controlled studies. I know they will be difficult to do and whether they should be done in clinical trials or the DILIN network or how we do it, I am not sure but we

desperately need controlled studies to see what is effective at treating these patients.

DR. CZAJA: I think the principal objective of developing therapies for idiopathic autoimmune hepatitis is to do just exactly what was mentioned, which is to identify the critical cell population as unbalanced and really immune tolerance to be overcome and to restore that imbalance. And that is why the key populations of regulatory T cells have been really at the forefront of these investigative efforts. And there is a very interesting Japanese animal model in which they take PD-negative mice and do neonatal thymiceray in those mice, creating really an absence of thymic-derived regulatory T cells. And then demonstrating a consistently developed from of autoimmune hepatitis, which can be prevented or ameliorated after the adopted transfer of regulatory T cells to this population. These animal studies, using infusions of the adaptive T cells are also being addressed in other animal

models and in patient populations. So, there is really speculation that there is going to be an effort to make these adjustments primarily through pharmacological means of bolstering regulatory T cell function, if, indeed, that is a problem, or to begin to supplement with immune cells that have been regulators indigenously present.

DR. BJORNSSON: What happened with the coffee break?

DR. CZAJA: I think we will have one more presentation, then we can have a coffee break.

PARTICIPANT: Mohammad for the NIH. I have one question and Jack brought a lot of papers or reviews about dangerous signal. And of course injecting antibody against PD1 CDR4, I don't see what kind of danger this can add to the model to make there might be a lot of hepatitis in liver injury. And the same thing to do to the 16 mice when you develop hepatitis, how this antigen in animal can produce liver injury. There is no danger still

from the immune system to make more allele to the blunt injury.

The second question is related to alcohol-induced liver injury. There is also antibodies against people 50 to 81. I didn't see a lot of discussion about this. The question is: does an aged population drink a lot of alcohol, probably in the north of Europe or US, probably have more liver injury because they have more some kind of damage because of alcohol. Is any study done seriously to compare it with more risk for DILI for alcohol drinker than non-drinker?

DR. UETRECHT: I don't think there is a significant increase in risk. And again, we tried to do studies with things like thioacetamide co-treatment to try to increase the amount of danger signal. But I think, and this is pure speculation, but going back to the exosomes, I think somehow the immune system can be very specific, so that in these -- again, pure speculation, but you can combine, in these

exosomes, drug-modified peptides and HMGB1 and other danger signals so that you specifically sensitize the immune system to that particular drug and it ignores other things that induce danger signals.

So, other inflammatory conditions in general and co-treatment with other cytotoxic drugs, liver cytotoxic drugs just doesn't do it. The immune system is smart enough, specific enough, that it responds to what it should. And almost always, it gets it right and responds with immune tolerance. I will bet you if we could look more carefully in the liver of humans that get isoniazid we only saw an immune response in those that had an ALT, when we looked at the peripheral blood. I will bet you there is an immune response in the liver of everyone.

DR. BJORNSSON: Can I answer this with alcohol? Actually the DILI method, alcohol was a protective factor against severity of liver injury, which is surprising. Is that correct,

Paul, in the first 300 patients, alcohol was a protective factor?

DR. CZAJA: Well, with that, I think we should break for coffee. And I would like to thank the speakers for their presentations and the audience. Thank you. 9:44 am

Coffee break

Session IIIB 10:09 am

DR. AVIGAN: I am going to ask the audience to sit down. We are going to start the second portion of this morning's session on immunity, an. We’re going to make a transition from some of the background pathology issues that we heard about this morning. They really set the stage for some of the regulatory and drug development questions that are right now very pressing.

And one of the reasons why I thought about in planning this session, initially this summer, was that we have new classes of drugs that are coming online that have as part of their profile,

autoimmunity as a side effect because in some sense that is how they work.

Avigan photo, biosketch, abstract

MA#1: So I am going to get the session started. I am Mark Avigan. I work at the FDA with John on critical path issues and I have a background in both hepatology and molecular biology. So, I am kind of an eclectic guy, but I am delighted that everyone is here and that we have an opportunity to talk about these very important issues.

MA#2: My talk today is about drug-induced immune injuries, why these are important. And of course, we are talking here about different kinds of injuries, different mechanisms, both with regards to liver as well as other organs.

MA#3: And what prompts our attention to this as hepatologists and liver injury people with regards to drugs is that there are now new drugs coming online? We will see more of these in the oncology space.

MA@4: I am going to talk about the challenges in definitions and regulatory implications of drug-induced immune injury and then turn my attention to talk about autoimmunity and autoimmune hepatitis, in particular, with regards to accounting for the diverse phenotypes and mechanisms, both with regards to particular drugs and individual patients who are susceptible. And I am going to then introduce the topic of the cancer drugs and we will hear more about this from David Berman and then talk a little bit about the challenges with regard to causality analysis where the RUCAM, as a tool, needs some work. And we are working with our colleagues on the NIH on this question.

MA#5: So, with regard to immune injury to the liver or other organs for that matter, there are, again, different molecular targets that come into play that incite these reactions that are either drug-associated or altered self-antigens, as we heard. From the point of view of classification in

simple terms, although there are commonalities between these broad pathways, there are two broad groupings of immune reactions or immune damage, immunological damage prompted by drugs.

One group of reaction pathways is immunoallergic pathways. And characteristically these have an onset within a few weeks of treatment. They can be very short. Multiple organs can be affected. And again, we think of these as the classic hypersensitivity reaction. So, we are talking about different mechanisms within this group of reactions. Fever and rash are not uncommon. We have heard about eosinophilia today as an example and re-challenge has significant risk.

On the other side of the coin are the autoimmune reactions. And again, they have some similarities in terms of what incites them. But autoimmune reactions are different in that typically their onset occurs after a more prolonged treatment. The type of injury that you see is more

subacute or chronic. Again, there are characteristic ranges of affected organs and these can depend on the specific drug and the specific drug signatures. We will come back to this point. And then for some, there are characteristic autoantibody profiles for certain drugs but this is not always the case. And there are some notable exceptions. From a public health perspective, there has been, of course, longstanding concern with regards to hypersensitivity reactions from drugs and these can be serious. These can be life-threatening. They can kill. We just had a conference a couple of weeks ago -- last week, on Stevens-Johnson syndrome, where patients end up -- these are very reactions, end up in burn units and have terrible reactions. But these are often discovered or determined, identified in the post-market phase because they are quite rare. So, there has to be large treatment exposure before you start seeing these reactions.

MA#6: And clearly, in this snapshot of safety alerts between 1996 and 2014 from the FDA, you can see that significant regulatory actions have been taken with regards to drugs and withdrawals and so on. Some of these regulatory actions have taken place after replacement of the problem drugs with drugs that have safer profiles.

MA#7: And likewise, there is a sizeable number of drugs that are labeled by FDA and then of course by the sponsors for autoimmune reactions. And this is just a very partial list, just to give you a sense of it. And different kinds of autoimmune reactions are relevant here.

There are lupus-like syndromes, drug-induced lupus erythematosus. I will refer to it as DILE. There is autoimmune hepatitis. And again, there can be disability associated with these kinds of reactions and, in some case, they can be, of course, life-threatening as well.

MA#8: So, optimizing our risk assessment and case management for this kind of problem is very

important. And again, to have an optimal approach in the face of this diversity for risk assessment and also to be able to learn more about them in research, we really need to have -- we need a number of things to set of place. We need to have a universal categorical criteria of reaction types. We have to have a nosology. We have to have a classification scheme that makes sense not just for the experts in pathology, in the pathogenesis but also for clinicians to identify patients, recognize them and so on. We need protective procedures to monitor patients and manage immune reactions when we see them. We need to have effective post-market surveillance strategies to tell and evaluate events when they occur, especially since many of these events are rare, so they will occur and be seen in the post-market. And we need adverse event descriptions and instructions to manage risk in labels and other tools with communication that are really optimal.

Now, in the face of these needs, we have to reconcile these real important challenges. And we go through some of these challenges. But one of the challenges, of course, is that some drugs actually can cause more than one kind of reaction. And we heard today about minocycline as an example of a drug that, in some individuals caused an immunoallergic reaction but in other people, they get a more classic autoimmune picture. So, different individuals can have from the same drug, different reactions. So, that has to be somehow -- that is one of the challenges that has to be incorporated in how we communicate risk.

MA#9: There are also variable temporal features of severity and affected organs for the same type of injury type. So, that is another layer of diversity and complexity that has to be communicated. So, for example, minocycline autoimmunity can include drug-induced lupus erythematosus. It could cause autoimmune hepatitis. It can affect other organs such as

thyroid, where you can get thyroiditis, other endocrinopathies and so on.

Another example is lamotrigine which can cause hypersensitivity of different organs, skin, liver, meninges, in different people, presumably with common mechanisms of injury.

So, another challenge in this group of challenges is that there are inter-individual differences which are hard to predict. So, there are co-determinants of risk that are idiosyncratic. They have to do with the HLA polymorphisms. They have to do with pre-existing antigen exposures that might have been primordial but have sort of set into motion a recognition of an antigen as foreign or an altered self-antigen and then the danger signals. That is, the concomitant, which we will come to in a moment.

MA#10: So, now I am going to focus more of my attention to the autoimmune side of that ledger that I showed you before and, of course, there are classic -- there are manifold manifestations of

autoimmunity from drugs and there are some classic presentations which overlap, to some extent, but not completely with what we have referred to as idiopathic autoimmunity.

So, in the case of drug-induced lupus, these signs and symptoms that I have listed here arthralgia, serositis, and so on are subacute and chronic cutaneous SLE, these are classic for drug reactions but, notably, many patients with drug-induced autoimmunity do not have some other features that are seen with idiopathic lupus, such as renal involvement for many drugs, CNS involvement and very serious, life-threatening skin reactions.

So, another feature of the drug reaction is that it is slow to onset after initiation of the drug. It is slow to resolve often, unless you intervene with steroids so that the clinical syndrome is a little bit different than what we see in the idiopathic from. And also, sensitization is not easily seen. Sensitization was seen with

immunoallergic reactions but not with these reactions.

MA#11: So, whether the liver is the target organ or you have other organs that are affected, there are certain common pathways across these different kinds of autoimmune injuries that come into play. So, there is a triggering mechanism that we heard about this morning, very nice presentations that initiate the reaction either through haptens of drug metabolites or through an alter self-antigen and there is a fair amount of data that we have heard about in previous meetings about the secondary stress signals that are the so-called danger hypothesis where concomitantly there is an infection or a heightened inflammation which, somehow, changes the regulatory network and makes susceptibility to initiation more complete.

And then the reactions through drug effects can be driven or sustained through drivers, through driver mechanisms, which I have listed here. And these include drug effects on a variety of steps

in immune homeostasis. And notably, one of the ones that we heard about today, which is a very important one to learn more about is the issue of tolerance. Certain drugs actually can change or perturbate tolerance and I will come back to that point later. But also but these pathologic steps actually afford an opportunity for interventions and prevention in certain individuals who are susceptible and need certain drugs. So, this is something that we had asked about before and requires more research.

MA#12: So, there are now over almost 100 drugs that are associated with lupus. The most well-known ones are, of course, are procainamide and hydralazine, where the rates of these reactions is extraordinary, particularly in patients who are slow acetylators or have certain HLA isotypes, the classic DR4.

But other drugs as well are associated with lupus more rarely but they need to be labeled and they need to be communicated and recognized by

clinicians when they occur. And interestingly, as we heard about before, there is a gender predisposition in females more than males but it is less pronounced in the idiopathic variety. We see these drug reactions more in older people but maybe that is because they are on polypharmacy.

MA#13: And here is a very partial list of drugs that have been linked to autoimmune hepatitis. And again, what is interesting is some of these drugs actually are more specifically reported as predisposing to autoimmune hepatitis rather than DILE. And some of these drugs have been removed from the market because of this effect or have not been introduced into the market.

Currently, we heard that the drugs that are being marketed currently that have this issue that is recognize, minocycline and nitrofurantoin but this is a moving target because now we are having these new oncology drugs coming online. We have more biologic agents, like the TNF-alphas that we heard about, where this kind of problem has been

recognized. So, the complexion of the drugs that are causing this problem over time will change.

MA#14: And there has, of course, been a long-standing interest in genetic susceptibility markers, not surprisingly. Of course, if they have utility, if they have good predictive value, not surprisingly, some of these as we heard very elegantly from Dr. Czaja, that some of these are overlapping or at least the HLA loci are overlapping with those that are implicated in the idiopathic forms of autoimmunity and autoimmune hepatitis. But there are specific isotypes for certain drugs, where there is an HLA connection having to do with antigen presentation. For example, minocycline and nitrocine polymorphism at the 30 amino acid position of the first open reading frame. A slow acetylator status rings the bell with regards to hydralazine. It has a similar pathway, actually, as INH. Again, so how some of these things have to do with the metabolism of the drugs, we don't completely understand.

And then of course, if we want to develop genetic susceptibility markers as clinical tools, as risk management tools, they have to have good predictive value for us.

MA#15: And so far, with maybe a few exceptions, individual loci, as markers or enrichment for risk have very small effect sizes. So, that their contribution of overall risk is relatively small. So, this is a kind of stumbling block.

But what we don't know actually, is how to compute the combinatorial effects of multiple interactive genetic loci, as well as other effects as well. So, this is difficult to study but it is an open question. And the modeling of risk effects of multiple loci and genetic loci, as well as non-genetic factors, and the challenges, the experimental challenges of how to do this is nicely captured by this diagram that was published a number of years ago by Teri Manolio, who is at the NIH who we had this conference with the other week.

MA#16: And basically, there are two important factors or two important variables that determine risk for a genetic locus. One is the effect size of the locus on risk and the other is its frequency in the population. And so you can from that make a diagram. And on the right side of the diagram are the kind of common variants that we often will determine by GWAs. They are frequently expressed in the population and they often will have a small effect size. So, on the one hand, they are easier to discovery in a case-controlled study design but they also are disappointingly, they have small effect sizes to be used as these single markers of risk.

MA#17: On the left side of the diagram are the rare alleles that are inherited in more of a Mendalian way and they may have high risk but they are hard to discover because you have to know where in the genome to look. You can't just have a pangenomic system method to discovery because there is a lot of false discovery in that method.

This kind of a diagram highlights the experimental challenges of determining the biosystem genomic regulators. What are the tradeoffs from an FDA perspective, from a regulatory perspective of when we consider the utility of markers and when they might enter into a label or into an instruction to clinicians.

There are different factors at play. And on the right side are the factors that favor a marker as a clinical tool, when the allele is common, when the test has a high positive predictive value, when the result strongly implicates treatment benefits versus risks, when there are few and expensive alternate treatments and when this adverse event is severe and will kill you if you get it. Those are the kinds of things that you would say hey, let's test for this.

MA#18: Now, it ends up that we do have some labels where we actually recommend genomic marker testing, but there are some nuances to this. Even with demographic groups, there can be variability

in the frequency of an allele, which then impacts the value of testing. An example is HLA-B*5701 for the abacavir hypersensitivity reaction, where the marker actually is very frequently expressed in Caucasians, in 5 to 8 percent of the population. So, you just have to test 20 people to prevent one bad reaction. That is a no-brainer. But if you go to East Asia, you know, Korea and places like that particular allele is very rare. So, you have to test over a thousand people to prevent one reaction. So, there is some variability in the utility of the marker, based upon allele frequency.

MA#18: Autoantibodies, are the sine qua non biomarkers of autoimmunity and drug-induced autoimmunity as well, they are not necessarily the mechanisms by which tissue injury occurs but they are manifestations of the dysregulation. Why there are different cellular components and isotypes among different autoimmune drug reactions is really not fully understood. We have heard a

little bit, we got some inkling of this this morning but it is still not completely understood.

Another frustration is that high versus low titers of drug-induced autoantibodies do not predict clinical significance of severity or injury. So again, the titer or the concentration of the antibodies don't really correlate with injuries. So, these are biomarkers but they are challenged in terms of what they really mean.

MA#19: The key point is made in this slide, which is that different drugs have different risk profiles for different kinds of autoimmune reactions, based upon what has been reported in the literature. So, for example, procainamide is very tied to DILI, as is hydralazine, less so to autoimmune hepatitis. But some drugs are connected to both and this makes it more complicated. Some drugs are connected to one target form of injury or one syndrome, even though they are connected in terms of their presumed pathologic pathways.

Another interesting point is that different drugs actually have different characteristic autoantibody profiles but these are not entirely specific, so that commonly the ANA, which is an immunofluorescent test and this has a homogeneous pattern, often what it reflects are autoantibodies to histones. And they are seen in many drug reactions with DILI, not necessarily all.

Some drugs give other characteristics of autoantibody, such as double-stranded DNA antibodies with minocycline or perinuclear antineutrophil cytoplasmic antibodies, which actually reflect antibodies to myeloperoxidase. Infliximab also gives a kind of particular set of autoantibodies, including DNA antibodies, cardiolipin antibodies.

MA#20: And when we look at drugs that induce autoimmune hepatitis, again, some of them are very heavily weighted towards autoimmune hepatitis and not to DILI and they have characteristic profiles of autoantibodies, which we heard about today, that

fit into either the so-called Type 1 autoimmune hepatitis category or the Type 2 autoimmune hepatitis category. The first four drugs on this list, actually, they were so tainted with risk for autoimmune hepatitis that they have either been removed from the market or they were never introduced to the market. We heard about tienilic acid before, and they make these characteristic antibodies, which you can determine in vitro or in cell staining from liver or from kidney in the microsomal traction. They turn out to bind to CYP2C9, one of the cytochromes and the dihydralazine CYP1A2, which there was a nice review, brief review that Paul Watkins actually wrote a number of years about the CYP1A2 antibody.

And then ipilimumab which we will hear about later is a drug which revs up T cells but doesn't really create any antibodies that are characteristic, at least so far, that we haven't discovered.

So, why do these particular drugs pick these particular cytochromes? We heard a little bit this morning about this idea of epitope expansion. Some of these drugs have a stop step where they meet the cytochrome in their metabolic clearance and so there is a physical proximity in the metabolism of these drugs with these cytochromes. And whether that has an effect on how the immune system ultimately decides to actually make antibodies against the enzyme, rather than drug is an open question but it may have something to do with this idea of expansion of the epitopes.

MA#21: And other findings with regard to autoantibodies is that they are often detected in individuals without liver injury. procainamide patients have an extraordinary rate of developing ANA, antineutrophil antibodies, even though many of them don't have a clinical syndrome. Likewise, infliximab, in a study of RA patients, 15 percent of all RA patients treated with infliximab actually have been found to develop double-stranded DNA

antibodies. And 55 percent with IBD develop ANA. So, of course, most of those patients do not have a clinical syndrome.

And we talked about, on the other hand, the point that autoantibodies can pick out, they have characteristic signatures for certain drugs which I have listed here. So, when we see a clinical syndrome and we see these autoantibodies, it is the circumstantial evidence that the drug is somehow tied to the reaction, but it is not foolproof. With regards to checkpoint inhibitors, as we will hear, there are autoreactive T cells that come into play. And perhaps in the future we will have good assays, not to measure autoantibodies, but to measure T cell reactivity in the presence of certain clones of T cells that are responding to particular drugs. And that might be the clinical assays of the future.

MA#22: Now, because autoantibodies are limited, we want to look for other potential dysregulated mechanisms as potential biomarkers. And there is a lot of literature about mechanisms

that come into play. One is the inhibition of DNA methyltransferase by certain drugs, procianamide and hydralazine, for example, which then basically unleashes gene expression through hypomethylation of a certain gene regulatory regions and then the expression in those T cells of certain molecules that enhance activity of the T cells. TH-2 cells are the ones that drive B cell autoreactivity. We heard a little bit today about this idea of reduced apoptosis, a defect that has been proposed with regard to clearance of cellular debris and perturbation there. And we heard a little bit from Jack about this idea of disruption of tolerance.

One of the mechanisms with regard to procainamide hydroxylamine which was nicely reviewed a number of years ago by Jack in one of his reviews, shows that there is a perturbation in a mouse model for positive thymic selection so that the T cells that are selected to be kept and recirculated are defective in some way and they

don't tolerate. They somehow activate. They don't have an energic reaction.

MA#23: So, I am going to close by just making a few points about these checkpoint inhibitors as a prelude to David Berman's talk. And I just point out that we are beginning to see more of these kinds of drugs at FDA, and we will see more of this in the future. I listed some of the molecular targets for these inhibitors. Because of the nature of how they work, they are linked to a high-risk for autoimmune organ injuries because they basically soup up autoreactive T cells and perhaps NK cells. That is how they work but they can also cause autoimmune injuries and we see a lot of them.

It is in the label but it is also in the post-market experience. The most common is colitis, but also hepatitis, liver failure, endocrine effects. And so there is a real risk level for life-threatening AEs, which you can actually see in clinical trials. You don't have to get a million patients exposed before you start

seeing them. Within a few thousand patients, you see a whole bunch of these reactions.

So, what we need to do a better job going forward is how to pick out patients to predict who are going to be the bad actors. Who are going to be more susceptible to autoimmune unintended reactions, rather than the reactions against the cancer cells.

MA#24: So, just to give a snapshot from my colleagues who where working this up from our spontaneous report database at FDA, and this is not expected because these reactions were actually seen in clinical trials as well, is that there is a certain percentage of patients, a certain number of patients in the spontaneous report who have been reported with colitis. The most common known adverse event in this category of adverse events, some with intestinal perforation and also cases of autoimmune hepatitis and hepatic failure.

Now, when we look at the cases with more focus, it turns out that many of the patients who

were bad actors actually already have underlying liver disease with, in this case, melanoma metastases to the liver. But there is a very striking temporality between the onset of serious liver function changes and the treatment step itself. So, there is a complexity of underlying cancer in the liver and then addition of a drug that actually, for these individuals, tips the balance not in their favor.

MA#25: And I just wanted to highlight an example of a case of interest that shows these complexities in the post-market database of a 60-year-old male. He has melanoma metastases with small lesions in his liver. He was apparently a good candidate for ipilimumab. This is the drug that is a CTLA4 inhibitor. And after the second dose, within three weeks, he developed flagrant liver failure with hepatic encephalopathy, hepatic cellular necrosis, very dramatic enzyme increases. And remarkably, because of the nature of this drug, there is no ANA positive -- ANA is not remarkable.

And the immunooglobulins are not elevated either. So, this is a particular feature of this kind of autoimmunity. The clinicians thought this was the drug reaction. They put the patient on prednisone and they put them on high-dose steroids. The patient didn't do very well and quickly died.

MA#26: The question for these kinds of drugs is that new drugs are coming online to treat cancer cells, basically through a therapeutic autoimmunity. The issue is how to find the sweet spot, what I have called an autoimmune Goldilocks zone, where we are actually aiming to find the right level of autoimmunity to deal with the cancer cell but not to harm our organs. And how to do this more elegantly is going to be the subject of more research in the future; how to pick out the patients who are susceptible, how to monitor them, how to early intervene, and modify their treatment course, and so on.

MA#27: In my last slide, I want to make some self-evident comments about causality with regards

to autoimmunity, where we are challenged using an algorithmic RUCAM score. And I just want to make these points, looking forward to perhaps more diversity RUCAM scoring, based on the drugs that are in question. The points I want to make are that the broad range of clinical presentations and timelines challenges the utility of a single algorithmic assessment of causality in these kinds of reactions in autoimmune hepatitis.

Current RUCAM criteria of causality are not in alignment with a late onset chronic autoimmune phenotype of hepatitis. Time/exposure effects, steroid responsiveness, histopathology, and serology bear attention for such an algorithm. Matching specific autoantibodies with certain drug-induced injuries as an algorithmic criteria for causality may have utility but requires case and control testing with validation studies.

And finally, in the future, a set of RUCAM-like scales might be established that would be appropriate to align with particularly

drug-related AIH scenarios. So, right now, we are sort of left with an expert opinion. But going forward and our colleagues at the NIH and DILIN have been thinking about this, maybe we will have more than one set of algorithmic criteria to employ, based upon the drugs that are suspected.

MA#28: So, then I am going to finish and go on to our next speaker, Dr. David Berman, who works at BMS. He is an expert immunopathologist who has been guiding different aspects of their program in oncotherapy. And he had a stint at the NIH working with Dr. Kleiner as an MD-PhD and we are very happy to have him.

Berman, photo, biosketch,abstract

DB#1: Thank you very much. I am going to talk about immune-mediated toxicity from a new class of therapies.

DB#2: I would be remiss at an FDA-sponsored meeting if I didn't note that I am an employee and shareholder of Bristol-Myers Squibb.

DB##: Historically, there have been three pillars for anti-cancer treatment: radiation, chemotherapy, and surgery. There is a new class of agents which you will start hearing about, or you may have started over the past couple of years, and that is immuno-oncology, which is harnessing the patient's own immune system to fight disease. This is a very exciting area. It is new; you are going to hear much more about it because there are more of this class of drugs. But one of the issues is, as was just pointed out, these drugs are intended to activate the immune system to attack the patient's own tumor. Consequently, there is the risk that the patient will have immune-mediated toxicity.

DB#4: There are some potential non-exclusive mechanisms why a patient who receives an immuno-oncology agent can develop an immune-mediated toxicity. The immune therapy could disrupt local or systemic homeostasis. The I-O agent could induce priming of a new T cell response

to a self-antigen. And perhaps even the immune system can induce a supraphysiologic response to commensal flora, for example, in the gut or in the skin and this could lead to bystander damage.

DB#5: Immuno-oncology agents can actually induce immune-mediated toxicity in almost any organ in the body, including the liver. And I know this is a liver meeting but I am going to focus mostly on the GI tract and the GI toxicities, and I will discuss why but I would like to come back to the liver towards the end.

DB#6: The drug that I am going to focus on for the rest of the presentation is ipilimumab. This is a monocolonal antibody that is being used to treat advanced melanoma, and it targets CTLA-4. And the reason I am going to focus on ipilimumab, or ipi for short, is because it is one of the I-O agents with which we have had the most experience. We have had it for 15 years in the clinic and treated over 10,000 patients in clinical trials. And now

there is a growing experience in the post-marketing use for advanced melanoma.

T cell activation typically requires two signals. The first signal is provided by the T cell receptor recognizing the target antigen in the context of an MHC molecule on an antigen-presenting cell. The second signal is provided by CD28, which binds to CD80 or CD86. Both of these signals are required for the T cell to be activated. And CD28 is called the co-stimulatory signal.

DB#7: CTLA-4 is normally expressed in T cells but it resides in vesicles within the T cell. And upon strong T cell activation, these vesicles fuse to the membrane surface, releasing CTLA-4, which migrates to the T-cell antigen-presenting cell synapse. And because CTLA-4 has a much higher affinity for CD80 and 86, it can actually out-compete CD28. And this turns off the co-stimulatory signal, thus down-regulating the T cell.

Ipilimumab, the trade name is Yervoy, works by specifically binding and blocking CTLA-4 on the surface of T cells, thus restoring CD28 co-stimulatory signal. CTLA-4 was discovered in 1988 by a French group and for the first five or six years, it was not really clear how important CTLA-4 was. And in fact, people initially, erroneously thought that CTLA-4 was another co-stimulatory receptor. It wasn't until 1995 that two groups deleted CTLA-4 in mice, showing an incredibly striking phenotype of death by three weeks due to massive lympho-proliferation in multiple organs. And this includes spectacularly, the pancreas and the heart. Interestingly, the phenotype of this immuno proliferation does not match the organs that we see typically in patients treated with anti-CTLA-4. Another interesting, unfortunate fact is that in adult wild type mice blockade of CTLA-4 by an antibody does not recapitulate the immune pathology that we see in patients, for the most

part. We can exacerbate chemically-induced colitis but we have been unable to really use mice or even cynomolgus monkeys as test cases for understanding the pathophysiology of anti-CTLA-4 toxicity in patients.

DB#8: This is a summary of the immune-mediated toxicity that we observed with ipilimumab or Yervoy. This is from the USPI and it is from the pivotal phase 3 trial. It is a table showing the incidence of severe to fatal immune-mediated toxicity. And you can see 15 percent of all patients who received ipilimumab developed some form of severe to fatal immune-mediated toxicity. The most frequent is enterocolitis but other organs involved included dermatitis, hepatotoxicity and, interestingly, endocrinopathy, among others.

Now, one question arises why do only 15 percent of patients develop clinically significant toxicity? It is not clear. Why do some patients develop enterocolitis, whereas others develop

hepatitis? Not clear. And the other interesting fact is that we tend not to see syndromes. We don't see ipilimumab-induced SLE. We don't see ipilimumab-induced rheumatoid arthritis. They tend to be organ-specific inflammation.

DB#9: Now, this is a summary of three key questions which faced us in the early development of ipilimumab but it can really be applied and probably will be applied to all new I-O therapies that are being developed.

First, can you design a management algorithm? Second, can you prevent the toxicity? And for Yervoy, the focus was really on GI because it was the most frequently severe and the most frequently fatal problem. And then finally, can you identify the mechanism of this toxicity? And that includes looking at the histology but also can we differentiate it from autoimmunity and from graft-versus-host disease?

Now, even when we started, we didn't fully expect to find a complete overlap with autoimmunity

with Crohn's or ulcerative because we know those are polygenic. They result from a gene environment interaction, probably. Whereas, with ipilimumab, we are specifically targeting a single pathway. But, nevertheless, we wanted to see if there was some overlap.

DB#10: So, I am going to focus on those three. First, I will focus on the management algorithm. There was a lot of trepidation when ipilimumab was first administered to patients because, remember that the mice who had CTLA-4 deletion died at week three and there were thoughts about patients. It just was not really clear. Thankfully, the toxicities were manageable. And through trial and error, an algorithm was defined.

First, recognition that these toxicities could be fatal and, therefore, the hallmark of the management algorithm was close monitoring. This is not a drug where you treat the patient and send

them on a cruise for six weeks to come back. You really need to follow these patients closely. Toxicities that are severe to life-threatening require corticosteroids and drug interruption or discontinuation, based on the management algorithm. Thankfully, the majority of patients do respond to high-dose corticosteroids and the majority do have complete resolution, although not all. And through trial and error, at least for ipilimumab, we had identified potential secondary rescue medications. For entericolitis, infliximab seems to do very well. And for hepatitis we used mycophenolic acid.

Now, I have been giving presentations on ipi toxicity for about ten years and for oncologists I always have to spend five or ten minutes explaining why we never wanted to use infliximab for hepatitis. But I think in this audience, based on the earlier discussion, I don't think you need an explanation about why avoided infliximab for hepatitis.

DB#11: Now, I am going to move on to how we could prevent the most severe toxicity. And what we came up with in discussions with IDD experts was the hypothesis that prophylactic oral budesonide could be used to reduce GI toxicity. And we chose oral budesonide because it has low systemic absorption. It is an oral corticosteroid and so we thought maybe this would dampen down the local immunity and not result in systemic immunosuppression.

Our primary endpoints, using the oncology CTCAE criteria was grade 2, which is essentially moderate to worse diarrhea. And we randomized patients in a one-to-one fashion to oral placebo versus budesonide in a double-blinded fashion and all patients received ipilimumab. Unfortunately, prophylactic budesonide did not prevent GI toxicity. And you can see here in this table 33 percent of the budesonide arm developed grade 2 or worse diarrhea compared to placebo. So,

unfortunately, budesonide cannot be used prophylactically to prevent diarrhea.

DB#12: Fortunately, in this study, we collected a series of biopsies and evaluations to try and characterize the pathophysiology GI toxicity. The first thing we did was pathology because I am a pathologist by training and we had all patients undergo endoscopy with biopsy one to two weeks after starting ipi. And we did one to two weeks because we really wanted to identify the incipient changes that were occurring in the gut, rather than waiting until patients had developed florid inflammation that was potentially secondary, rather than -- and that would obscure the primary pathology. One in four patients did have inflammation by histology. Similar numbers had inflammation by endoscopy. The histology included both acute inflammation and chronic inflammation. And there was no significant association between patients who had inflammation at biopsy and subsequent enterocolitis. We also had

this reviewed by an expert gastropathologist who found that the histology did overlap, somewhat, with IBD but it is was distinct. For example, there was some overlap with ulcerative colitis from a histologic pattern but the location and the endoscopic findings did not really match what is typically seen with UC.

The hallmarks of Crohn's disease were present in some patients but they were not consistently observed in all patients. And, interestingly, there was a distinct pathology from graph-versus-host disease. So, we could not clearly assign it to any of the classic buckets that previously existed. Just as a point here, I will take a second and little diversion to talk about terminology. We have actually gone through a whole series of terms to describe this. In fact, when the drug first started, the term used was autoimmune toxicity. We then evolved into immune-related. And then finally, when working with the FDA, we actually came up with the term

immune-mediated. And we actually moved away from calling these autoimmune toxicities, although they may very well be autoimmune toxicities, was that we found -- we were concerned that some of the doctors or the emergency room doctors who would have seen these patients from a secondary standpoint would confuse these with classic autoimmune toxicity that might treat them differently if they just got a report that this patient had autoimmune enterocolitis. So, we have actually moved away not from a mechanistic reason but just from a medical information to calling these toxicities immune-mediated.

DB#13: We also collected fecal calprotectin in all of these patients at regular intervals. This is a neutrophil-derived protein that is shed in the stool and can be a marker of disease activity for inflammatory bowel disease. And we found that ipilimumab did induce an increase in fecal calprotectin over time but it was not specific.

And I have three examples of patients shown here. These are tables. On the x axis is time. In those little triangles are doses of ipilimumab. And the y axis is the amount of fecal calprotectin. This first patient did have an increase in fecal calprotectin but actually had no immune-mediated enterocolitis. The second patient did, indeed, have an increase in fecal calprotectin that did precede severe or moderate enterocolitis. So, that was what we had expected. But the third patient had a severe enterocolitis with no elevation in fecal calprotectin prior but did have an increase in fecal calprotectin after the enterocolitis had resolved. So, it was really non-specific and cannot really be used to monitor or to predict.

DB#14: We also looked at humoral responses to enteric flora. These antibodies, which are to either microbial antigens or to pANCA at the time were being used in an exploratory fashion to try and differentiate Crohn's disease and ulcerative

colitis. I know that they are not completely validated and specific but we felt that they would try to at least give us directional support as to whether these were more of the CD or UC type of picture.

We found that ipi did induce an increase but it was non-specific and could not really be used to classify the patients. I will discuss the data in a second but I will point out that we also looked at similar humoral responses to tumor antigens, which are antigens that are only expressed in tumors. We found a very similar phenomenon, that ipi would induce fluctuations in humoral response to these antigens. That probably has to do with the mechanism of action that ipilimumab not only activates CD8 T cells but also activates CD4 T cells and that probably helps in enhancing a plasma cell or humoral response.

So, for the data shown in the table here, each column represents a different antibody to a specific antigen. And we present these by the

number of patients by worst grade enterocolitis. We had 115 patients treated in the first row. So, including any grade for patients who had enterocolitis and who didn't. And you can see that out of those 115 only 10 to 25 percent actually had an increase in humoral responses to these antigens. Interestingly, of those who had an increase, the majority actually never had any enterocolitis and you can see that in the second row. But 61 patients had no enterocolitis. And so you can see in the first column, out of the 18 patients who had a response to I2, 13 out of the 18 actually never even had enterocolitis. And finally, in the last row, of those patients who did have enterocolitis, there were 42, only a minority actually had a positive humoral response. And the frequency probably matches the general population as well. So, humoral responses could not be used to predict, nor could they really be used to classify the pathophysiology.

DB#15: I will now turn to hepatitis. I know this is a liver conference. We have done more work on enterocolitis A) because it is potentially more severe and life-threatening; and B), the biomarkers on the assessment tends to be much easier. Patients can have endoscopy fairly routinely because there are fecal biomarkers. There is a lot of interest now in the microbiome. We can look at humoral responses. For hepatitis, we are limited to liver biopsies, but most of these patients who have end-stage cancer don't want to undergo a liver biopsy. We are limited to serologies, to LFTs, which we do monitor but that doesn't shed light, for the most part, on pathophysiology. We have been limited to try to explore the pathophysiology but, increasingly, I do think there is going to be a need to understand what is going on. Our biggest piece of information comes from a case series that Dr. Kleiner reviewed. He is the world's expert in liver toxicity from ipilimumab because he has seen five patients who

had severe immune-mediated hepatitis from ipilimumab. And what he observed is that these were really a non-specific inflammatory pattern. And the histology overlapped that with what you could see with acute viral hepatitis and drug reaction. And he concluded that this really required clinical pathologic correlation. Now, the majority of patients with ipi-induced hepatitis will resolve to high dose corticosteroids. Those who don't may respond to mycophenolic acid.

Many times, these patients have metastatic melanoma to the liver and it can be hard to differentiate whether this is a mass effect or is really ipi-induced, or really an immune-mediated picture.

But other immuno-oncology agents that are being developed are likely to have a different type of hepatitis that may be not responsive to corticosteroids. Also, these immuno-oncology agents are going to be given together in doublets,

they already are, and perhaps even triplets in higher order combinations.

And for me, at least, hepatitis is the most concerning of the immune toxicities we see because it is such a key organ. With enterocolitis if it is not responsive to corticosteroids, the surgeon can always go in and do a colectomy. But if we don't have the appropriate algorithms for hepatitis, this is, obviously, a major problem in these end stage cancer patients.

DB#16: We have also looked at dermatitis, which is less of a problem, although fatal events have been observed. And this is a case series from the NCI of eight patients who had immune-mediated dermatitis.

I should mention that in those five cases, we had excluded viral etiology. We had excluded other concomitant drugs. In this case series, we excluded other concomitant drugs that may have caused the dermatitis. But the histology and the clinical pattern really represented a typical drug

reaction. There was predominately a T cell infiltrate. Interestingly, some of these patients had eosinophilia in their blood. And it was distinct from autoimmunity and GVHD.

DB#17: As I mentioned, there are other checkpoint receptors besides anti-CTLA-4 that are being developed. There are other co-stimulatory agonists that are being developed that target receptors such as CD137. So, you will be hearing more about these, guaranteed, over the next several years. This does lead to an interesting academic point in that we are intervening by targeting single molecules in the immune system. And for the most part, entry of these patients into clinical trials requires no history of an autoimmune disease. So, from an academic standpoint, this really represents and experiment in patients where we are manipulating single immune pathways and, potentially, by combining multiple pathways. And I think that this may help shed light on autoimmunity, maybe.

This also raises another related question. Does the safety profile of ipilimumab --- mostly enterocolitis, skin, and liver --- shed light on the role of CTLA-4 in preventing autoimmunity in those organs? It is just a question.

DB#18: This is my last slide. Immuno-oncology is an emerging treatment modality. It has already demonstrated survival in at least two tumors. For ipilimumab, the enterocolitis picture, and the hepatitis and the skin appears to stem from classic autoimmune conditions, but more study is needed about the mechanism of action of these toxicities. As was mentioned by Mark, we do need to be able to predict who is going to be at risk. And that probably represents the other hand of understanding the pathophysiology. And once we understand what is really happening, we might be able to identify who is at risk. Thank you very much. (Applause)

Moderatot Session IIIB-6

DR. AVIGAN: Thank you. That was terrific. The last speaker for today's morning session is Arie Regev, who is with Eli Lilly. He is one of their leaders in liver safety. He has an academic track record as well from the University of Miami and before that in Tel Aviv.

He is going to tell us about a hypersensitivity reaction to a drug in development that is very interesting. So, we are going now into the immunallergic arm of that scheme that I showed you.

Regev photo, biosketch,abstract

AR#1: Thank you, Mark and thank you all for being here and sticking with it. This is going to be a little bit of a detour to the left side of the first slide that Mark showed, which is a hypersensitivity allergic-type of reaction.

And I am going to start with just a few general comments, which I think could be summarized in probably two words that were repeatedly mentioned this morning: It’s complicated.

AR#2: There is an accumulating amount of data, but our understanding of the actual mechanism underlying both what we call immune-mediated and metabolic-type drug-induced liver injury, our understanding is still incomplete. And there is a basic approach to separate drug-induced liver injury to two big groups, one of which is called idiosyncratic and the other one is called either intrinsic or sometimes dose-dependent. But within the group that is called idiosyncratic, we do know that there is a tendency to see those reactions in patients who are getting medications in larger or higher doses. And 50 milligrams a day has been mentioned in several places as a cutoff. Actually, 10 milligrams has been mentioned as a cutoff as well for a higher number of drugs represented in these groups.

What is very unusual in the groups of patients that are seen with idiosyncratic drug-induced liver injury is what we call a dose-dependency curve. And this is pretty rare to see and almost

not reported in the literature. And the aim of this short presentation would be to actually show you a group case series of patients that seem to be doing just that.

AR#3: So, this is a group of patients that were treated with an anti-inflammatory drug in development within Eli Lilly. The name of the compound was an mPGES-1 inhibitor. To make a long story very short, the point about this particular molecule is that in the prostaglandin pathway but lower than the COX-1 and COX-2 inhibitors, you can see it in the red in the right lower part of the slide on the pathway to prostaglandin E2, which is the main mediator og pain and inflammation. This is where this particular molecule was supposed to hit as an anti-inflammatory, anti-pain drug.

AR#4: And this was a Phase I study that I will describe to you in a little detail, just so you can get the data in the correct perspective. It was a double-blind dose-escalating study of 28-day duration. Hepatic biochemical tests were done at

least once weekly. And there were five treatment groups.

As you can see, there was a placebo group that included 6 patients. There was a comparator group of another NSAID, which was celecoxib 400 milligram once daily. And there were 3 what we call for short LY, which are the study drug molecules: one 25 milligram group of 8 patients, a 75 milligram group of 10 patients, and the last one was a 225 milligram group of 9 patients. I would tell you here very briefly that we were actually planning on going for a fourth group, which was supposed to be a 450 milligram group, that was stopped early.

AR#5: So, a little bit more about the design and the outcome of the study. There were a priori-defined stopping rules, based on the FDA guidance. Patients were basically healthy, as far as their livers. They were healthy volunteers with no alcohol drinking history. And their plasma and urine were analyzed using HPLC and HRMS

to determine metabolic profile and assess for reactive metabolite formation.

AR#6: And everything looked pretty nice and dandy until we had to suddenly terminate the study because 2 cases of DILI were discovered in subjects who received 225 milligrams for about 19 days. And I will show you these 2 patients very soon. But as we started the study, we immediately looked at all the other patients, interviewing them closely and taking blood samples. And we discovered that there were 4 more cases already showing significant reactions. So, we ended up with 6 patients, showing drug-induced liver injury. And just so you know, to give you a little bit of a spoiler, all 6 subjects recovered, following the discontinuation but it wasn't a significant period.

AR#7: So, the patients affected with drug-induced liver injury numbered 6, of which there were 4 females, 2 males, ages 32 to 59. They

all had a normal hepatic biochemical tests on enrollment at baseline.

AR#8: The presentation was between 16 days and 34 days, a mean of 22 days after starting the study drug. And again, they were caught in different times because we stopped everybody on a certain date and then looked back to see how long they were treated.

AR#9: Symptoms included epigastric pain, fatigue, nausea, low-grade fever, and rash. And you can see here that two of the patients that had rash actually had urticaria.

AR#10: Now, going to the ALT levels. So, as you can see here, all six cases had ALT levels of more than three times upper limit of normal. Remember, they were enrolled with normal levels. Six cases, actually had -- 4 cases had more than 15 times upper limit of normal for ALT and one case had more than 45 times upper limit of normal of ALT.

AR#11: Alkaline phosphatase and total bilirubin, on the other hand, did not exceed 1.5

times upper limit of normal. There were mild increases but nobody reached 1.5 times upper limit -- or not exceed 1.5 times upper limit of normal.

AR#12: Eosinophilia of more than 10 percent was seen in 5 of those subjects, bringing us into this area of hypersensitivity type reaction. And in 2 subjects, it was more than 20 percent eosinophilia count. Viral serology for hepatitis A, B, C, D, and E actually no D but E was negative. Antinuclear antibody, anti-smooth muscle antibody, ultrasound performed to each one of these patients, they were all negative. So, we were pretty much left with a very clear picture of drug-related phenomenon.

AR#13: A little bit more about the clinical course of these patients. So, the things went a little bit on the dramatic side here. The two first patients were worrisome. They were very symptomatic. They had very high ALT levels and they were hospitalized by the principle investigators. And in the hospital, they were

treated by hepatologists who decided to treat them with N-acetylcysteine. And both of those patients underwent liver biopsies very soon after they were admitted. And again, I am not sure this was completely indicated, N-acetylcysteine but, nevertheless, it was given and they showed improvement after that, which we will never know if it was related or unrelated but since the others improved without it, it is very likely that they were unrelated. You can see the course of the 2 that were hospitalized, the ALT changes and the course of those that were not hospitalized and did not have biopsy. These data were published by the hepatologists who were treating them in the hospital. I'm not going to tell you where it was.

AR#14: And the liver biopsies were actually published as well. And you could see very clear zone 3 necrosis with numerous portal and lobular eosinophils. If you look at the right upper hand, you can see pretty clearly a few eosinophils. In the lower left side, it is a smaller size but both

lower frames have a lot of the eosinophils at the same time and you can see maybe even at the beginning of a granuloma-like structure in the right lower frame. There was no fibrosis. There was, interestingly, some cholestasis. If you look at the right upper frame, there is a very distinct area. I should have some kind of an arrow but I don't know where it is. But there are distinct areas of cholestasis in that area.

AR#15: So, looking at the dose relationship, we noticed a very interesting observation. In the placebo group, there were no reactions. In the comparator group, there were no reactions. In patients that got LY 25 milligrams daily, there were no reactions. In the group that got 75 milligrams daily that were 10 patients, there was 1 patient who had an increase of her ALT and an increase in eosinophils. So, a similar type, one of the milder presentation. On the other hand, the group that got the 225 milligram, which was 3 times higher than the 75 mg dose, out of the 9, 5 patients

had significant drug-induced liver injury, which was, in most of the cases, severe. And that comes up to a 56 percent of the group that was treated.

AR#16: So, we, of course, did not continue on to the 450 milligram dose, but it is probably likely that we would get as high as 100 percent with that dose. And this is not a usual observation for a hypersensitivity type hepatocellular drug-induced liver injury. Evaluation of the dosing groups demonstrated a clear trend, as you can see, of increasing likelihood with increasing dose. But despite this trend, plasma concentrations of LY in DILI patients who had the same dose was basically comparable. And the exposure was within the prediction range, based on the single dose data.

AR#17: I don't have a lot to show you here but this is a simulated steady state concentration. And you can see the patients who received 225 milligrams were in a completely different zone, as far as exposure, compared to patients who received the 75 milligram dose.

AR#18: And the behavior of eosinophilia also followed the same trend. So, this was a very strongly dose-dependent presentation and manifestation. So, including eosinophilia, eosinophil count followed the same pattern.

AR#19: Then we did various analyses and tests. And one of the interesting findings was IgE levels that were significantly elevated in the DILI cases, compared to patients who did not develop DILI. There was no difference with IgG, IgA, and IgM. I remind you, ANA and ASTHMA were also not elevated. This was not an autoimmune type of phenomenon but they did have a significant increase in IgE.

AR#20: We did look for metabolites, trying to understand the mechanism. There were no unique human metabolites identified, compared to animal studies. Profiling of human plasma using LC and MS revealed the presence of LY and three main metabolites, which were called M1, M3, and M5. In all plasma pools, the parent drug was the predominant drug-related component. M3 was

generated from hydrolysis of an intermediate epoxide and M3 was the most prominent metabolite observed and the only one that was observed across all plasma samples. And based on the LC/MS ion intensity, the relative percentage of M3 was less than two percent in patients that received 25 milligrams and 75 milligrams but was between two to ten percent in the 225 milligram group.

AR#21: And a few final comments. We know that this type of allergic/hypersensitivity phenomenon has been described from various drugs. We have seen a few in the previous talk and in other talks. And there is an interesting use of nomenclature. Different people in different disciplines call these phenomena in different ways and give them slightly different definitions. And we hear terms like DRESS syndrome, which is drug reaction with eosinophilia and systemic symptoms. We hear DIHS, drug-induced hypersensitivity syndrome, AHS, which is the anticonvulsant hypersensitivity syndrome. They are many terms used for very

similar conditions with slightly different definitions. But in most cases, immunoallergic, features are believed to be associated with worse outcome in DILI patients. Enhanced by the way in the discontinuation rules of the FDA guidance, eosinophilia is considered one of the reasons to discontinue early when ALT crosses three times upper limit of normal.

In a very recent study just published by the DILI group, there were immunoallergic features. Two out of the three that you see here at the bottom of the slide were present in 11 percent of the patients with hepatocellular DILI. But of course, these are rare phenomenon and there is no mention of a dose-relationship curve. This is a pretty unusual observation.

AR#22: To summarize, a dose-response relationship has rarely been described with immune-mediated DILI. There are partial descriptions about a few drugs, but still, a very rare presentation, which suggested is not a

complete dose-response curve. This case series described 6 patients who presented with acute hepatocellular hypersensitivity-type DILI, which was strongly dose-dependent. Basically, we could have reached, with the next dose, probably 100 percent drug-induced liver injury frequency, which is unusual. You would probably have one other or two other drugs to mention with such a phenomenon. Tylenol will be the prototype for these type of response.

DILI occurred in about 56 percent of patients receiving the high dose. Exposure was significantly higher with higher doses but was not different within the same dose cohort. We do not know what would have happened, if we had continued treatment for one more week. It might have reached many more patients.

DILI patients were more likely to be older and female, even though we have a very small number than patients who did not develop DILI. And finally, although a specific metabolite may be involved with

the DILI mechanism here, additional work may be needed to clarify its role.

AR#23: And I thank you for your attention. Thank you very much.

Session IIIB Discussion

DR. AVIGAN: We are going to open this up for questions. And we have a few minutes and then we are going to go on to a second mini session with Paul Watkins on the consortium idea that we have been discussing. Ten minutes? Okay, so we will give ourselves maybe 15 or 20 minutes, max.

DR. TILLMANN: I have two questions to ask, two questions to Arie. One is: did you look for whether the metabolites were differently distributed among the DILI and non-DILI? In the case series were the metabolites different?

DR. REGEV: They were not but I can bring you -- or correct me when I am saying something that is not completely accurate. So, she is giving me a thumbs up. They were not, as far as we know.

DR. TILLMANN: And for the skin reaction, it looks like perhaps they one needs to distinguish rash as an immunoallergic feature from a severe skin reaction, which probably might explain why I know we are saying it is good and you are saying

it is bad. Because the patients probably had bad skin reactions when they have a bad outcome.

DR. REGEV: Yes, I think it is a good comment. I think there is a definition thing. And clearly, one is associated with what we call severe skin reaction, like Stevens-Johnson syndrome. Those have been very clearly shown to known to have bad prognosis. The others, hypersensitivity reaction is being described in two different ways in the literature. Some say it is a bad predictor and recent, a few articles say maybe it is a good predictor. And even biopsies having eosinophils have been said to be a good predictor and a bad predictor but different outcomes. So, I think there is still to be learned about this. I agree.

DR. REGEV: So, I am unable to present exact data about preclinical studies but I can tell you, in general, that the answer is yes, there were some findings in animals. They were completely different from what we saw here, as far as the

pattern, timing. They were completely different but they were not clean animal studies.

DR. WRIGHT: Terry Wright with Genentech. My question is to Drs. Avigan and. Berman. Checkpoint inhibitors PD1, PDL1 may be associated with somewhat less immune-mediated injury. And there was a suggestion from the ipi data that the patients who have metastatic liver disease may be at increased risk. My question actually relates to the risk quotations with liver disease and some with new checkpoint inhibitors, NASH, which is so common, hepatitis B, hepatitis C I know has been excluded from many of these trials but we are now looking at the use of these trials in patients who have metastatic carcinoma. So, my question relates to sort of what do we know about the risk of these new drugs in the setting of patients who have liver disease either viral disease or nonviral disease.

DR. AVIGAN: You are asking about ipilimumab, which was sort of a poster child. The adverse events which looked immune-mediated were

seen in clinical trials. They were actually quite frequent. They were clearly drug-related. They were published in *The New England Journal* article back in 2011. The registration trial was nicely published with regards to the catalogue of adverse events.

And your question about other ligands, which have similar effects but you are saying may be less than PD1 to PD1 ligand and there actually are now new therapies also coming online with regards to genetically engineer lymphocytes, which we will see more of and may have similar catalogues of adverse events. I think that the drugs that are approved so far labeled similarly across the products, whether quantitatively have similar risk effects where there is important nuances, I don't think we have that data yet.

DR. BERMAN: A couple of perspectives. I'm not sure if we actually ever published this data but we looked at whether baseline liver metastasis was a risk factor for developing immune-mediated

hepatitis. And the answer was no. Patients who had baseline liver mets were not at increased risk of developing. Because of our concern about the liver toxicity, we excluded hepatitis B, C. But it turns out, and we don't have definitive data, there are case reports that you can look in *PubMed*, a case series of patients with hep B or C who were treated with ipilimumab and actually did fine. We just did not study those comprehensively but you can actually look at the literature for that. And you are probably aware that a lot of these checkpoint targets are also being investigated for virology. There have been a lot of preclinical work showing that these checkpoint inhibitors can also restore T cell exhaustion and chronic viral diseases. So, of course, there is interest in hepatitis B, at least.

DR. AVIGAN: I just want to add one other point, which is an important point that was raised by David, also, which is combinatorial therapy. So, the combination drug that was mentioned by somebody

was a BRAF inhibitor. I think it was you. The drug was a CTLA-4, it is the animal model, but also of a BRAF inhibitor. So, when we start tinkering around with a biosystem network and you are worried about the canoe going off the edge of the cliff, to some extent, you don't know exactly how the homeostasis controls really work for compensation. But I think the more combinatorialism you introduce, the more uncertainty there is in who might be a bad actor.

DR. REGEV: Can I ask David, from a mechanistic standpoint, you were not expecting a reactivation of hepatitis B as a side effect for this drug, or were you?

DR. BERMAN: No, we were not but we were concerned that anything that would induce inflammation of liver that ipilimumab would exacerbate and cause worse toxicity. That was the concern, not reactivation.

PARTICIPANT: Thank you. I think we need to be very careful when we use the terminology. So,

I was following up the case that Mark Avigan presented ipilimumab and drug-induced liver injury, 1700 in ALT or something. The patient didn't have any autoantibodies. So, there were no features about autoimmunity. And to put this patient on 18 milligrams of prednisone, I don't think there was an indication for that. I don't think that for metabolic idiosyncraty, there is no role for steroids there. So, the fact is that even though some patients develop autoimmune hepatitis from this drug doesn't mean that everybody was drug-induced liver injury.

DR. BERMAN: May I just make a comment on that? Actually, I thought 80 milligrams for this patient was too low. This patient should have gotten 125 or 250 milligrams.

PARTICIPANT: Why? There were no features of autoimmunity.

DR. BERMAN: No. Okay, this is exactly why I stated earlier what I said before, which is about terminology. We did not want to call these

autoimmune for a variety of reasons. But it is called immune-mediated. And what we found in the clinical trials is that early intervention with high-dose corticosteroids can rapidly reverse the toxicity.

PARTICIPANT: How do you know? There is no control group.

DR. BERMAN: No, there is no control group but the reason there is no control group is because the toxicity can be so life threatening that you really can't give a watch and wait versus high-dose corticosteroids. I think that this is actually an interesting point. And there is probably an education component here that has to be about -- and it is not just hepatologist. I think as more patients as endocrinologists and as gastroenterologists see, there needs to be more type of education about what is going on and why is this different from how you would normally treat that.

PARTICIPANT: You know people said it was unethical to do a plus equal control trial with ursodeoxycholic acid in PSC because everybody knew in Germany, everybody knew it helped. It was found out that those who received active treatment had worse outcome with high doses. So, I mean we are hearing clinical medicine. If you are going to propose a huge dose, you need some control data, not because you believe it.

DR. AVIGAN: I was going to say that your point about nomenclature is correct. We probably need to evolve our nomenclature. Because I tried to actually agree with your point in what I was saying, which was if we call this autoimmune, and maybe that is a bad term, it is a different kettle of fish. There are no autoantibodies, et cetera. But the question then becomes there seems to be a souped-up autoreactive T cell mechanism, which is part of how these drugs work. So, the rationale for steroid use here has to do with the effect of steroids on such cells, in terms of their activity.

PARTICIPANT: Any theoretical possibilities that do not turn into be a real thing, unfortunately, even though -- just a small comment that is the case that Arie presented. I think even though it killed your drug, it didn't kill any patients. So, it doesn't mean that it is serious. I think the skin reactions in the DILIN paper is not immunoallergic. I mean Steven Jones is not immunoallergic. It is something else; it is nomenclature.

DR. REGEV: Right, right. I agree. And just for technical regulatory standpoint, all of these patients crossed what we call the FDA-recommended stopping rules because they were all significantly symptomatic and ALTs were as high as 45 times the upper limit of normal. There was no real practical way to continue treating them. And of course, this was not a life-saving drug. It was an NSAID. But, I agree with your comment. It is point well taken.

PARTICIPANT: I have a question for Dr. Regev. And for that compound, do you find some reactive metabolites. Are the metabolites are they reactive or stable metabolite?

DR. REGEV: Well, do you want to comment on this? It was what we considered a reactive metabolite but we have a person right here that could elaborate.

PARTICIPANT: What we saw in humans but also saw previously in rats and dogs, which are Arie had correctly said, the etiology of the liver toxicity, there was some liver toxicity in the rats, very minor. There was something more severe in the god but it was hepatocellular degeneration not even necrosis. It put our focus on lookING at liver very intensively when we do this clinical trial, the actual presentation and progression was, as you saw, completely different than what we saw in animals. But, all that said, the animal metabolic profiles were almost identical and they did show bioactivation in all circumstances.

So, we have this one ring. It gets epoxidized. It blasts apart. We got cysteine conjugates. We got glutathione conjugates. In looking back on it in retrospect that we maybe should have been a little bit more cautious about that, seeing it already in the animals. But you see all those metabolic pathways, all the bioactivation and the animals did okay. The dog data emerged after three months of chronic dosing. There is no way we ever saw that hepatocellular degeneration. It is frustrating to be in the preclinical space and not be able to recapitulate, even when you have all your metabolites covered and an understanding of the clearance pathways that we were not able to figure out what was going on.

DR. REGEV: And just to stress this point, so dog studies showed first response after three months of treatment. It was mild alkaline phosphatase elevation. So, just to show you how poor translational quality we have. But that was the reason. Since this for us showed the liver as

a potential target, this is why we checked liver test so often and this is why we were so prone to discontinue when we saw the first signs. I mean this was significantly sick patients. But yes, we did have a few warning signs in the animal studies.

DR. AVIGAN: We have just a few more minutes.

PARTICIPANT: Here is a question for Dr. Berman. Why infliximab and not mycophenolic acid. So, maybe some pretty severe case induced liver injury caused by the activation of CD8 cells and the depleting CD8 cell would help prevent the sever cases. Do you have some experience or some hypothesis about phenophytic assay treatment?

DR. BERMAN: So, you are asking whether we actually have patients treated with mycophenolic acid?

PARTICIPANT: Yes.

DR. BERMAN: Well, yes. Yes, we have. And actually, interestingly, it is a balance, as Mark said, which is we don't want to deplete the

antitumor T cells. We want to deplete the autoreactive T cells. There is always a balance.

PARTICIPANT: Published in literature?

DR. BERMAN: Yes, there was published literature.

DR. AVIGAN: I mean I just have to say that part of the problem here is that the good cells are also the bad cells, to some extent. It is kind of like inducing graft-versus-host disease growing in our transplant patient to kill CML cells. But you know there is a kind of balance, which may be actually more quantitative than actually what is specifically being targeted.

PARTICIPANT: So, I guess what you need is a complex nomenclature. Is that what you are talking about? Okay. Anyway, I have a question about the T cell infiltrates or the lymphocyte infiltrates. Did you actually look at that a little closer? Are they CD4 or CD8 on both examples and are they polyclonal?

DR. BERMAN: In the liver?

PARTICIPANT: Liver, skin, whatever.

DR. BERMAN: Yes, I don't remember the data. I think that was published, at least. I don't remember it offhand but from a clinicality standpoint, no, we haven't looked at that. Yes, that was published. I just don't remember it offhand.

PARTICIPANT: So, I am not quite sure about the autoantibodies titer is not collated with the injury. For the model that Jack Uetrecht about it in last fall lab, there was clear correlation between our antibodies in the serum of the animal and ALT. That is one thing. The other thing and the study was done 25 years ago when my Ph.D. would be the hydralazine with autoantibody against P4501A2, it was found that when we would stop the drug for a few months, each time we test the sera, it is dropped in the titer of the antibody. It is not clear to say yes and no because the patient you have like maybe 10, 20 and there are different times

that you take the serum and it is very hard to make the conclusion. That is one question.

The second and my comment, the question about the oncology drug. Did you look at any immunosuppressive cells that you see that may be dropping in the liver in this patient? Because in the cancer, you have these immunosuppression and that is probably going to give us some ideas about how the hepatitis could be.

DR. BERMAN: Yes, I think that we don't but that work absolutely needs to be done. And if you know how to do that without being too invasive, I think the problem is actually getting the samples. You know these are end stage cancer patients. They usually don't want to have a biopsy, unless they really have to. Nobody wants to, end stage or not.

PARTICIPANT: Two quick questions. The first one is in the healthy volunteer study we just heard about. Did you do any skin tests either before, during or after in those volunteers? And what do you think about that?

DR. REGEV: You are referring to the study that I -- no, this was as surprise for us. So, no, we didn't have skin biopsies. And the skin conditions resolve very quickly. So, we don't have data. But in general, we took the picture to be a pretty classical hypersensitivity type syndrome with eosinophils, rash, urticaria, and the eosinophilic infiltrates. So, we didn't go after the skin lesions themselves.

PARTICIPANT: I was thinking then you could use it. I mean, obviously, you are still interested in the topic. The question is how you get around it and avoid that hypersensitivity. And that might be one way to approach the system.

DR. REGEV: That is a good point. And we have had many discussions on second and third generations that, unfortunately, I am not able to discuss.

PARTICIPANT: The second question was in the -- I can't really pronounce it, the modulation of that system. There is, obviously, always a worry

when you use an immunology activating agent. And I think the CD28 story is very cautionary. But you also have the chance to use your own antidote in that system and fine tune and regulate. So, obviously, you want to treat the cancer but it is a balance. How do you -- did you think about that? And what do you think about that as an idea?

DR. BERMAN: Yes, so we have anti-CTLA-4 ipilimumab and then we have CTLA-4 Ig, which is Orencia used to treat rheumatoid arthritis. We actually thought as using that as an antidote but we were worried about antibody complexes forming, causing other trouble. But we have actually -- I mean we have jokingly talked about it more than anything.

DR. AVIGAN: I think we are going to end at this point and ask Paul Watkins to come up and give us a little summary of our meeting yesterday.

DR. BERMAN: Thank you very much.

Session IIIC Discussion 11:30 am

Watkins photo, biosketch (no abstract or slides)

DR. WATKINS: Okay, we had a meeting last night at 8 pm. I think about a third of the audience attended, with which we were delighted, It was a show of support to talk about the potential of starting a Liver Safety Research Consortium.

Now, I don't have to, in this group, say that the major adverse event that historically caused drug abandonment in development has been cardiovascular but liver is right behind it by a couple of percentages. And there is now a regulatory path forward for the major group of cardiovascular adverse events, which is searching for data on torsade de pointes, which involves an electrocardiographic prolonged QT study. There is no equivalent path for drug-induced liver injury. The question is whether we should clone a very successful organization called the Cardiac Safety Research Consortium. It was launched in 2006 through an FDA critical path initiative memorandum

of understanding with Duke University to support research into the evaluation of cardiac safety of medical products. And really what got this going was the creation of an electrocardiogram warehouse. Norm Stockbridge really was the central person who dictated that ECGs had to be in a standard electronic format, so they would be comparable from one organization to another and then had, over time, accumulated these electronic ECGs, initially, in the prolonged QT studies. So, this opportunity to look and analyze this aggregate data in a precompetitive fashion across the companies was really what started the cardiac research safety consortium.

Now, the mission of this consortium is to advance regulatory science specifically related to precompetitive cardiac safety issues, through the collaborative process of a public-private partnership across interested stakeholders, with many participating pharmaceutical companies. And in addition, Quintiles and a couple of contract

research organizations and some medical device manufacturers are partners in it.

And the ECG warehouse is only what started it but the companies actually own their own data in it. And my understanding is the data of the actual ECGs did not come from the FDA but came from the individual pharmaceutical companies to set this up. And then release of the data for additional analyses represents the collaborative effort of scientific good will within this consortium. A scientific oversight committee has been formed to evaluate proposals for use of the released ECG data and to foster collaboration within the research community. They have published over 30 different white papers that have been very influential in determining practice but also regulatory approaches to evaluating cardiac safety. And a lot of them, initially, were around arrhythmias and prolonged QT in this ECG warehouse data. And so this is a fairly recent publication, "Can thorough QT:QTc study be replaced by early QT assessment in

routine clinical pharmacology studies? Scientific update and a research proposal for the path forward." A long list of authors that include regulators, include industry and academic leaders.

But over time, they have really drifted from that initial focus to really look at broad areas of cardiac safety and they developed a relationship with the American Heart Journal to get sort of accelerated access for publication of these white papers. So, a centralized adjudication of cardiovascular end points in cardiovascular and noncardiovascular pharmacologic trials: A report from the Cardiac Safety Research Consortium; Assessment of drug-induced increases in blood pressure during drug development and again, a report from the consortium.

So, is the time right to start a Liver Safety Research Consortium? Analogous, somewhat to the ECG database, the warehouse, John and Ted Guo have been accumulating data in the eDish format that I thought was liver test data on 150,000 patients.

John said last night it is much more than that now. And we learned last night that the trigger to ask a company to put the data into the eDISH format, to submit to the FDA, is really the NDA reviewer. So, when any medical reviewer raises a liver safety concern, that is the trigger that leads to a request for these data to be submitted in a standardized format.

You have seen the classic eDISH plot. It is not the ideal dataset to begin to answer all the questions we want but it has, not only the peak ALTs and bilirubins, but has also the four traditional serum liver test chemistries, serially over time for every single subject or patient, represented by a single point. So, you can click on it and get the time course of all liver test data for that person.

The FDA cannot release these data, which are confidential property of the companies submitting them. We would have to get it directly from the companies who are willing to volunteer data from

the comparator or placebo-controlled group. That would involve, somehow cutting out the data from a proprietary drug, if you wanted to do that. And in some cases, a comparator may be a proprietary drug but I am told that is a minor issue and most of the data that is in the eDISH format.

So, you can begin to ask questions like what is the incidence of ALT elevations at various levels in a placebo-treated multiple sclerosis population or congestive heart failure and begin to perhaps get the data to have disease-specific reference ranges. Again, not ideal. These are not -- you know -- people are on multiple drugs. But I think the consensus was this would be a valuable starting point. Certainly, if companies weren't willing to forward this data, it would be a tremendous challenge to get more in-depth data.

So, the other point is that the climate is changing for liver safety evaluation. A requirement that all NDAs be in eDISH- compatible format would create a great opportunity because it

would become much easier to compare data across different companies. And there is evolution of new data management and commercial analytical tools, such as a Spotfire and JMP Clinical and I think JReview as well that are now designing themselves to be able to use that data and extract it in a very efficient way. They have some marvelous visualization tools that I think will transform the ability to analyze the data. And I think it is important that experts such as those in this room have not only a front row seat but actually be involved to see that there is appropriate interpretation. My own personal interest is in the biomarkers, which you will hear about but I think the new genetic biomarkers are going to revolutionize the assessment of liver safety.

We heard yesterday that SAFE-T, for instance, is moving to try to get context of use of a variety of different biomarkers. This is just the beginning. But we know now that the interpretation of those is not as simple as initial

hypotheses and they are going to have to applied to thousands of patients across multiple diseases, multiple drugs, to really get the most accurate assessment of how useful these will be. But I believe it will revolutionize the assessment of liver safety.

But what that means is now companies need to start deciding when and what to save, perhaps just when a potential liver signal is detected. It is it serum? Is it plasma? We will hear an example of urine. How to store them. How to process them, for that matter.

And then how to make sure they are linked to the relevant phenotypic data so that years after the fact, when the team has moved on, maybe even the drug is abandon, it is very easy to go back and find those specimens, find the cases, find match controls, which all should be very easy to do with the new data management tools that are coming online. The initial leaders that have basically stepped to the floor, is me on the academic side,

Mark Avigan and John Senior on the FDA side, and Michael Merz on the industry side.

We have full cooperation with Duke University to synergize this with the Cardiac Research Safety Consortium and do economies of scale, wherever that is possible. They will share all their contractual agreements and lessons that they have learned.

What came out last night was endorsement, I think, essentially universal, to move forward to create a concise document, which will outline the objectives and deliverables of the Liver Safety Research Consortium. And we will be putting that together. The idea was to start small and direct because there are many areas this could go into.

And the first would be precompetitive analysis of comparator eDISH data, getting what the disease diagnosis was of the population and inclusion and exclusion criteria. Establish guidelines for biospecimen collection and storage and linkage to appropriate phenotypic data. And

organize think tanks to prioritize topics for liver safety assessment for white papers to work towards, including DILI and chronic liver disease, oncology, other special situations, pediatrics, but not to have them in the initial mandate going forward.

I know John has a couple comments to make but that is where we stand. Again, the plan is we all come up with a two-or-three-page document. Everyone attending this meeting is going to get it. You will also get my complete slides that I showed last night. And we will begin the dialogue of moving forward with this and see what sort of cooperation we get.

I think our partnership with the Cardiac Research Safety Consortium and the individuals from those companies already involved will be helpful and we will pursue that. John.

DR. SENIOR: Paul, thank you so much. I think a point of caution. You mentioned CDISC which was a good standardization idea but if we

exclude other data than CDISC, we may miss some very important information. When a patient gets sick at a study site, often the investigator will use the local or hospital lab to get data fon following the patient and immediately find out what is going on. If those data are excluded because they don't meet CDISC standards, we may miss the boat.

Currently, the requirement for submitting eDISH data is that the sponsors send us **all** the data, not just that in CDISC format, not just the standard lab data, but **all** the data, including the local labs. We heard yesterday that local labs may have different upper limits of normal and all of that. We can't worry about that. Let's look at the data, whether they are standardized or not. We cannot afford the delays of waiting for standardized results.

Next, probably one of the most specific biomarkers is the clinical appearance of symptoms, described in clinical narratives. Now, we maybe ought to take a better look at symptoms from the

patient and educate physicians, medical students, everybody to be on the lookout for symptoms because they may be very specific. The whole business of routine monitoring is, I think, a failure. Why? We are looking for something that for any given drug is rare; If you do routine monitoring, all you get is normal, normal, normal, normal. And people get very weary of looking at normal results. And it is very expensive. It is very inefficient. It is much better to start with a problem and then zoom in and get the data, not by routine monitoring but for cause investigation.

The technique of using the postage stamp device for point-of-care fingerstick ALT estimate may be something that is cheap and available. You heard it described by Nira Pollock yesterday. And it may be an idea whose time has come. You heard from Arthur Karmen from yesterday; he speeded up the measurement of transaminase activity from several days down to five minutes. But, it still takes five minutes after it gets to the lab. So,

you draw the blood, you send it off. You don't really know the results until later today or tomorrow. It is too much time lost. It is a very good idea to have an immediate value, even if it is not all that accurate. Even if it is only going to tell you the patients like in the normal bucket, or the intermediate bucket, or the high bucket. That is close enough to start looking closely, to start for-cause monitoring.

I also want to say something about Duke, which Paul mentioned as the site for the cardiac safety consortium. It just so happens that Duke has come to the FDA. I am speaking about Dr. Robert Califf. He is now the Deputy Commissioner, a pretty high position, Deputy Commissioner. He is in charge of CDER; in charge of CBER; in charge of CDRH, and in addition he has tobacco to worry about. But he has a lot of power and he is already on the team. So, he has come to the FDA, just started a couple of weeks ago.

DR. CZAJA: Yes and he was, I think, the key individual that got the idea to bring the Cardiac Research Safety Consortium to life and seat it at Duke.

DR. SENIOR: He is a world leader in clinical trials and I don't know what he hopes to accomplish at the FDA but I think he has big ideas.

DR. CZAJA: He is also on the Advisory Board of our Institute, by the way.

DR. SENIOR: Right. And maybe Paul can set forth his proposal this on two pages, but he should give himself a little room, maybe three or four.

DR. CZAJA: A little more room. Okay, Anna, I don't know if you can one-up John, but do you have something quick to say? Because we have to go to lunch.

DR. SZARFMAN: Can you hear me?

DR. CZAJA: Yes, perfectly.

DR. SZARFMAN: Yes, I work with clinical trial data of spontaneous reports, et cetera. There is another issue that we need to discuss. I am a

board certified clinical pathologist. I talk with people that run central labs and they generate the most accurate results because otherwise they would not be accredited. The problem is that the data in practice is being transformed into 800 formats. Then we receive the data, and I transform the data in about 2800 different formats. And we hear in observational studies a few weeks ago that there are 50 different formats for -- there was a statement that the data is being transformed by statisticians that have not been -- If there is a way of improving the quality, maybe by directly accessing the data generated from the best machines and avoid doing manual transformation and this procedure will improve the quality of the data we get.

The second thing that has happened, because the computers that are connected to the instruments in central labs and local labs, they can be programmed to generate --

DR. WATKINS: Just one thing, Anna, and then we can continue this offline. But I think right now the focus of the Liver Safety Research Consortium would be on clinical trials in a drug development setting. It was brought up last night, you know post-marketing, et cetera but I think the initial focus will just be on Phase I through III clinical trials.

DR. SZARFMAN: I am talking about clinical trial data.

DR. WATKINS: Okay. All right, so let me close this session and break for lunch, but I would just like to give a round of applause for John, who is just incredible. (Applause) Please be back here at 1 o'clock for the afternoon session. I hope everybody will attend. 12:07 pm

Lunch break

Session IVA 1:03 pm

Moderators – Paul Watkins and Gyongyi Szabo

DR. WATKINS: Welcome to the afternoon session. My co-chair is Gyongyi Szabo, who is going to be our first speaker. I will introduce the speakers in the first half and she will introduce the speakers in the second half.

And so, without further ado, Gyongyi Szabo is the vice-chair of research in the Department of Medicine, a Professor in the Department of Medicine, and also Associate Dean for Clinical and Translational Research in the school of Medicine and Director of the MD-PhD program at University of Massachusetts. She is also the current president of the American Association for the Study of liver Diseases. And you can see why I told her husband recently that I am a fan of hers and, of course, he said he was, too. And by the way, all that stuff is besides being an international leader in research into molecular mechanisms underlying a variety of liver diseases. So, here she is talking about microRNA-122 uses and applications.

Szabo photo, biosketch, abstract

GS#1: Thank you, Paul. Thank you for the nice introduction. I would like to congratulate Dr. Senior and thank him for the invitation to give me the opportunity to talk about this today.

GS#2: A few years ago, I became interested in microRNAs mostly because, as you all well know and talked about during this conference, we have very poor markers of liver injury in our armamentarium. Currently and for many, many years, use of transminases certainly gave some information for us in clinical practice but have very severe limitations. They are not specific. They really don't correlate well with the progression of liver disease, cannot distinguish between inflammation and liver injury, inflammation or fibrosis, and, certainly cannot distinguish between drug-induced liver injury and other type of liver injuries. So there is clearly a need for more specific and stable biomarkers. And I do like to hear that that work

is being undertaken in new biomarker discoveries for liver disease.

GS#3: So, one of the potential targets and candidates for biomarkers could be potentially microRNAs for several reason. The microRNAs regulate various genes and they also are found in a very stable form in cell-free body fluids, including the serum and some of the microRNAs actually are packaged into small vesicles, either exosomes or microvesicles, or apoptotic bodies and can be found in the circulation. Therefore, all of these characteristics make them attractive new non-invasive biomarkers.

GS#4: For hepatologists, microRNA-122 is particularly exciting because very uniquely this particular microRNA represents about 80 percent of the entire microRNA pool in hepatocytes. Now, if you consider that there are more than a thousand different type of microRNAs, it is pretty remarkable to have one in that high kind of propensity in liver cells. But it turns out that

microRNA-122 regulates various mechanisms including cholesterol biosynthesis and it has been identified as major host factor in hep C viral replication. And I am not going to talk about that part today.

But interestingly, there has been a lot of attention to microRNA-122 changes in liver diseases, particularly in the circulation, in the plasma and serum compartment. And various studies demonstrated that in drug-induced liver injury there is increase in the serum levels of microRNA-122. It has been shown to increase in chronic hepatitis C infection and also in non-alcoholic fatty-liver disease and in hepatocellular carcinoma. So, it certainly marks at the same time that this is possibly and very likely not going to be a specific marker but certainly deserves additional attention.

GS#5: If one looks at, for example, acetaminophen-induced drug liver injury, in a mouse model, what we find is that on the left

various time points and changes in ALT levels in mice. And, as one would expect, a few hours after a sublethal dose of acetaminophen, ALT levels increased. But at the same time, if you look at microRNA-122 levels in the same plasma specimens, then it appears that at one hour, microRNA-122 shows a significant increase at the point when ALT hasn't changed yet, suggesting that potentially the timing and the sensitivity of this marker could be a little more sensitive than ALT.

GS#6: Also in a different study in a rat model, in a fulminant hepatitis model of Wilson's disease, investigators found that kind of the similar phenomenon that on the top panels you see on the left, the microRNA-122 increase that is at week ten is already significantly increased when AST is still normal. And at a later time, again, the ALT and bilirubin changes show differences but really, the microRNA-122 shows up and increases earlier on, suggesting that this could be an early marker.

GS#7: Definitely changes in the serum microRNA-122 levels in various model of liver injury appear to correlate with ALT. So, on the left of upper part is an alcoholic liver disease model in mice; in the middle, acetaminophine-induced liver injury; and on the right is an infectious and inflammatory model in mice that is an autoimmune disease induced by the CpG, DNA and LPS administration.

The extent of the increases and even the magnitude of microRNA-122 changes are different between the different models. And the highest kind of level both in ALT and miR-122 were found in the APAP model, where there is the largest extent of hepatocyte damage.

In chronic hepCV infection in humans, we also found that there is a linear correlation between ALT changes and microRNA-122 in the circle they think of plasma in patients.

GS#8: So, moving on to a different kind of model, actually we were interested in the role of

microRNA-122 in the non-alcoholic fatty liver disease. And here, again, if you use a mouse model of methionine-deficient diet or a control diet, what we find is that over time between one to eight weeks of administration of this diet that induces massive steatohepatitis and actually fibrosis by week eight, we find that increasing the serum microRNA-122 but, interestingly, the correlating levels of liver microRNA-122 actually were decreased. So, that really was intriguing to us and made us question the potential role of microRNA-122 in the liver. So, microRNAs are included by DNA and, essentially, in the biosynthesis there is a pre-microRNA-122 form. And that essentially indicates the formation of new microRNA-122. And interestingly what we found was that this pre-microRNA-122 was severely reduced, compared to normal animals in the mice with steatohepatitis. And one of the factors that actually drive the promote the region of microRNA-122 have an HNF6 side, which essentially is one of the promoters and

inducers for microRNA-122. Interestingly, we found that that was reduced also, suggesting that there is a transcription regulation of microRNA-122 in this model of non-alcoholic fatty-liver disease, leading to the lower levels in the liver. In addition to the regulation of cholesterol synthesis, relatively little is known about the role of microRNA-122 in hepatocytes liver diseases. So, various studies show that there is new 122 expression human NASH in the liver.

And then it has also been recognized that if you look at gene sequences, we found that there are potential putative targets of microRNA-122 that included the MAP3K3 kinase and the hypoxia inducible factor 1 alpha, HIF-1a. And it is also known that HIF-1a actually contributes to the steatosis and actually regulates steatosis in alcohol-induced liver disease but also in other conditions and it has been implicated in NASH.

GS#9: And another kind of known background is that the MAP3K3 actually regulates NFKB in cell

survival and tissue remodeling processes. So, these potential correlations led us to the hypotheses that potentially the decreased level of microRNA-122 in the liver in NASH could have some specific pathogenic roles.

So, to explore this, we started at evaluating the MAP3K3 kinase and we found that at the messenger level it was increased in the non-alcoholic fatty-liver disease model. And it was increased at the protein level not only in the total liver but also in isolated hepatocytes. Now, I showed that potentially these MAP3K3 kinase is a target of microRNA-122 regulation. And so that question be used an inhibitor of microRNA-122 in isolated hepatocytes. And then we found that if, indeed, we inhibit microRNA-122, then the levels of the MAP3K3 actually increased. I probably should clarify that there is actually most of the microRNAs act in a way that they inhibit the target messenger RNA. So, in this case, if microRNA-122 is reduced, that means that the inhibition of the

MAP3K3 is really meaning that then it is expected that by limiting microRNA-122 we actually find the metric K3 kinase RNA being increased. That suggests that microRNA-122 targets the MAP3K3 kinase.

GS#10: Now, bouncing from this MAP3K3 is NF kappa B, which is another major regulator of inflammation. And in it, we find that in the MCD diet-induced model, in the liver there is a massive induction of NF kappa B and this also is seen in the nuclear binding level in the total level but also in hepatocytes and that is on the top right side. And if we inhibit the MAP3K kinase, then we can actually attenuate and NF kappa B activation, suggesting that, indeed, there is a causal kind of relationship between these various kinases and regulatory factors.

GS#11: The other potential target for microRNA-122, as I told you earlier, is HIF-1, the hypoxia inducible factor 1. And this is interesting and potentially clinically relevant because those of you who treat and see patients with

non-alcoholic fatty-liver disease, many of them actually have sleep apnea. So, hypoxia is happening at the macroscopic or physiological level. But there is also a lot of speculation that even at the liver tissue level, hypoxia could, potentially play a role.

What they find is an upregulation of hypoxia inducible factor of 1 at the RNA level and on the right top side, you can see that there is an increase in the activity of HIF-1 because this is a nuclear regulatory factor and there is increased DNA binding of HIF-1 in the steatohepatitis model. Now, HIF-1 regulates various process and one of the targets of the HIF-1 is lysil oxydase that plays a role in fibroids and tissue remodeling and vimentin is another one that also is in tissue remodeling and the transformation.

And as you can see, both the RNA levels of vimentin and also the immunohistology staining suggests that the protein levels are increased in mice with steatohepatitis compared to controls. To

come back and show the causal relationship here, we used an anti-microRNA-122 SINRA transected to hepatocytes in the left upper corner you can see that the HIF-1a levels actually are increased when we inhibit microRNA-122. Therefore, essentially leaving the repression effect of microRNA-122 on the HIF-1. And on the right-hand side, you can see that the same things happens at the biological activity in the nuclear binding.

GS#12: And the same thing happens in hepatocytes on vimentin, if we inhibit microRNA-122, then the SRNI against microRNA-122 and not the control increased the vimentin levels at hepatocytes. That kind of left us with the conclusion that microRNA-122 in non-alcoholic steatohepatitis has multiple roles. First of all, it appears that there is a reduction at the transcriptional level by reducing the pri-microRNA-122 levels, most likely through HNF6 and potentially other mechanisms. And this leads a reduction in the mature microRNA-122 in the

liver. But at the same time, there are some mechanisms that are not very well known but certainly result in increased levels of serum microRNA-122 so that kind of contributes to this consistent dichotomy. It appears that in the liver the microRNA-122 actually has, in addition to cholesterol metabolism appears to regulate HIF-1 alpha and the MAP3K3 kinase and those processes can contribute to inflammation, fibrosis remodeling and certainly the circulating microRNA-122 potentially could be at least one of the biomarkers indicating liver damage.

GS#13: What I wanted to come back to is that the microRNAs in the serum are often actually packaged in exosomes. And as exosomes are small extracellular membranes vesicles on the size of 50 to 100 nanometer in diameter that are produced by most cell types.

GS#14: They are found in the extracellular space and various biological fluids, not only serum, saliva, and in all kinds of other biological

fluids. They contain various nucleic acids and proteins and among those are microRNAs. There is increasing evidence that these exosomes actually can function as kind of messengers between cells and potentially may get to various organs and could be having a beneficial and harmful pathological effect. Certainly hepatocytes are one of the sources of exosomes that can also be targets.

GS#15: And indeed, there are various recent publications that indicate that exosomes could be considered as like biomarkers of liver disease. So, for example, in various types of liver injury, the presence of an increase in exosomes have been noted in various biological fluids, as described here. Many of those microRNAs actually did contain microRNAs as well. That is certainly of interest.

GS#16: So, we ask the question if exosomes serve as therapeutic vehicles and could potentially these actually have some function and effect. And the way we approached this was that we took a B cell line. So, there are B cells that

produce large amount of exosomes after stimulation at IL-4 and CD40. And then we took those exosomes and isolate them. Now one of the characteristics of exosomes is expression of CD63 that allows the purification of these exosome compartments.

GS#17: And then we used those exosomes and either loaded them with various microRNAs. Or particularly for microRNA-155. That was the kind of system that we used or we used an inhibitor of micrRNA-155 and these kind of modified exosomes were then tested for functional activity.

GS#18: We tested them by delivering this microRNA-155 inhibitors to macrophages and that was because normally microRNA-155 actually can regulate inflammation or they tried to deliver a precursor of the microRNA-155 into hepatocytes and this was two hepatocytes were chosen as targets because typically hepatocytes microRNA-155 expression is very low.

So, what we found was that if took macrophages and stimulated them with LPS and that

is the first two bars on the left compared to the one very much to the left, no treatment. Then LPS stimulation induces a lot of microRNA-155 in this side. And on the right-hand side in that kind of graph, you can see that this goes along with an increase in TNF production.

And now if you look at the last two bars in each of these panels, it shows that if we use a control inhibitor-loaded exosome, nothing really happens. But if we put a microRNA-155 inhibitor into the exosomes and put these exosomes on the macrophage in the presence of LPS, then actually we can inhibit TNF production.

GS#18: And that suggests that, indeed, these exosomes could be actually vehicles to bring on to us a type of modulation. In this particular case if this was an inhibitor, again with the microRNA-155 and this inhibitor actually was biologically active. I don't have enough time to go into details but it was shown as in our publication that actually what they find is that

the exosome-mediated delivery of these inhibitors is more efficient than just doing a regular transpection inside with an inhibitor, which I think is very intriguing and certainly brings a little more attention to the exosomes in this system. The opposite side of this is that we actually made exosomes and then and loaded them with microRNA-155 precursor, essentially to see what was the effect of these exosomes on hepatocytes that normally don't express much microRNA-155. We injected these loaded microRNA-155-loaded exosomes into mice and then we evaluated the liver and also isolated hepatocytes for the expression of microRNA-155. And these were mice that were microRNA-155 deficient. So, normally they didn't have natural microRNA-155. By giving these exosomes loaded with miR-155, we found that we couldn't detect the miR-155 in the liver of these knockout mice. And if you isolated hepatocytes that the miR-155 actually was found in hepatocytes, suggesting that, indeed, again, these

exosomes are capable in vivo to deliver these either inhibitor or a precursor for macroRNA into the liver and into hepatocytes.

GS#19: To summarize, I want to leave you with the idea that there is evidence that exosomes actually could be therapeutic vehicles. It could be that depending on, so on the left side with the black kind of RNA and microRNA, that is an inhibitor. And if we put that into an exosome, then actually that has an effect on macrophages to potentially inhibit the microRNA-155 activity and the contrary of this, if we take the exosomes and put the precursor on it with the blue kind of microRNA marking, then that potentially can deliver a functional microRNA to tissues in mice and particularly to hepatocytes. That suggests that certainly exosomes are a new and exciting area from the standpoint of cell-to-cell communication or potentially, organ-t- organ communication. They also potentially deserve to be evaluated as therapeutic vehicles.

GS#20: And I want to thank our funding agency and my colleagues who contributed to this work. Thank you. (Applause)

DR. WATKINS: That's great. We have time for some questions. I have just one question starting off: miR-122 is more sensitive early on in acetaminophen injury. And I think there are some data that they might be actively eliminated from cells before they die, suggesting that this might be an adaptive response for the cell to get rid of miR-122. And if I were smart enough, I could have figured out a mechanism in what you said why that might be adaptive. A hepatocyte is being challenged by a toxin. Why might it want to dramatically reduce its content of miR-122? Would you understand that?

DR. SZABO: I do understand your point, nd I think it is a very interesting one. I'm not sure that I actually thought about it that way but I certainly think that is a consideration. To think about that you know maybe when the study is damaged then having all this microRNA-122 is not good

anymore and then it is a definite mechanism to kind of get rid of it by filling out of the circulation. I think the way I would approach this question and I cannot answer -- I am not aware of any data that would support or kind of disregard the aspect that you are bringing on. But another consideration is that could that be the injured hepatocyte is trying to send out some message to some other cells or non-injured hepatocytes or to any other organs in forms of by releasing these microRNAs. I think that is the question that we were mostly interested in. And in fact there is a difference for that for example, this is data that is under consideration for publication that for example if you put the alcohol on hepatocytes or in vivo in alcohol liver disease, we find that there is an increase in the circulating number of exosomes and these exosomes actually contain microRNA-122. And that appears that actually can regulate the function of monocytes and macrofages. And normally monocytes and macrofages, microRNA-122 can be very

detectable. So, I think that is kind of a fascinating possibility that maybe these damaged hepatocytes use the exosomes to actually alert other cells or modify functions.

DR. WATKINS: Will Proctor.

DR. PROCTOR: Yes, Will Proctor from Genentech. Great talk. I have two quick questions. In your NASH model, have you done work to really show they are in exosomes or are they in protein complexes? And there is some discrepancy in the literature that maybe R-122 is predominately in the protein complex form versus vesicular form or exosome form.

And then my second question is more of a practical application. In terms of standardization and normalization for circulating microRNAs, where we do a lot of work in preclinical inbred strains, where we are treating controls with a toxin in our disease state and then we know there is a larger spread, potentially, in humans and there is no consensus on disease state age and what controls

we should use, besides maybe an exogenous spike in volume put into the RNA traction.

So, just those are the two points that maybe you could address.

DR. SZABO: Right. From the NASH work, I think we haven't used the immunosuppression agent to look at if the microRNA-122 was in complex with argo 2. We just published a study in *Hepatology* that was evaluating similar questions in hepatitis C infection. And what we found was that exosomes that are produced by hep C infected hepatocytes, we find that that there is double-stranded or single-stranded RNA in these complexes. Those actually are ready to infect the named hepatocyte, even if you just use exosomes. But we haven't looked at NASH.

In terms of standardization of exosomes, that is a very valid question and there are a lot of meetings going on. For example, there is the NIH Extracellular RNA Consortium that was initiated about I think one and a half or two years ago now.

And one of the working groups in that consortium is evaluating this very question. In fact, there is a big meeting on International Extracellular Vesicle meeting that is going to happen in a few weeks here in Washington, D.C. I don't know if these are being evaluated.

DR. WATKINS: You can go next and then Elliott.

PARTICIPANT: Yes. Exosome when you have the microRNA inside of them, how are you going to be sure that they are going to go the liver? They could be going to other organs. And miR-155 has many leukocytes, so it may be affecting in all one place. The other thing is microRNA can hit, you can have five, six, seven microRNAs in the same spot. So, if you deplete one, what are the consequences on the other microRNA composition for the same side that are balancing?

DR. SZABO: These are very good questions. We did a study where we took microRNA-155 containing serum and exosomes and put it into

miR-155 knockout mice, and then evaluated the expression of miR-155 in various tissues. After IV injection, the liver had the highest amount of microRNA-155 with very detectable levels in some of the tissues as well. So, your point is very well taken. It is not only going to deliver, obviously. In terms of the cross-regulation of the various microRNAs it is a very valid question. I think that a beauty of the microRNAs, when one looks at them as a therapeutic target, that microRNAs by immune microRNA are never going to have the kind of total inhibition of any of the target genes, which I think in many cases could be an advantage, but it will depend on what you target. And in terms of compensatory microRNA changes, certainly, that is a possibility.

DR. WATKINS: Elliot.

DR. NORRY: This question is from a drug development standpoint. I am wondering if putting the logistics of availability of the tasks and standardization of results, do you think that we

are at the point where, for diseases like myositis or muscular dystrophy, ALT is really not a reliable measure of liver injury, in that it is affected by the disease itself. Are we at the point where miR-122 could be used as a surrogate measure of liver injury?

DR. SZABO: Well, I think that is a very good point, although I am not an expert in skeletal muscle or any of this. But I think it is a relatively easy experiment to do that. I mean in the baseline expression of microRNA-122 is much lower in any other time. So, theoretically I think that that could be a very good marker to distinguish between liver injury versus some other source of particular increase in AST.

DR. NORRY: Thanks.

DR. WATKINS: Jim Freston, last question.

DR. FRESTON: To extend that question, there are conditions where Kupffer cells are jammed, hemolytic conditions, anti-parasitic drugs, in which with the saturation of the Kupffer cells, the

elimination of half-life of the transaminases is prolonged and so it may cause a false elevation of transaminases that looks like liver injury. Could microRNA-122 be used in that circumstance to exonerate liver injury?

DR. SZABO: That is a really interesting concept that I must admit I never thought about.

DR. FRESTON: And phospholipidosis isanother example.

DR. SZABO: Right. I don't think that the levels of microRNA-122, at least to my knowledge, I am not aware of publication that would have looked at miR-122 in the circulation in those kind of conditions in comparison to transaminases. I think the role or the effect of microRNA-122 on Kupffer cells is not known. So, I think what you are proposing is that could that potentially modulate Kupffer cell functions. And at this point, I don't think that anyone looked at that.

DR. FRESTON: Thank you.

DR. WATKINS: Right. And you know there are new technologies now that are able to profile microRNAs, including a one company now that has the ability to do 63 together and is charging $125 a sample. I won't give an advertisement. So, the technology is ramping up very quickly in this area.

Okay, our next speaker is Paul Hayashi. Everybody calls him Skip. He is an associate professor of medicine. At the University of North Carolina, he is a hepatologist. He has also been a critical worker in the Drug-Induced Liver Injury Network in our almost 40 publications. If you track over time, Skip has moved up the author list right up to the front. And I have sort of drifted back. I think we passed about a year and a half ago in the thing. He is going to talk to us about one application of the incredible DILIN database that has some regulatory implications. Where am I here? Oh, yes, DILIN experience with Hy's Law in patients with preexisting liver disease. Skip.

Hayashi photo, biosketch, abstract

PH#1: Thank you very much. First of all, I thank John Senior for inviting me and Paul, of course. I have no financial disclosures. I will disclose that, as Paul said, I am a clinician. So, I am not well versed in the ways of the FDA or industry but I am learning a lot. If I say something stupid about your field of interest, please step up to the mike and publicly humiliate me in front of my peers. I will try not to take it personally.

Paul and John asked me to talk about this and it was really an exciting question and I realized there are absolutely no data in this area. So, that is good and bad. It means that the background is bad and there is not much to say in terms of background but I will do the best I can.

PH#2: This is the outline. I will be talking about Hy's Law and backtracking just a little bit, with a few slides about making sure we all have it right and we know what we are talking about in

regard to Hy's Law and its derivations. I'll talk a little bit about the track record, which has been alluded to here quite a bit in the past two days. And then quickly go into sort of chronic liver disease outcomes in relations to Hy's Law and in the DILIN experience. And then lastly, let us look at the new data that we just started putting together in the last several months. It is very preliminary but it will be getting right at the question that I have been asked to address: Hy's Law in chronic liver disease within DILIN.

PH#3: First of all, I thought it was probably appropriate to go back to the man himself, in his last addition of his textbook. And this is what he said: "Drug-induced hepatocellular jaundice is a serious entity. The mortality rate is from 10 to 50 percent." We have seen that a lot. On the facing page, there is actually a table that I slimmed it down quite a bit, but he did put parameters on the enzymes. The AST and ALT were 3 to 50 times the upper limit of normal for

hepatocellular injury, and he did put parameters on the alk phos, which was less than one to three times the upper limit of normal. You notice that he did not put any parameters on jaundice. It was a clinical call there.

PH#4: This is Hy's Law according to the FDA and this is lifted straight from their guidance for industry.

PH#5: So the AST and ALT are again, greater than three times, bilirubin there they did put a hard stop parameter of two times the upper limit of normal but they did not with alk phos.

PH#6: Basically they just say initial findings of cholestasis elevated serum alk phosphase and no further guidance there. And then there is obviously no reason for other liver biochemistries to get at causality here.

PH#7: So, these are Hy's Law's other derivations. This is the top one which is our DILIN group and this is what we have used when we looked at this. Again, the ALT and bilirubin look

very familiar. We do put a hard stop at alk phos less than two times the upper limit of normal. I also put up the Spanish and South American DILI Registry. They used a little bit different in two things. This is their most recent paper which I am sure some of the authors are out there and this was published last year. ALT and bilirubin are, again, the same. But they either used excluding other cholestatic causes but then they also used a new derivation which is incorporating the R-value. And here what they did was they took the AST or ALT, whichever was higher, and they put it times the upper limit of normal divided by the alk phos times the upper limit of normal and it had to be greater than five. And so they make the argument the alk phos sets a stand alone could probably be done away with and if you could just use the R-value. And their performance, at least in their study was better. Their RC curves were better for this, as opposed to a straightforward Hy's Law.

PH#8: So what about the track record in drug trials? This was shown yesterday quite a bit. I won't go into it much. This is bromfenac, troglitazone, and ximelagatran. So, these are sort of triumphs of Hy's Law that seem to pan out for post-marketing for the first two and then, obviously, the first one was not approved but later withdrawn from other markets.

PH#9: What about in registries? Hy's Law does very well in all the registries, really. This is the DILIN experience, 13.4 percent if you met Hy's Law in a hepatocellular injury, you had a 13.4 percent positive predicted value that you were not going to do well. That was mortality as an outcome.

Now the Spanish/South American Registry is very similar. Again, I told you they use two different models. They used a straightforward Hy's Law but also this modified one where they used the R-value. And there again it is between 8 and 9.6 percent.

I do want to point out one nuance here. You know in the DILIN we use mortality. But if you read the paper carefully in the second one, they use mortality but they also use acute liver injury. In other words, bad synthetic dysfunction. And I will come back to that. I think that is important. You know, what are we defining as a bad outcome? It is a little different between the two.

And then the Swedish Adverse Drug Reactions, Dr. Bjornsson's registry out there. It shows you the number. This is how Hy's Law is panning out. It is somewhere around 10 percent, give or take a couple percentage points.

PH#10: A word about chronic liver disease in DILI. Again, going back to what Dr. Zimmerman said, it is remarkable how much what he said did pan out over time. He said that there as a stubborn misconception that susceptibility was higher in patients with chronic liver disease. And he also said that addition to DILI to chronic liver disease

would be troublesome. I get the feeling that is the general feeling across the field.

There are some data to support it. For example, the statin data suggest no increase in susceptibility, but on the other hand, there are some data that suggests that maybe it is a problem, for example in TB. When you monitor for TB, it is different. If you have chronic liver disease, you are monitoring ALTs. Or if you don't you are just going on symptoms.

Before I go into some of the newer data that we have in relation to Hy's Law in our chronic liver disease patients, it is good to review what we do in DILIN and what comes out adjudication. Basically, three of our members get together and independently score these cases and then come to consensus. This is the scoring system. I just want to go over it real quickly again. One is definite, greater than 95 percent likelihood beyond reasonable doubt that this is DILI. Two, highly likely, 75 to 95 percent, and probable 50 to 74

percent, based on the legal language in a court of law. So, basically, what we would say is that three or better would be enough to convict here. I highlight those because the rest of this data, just keep in mind, will be only dealing with cases that met those scores.

PH#11: I will just talk a little bit about some backdrop data. This is the idiosyncratic DILIN experience within the first six months, morbidity and mortality. And I just want to give you an idea. We do have a measurable rate of bad outcomes. And these were 660 DILI cases, a six-month follow-up. We have the survival curves based on three different groups. Basically, liver transplant is the worst group, the solid black line. And we did a fair number fairly early on. Liver-related death is the next line, And then non-liver-related death is the line that lingers out a little bit longer. And I suspect those are a lot of the cancer patients.

PH#12: Within this study, there are some hints that Hy's Law is still a player or helpful. We didn't break it out in this paper, as I will in a minute. But basically, preexisting liver disease was more common in those who had a death or transplant outcome. As you can see, 24 percent versus 11 percent. And if you restricted it just to liver-related death or transplant, again, it was statistically significantly higher for those with preexisting liver disease.

Again, making Dr. Zimmerman's comment that it would be troublesome seem to be somewhat true here. Now, Hy's Law was also more common in those with death or transplant outcome. Again, 46 to 26 percent and if you just restricted it to liver-related death or transplant, it was 53 to 26 percent, both statistically significant.

Now, when we looked at it as a multivariate model, both chronic liver disease and Hy's Law fell out of the multivariate model but I have to say there is a lot of collinearity here. Because you

can see for Hy's Law, for example, ALT and bilirubin both stayed in the model. And for chronic liver disease, low platelets and low albumin both stayed in the model. So, I suspect if you took them out, then Hy's Law would slip back in and so would chronic liver disease.

PH#13: Okay, so this is the preliminary data that we have predicting fatal outcome in Hy's Law. This is a cohort of now 894 patients, again, all definite, highly likely, or probable. And what I did was we looked at two groups, obviously, those with chronic liver disease going into the DILI and those without chronic liver disease. I later will subgroup them as viral hepatitis and, as best as we can tell, NAFLD and unexplained elevated liver biochemistries.

PH#14: So, in the outcomes, this is where I come back to this. Now, in this analysis, I did do it both ways. Four is just actually you start to show liver failure; you develop ascites, encephalopathy, but you make it; you don't get

transplanted. You survive it. Five, of course, is death or transplant. I did a model on both but I am only going to show you the five data.

PH#15: These are deaths or transplant. And I did it two different ways. All-cause, any time during follow-up. So, 1: you get transplanted anytime or die for no reason. And then 2: liver-related death within six months of transplant within six month.

PH#16: Demographic,clinical characteristics: it is a busy slide. But I will just highlight the fact that there really was no difference between a non-fatal, fatal, and total, except for age. As you might expect, the fatal group was a little bit older.

And then as far as chronic liver disease, individually, there was no real statistical difference. Even Hy's Law did not necessarily meet statistical significance, when we looked at liver-related within six months or liver transplant within six months.

PH#17: If I expand it a little bit to follow-up at any time, death or liver transplant at any time, then Hy's Law does come back and is statistically significantly higher. So, again, if you got transplanted in month seven, I don't know, then that may be clinically significant or maybe that should be in there as a predictor for Hy's Law. Okay, what I am going to show you next is a series of slides. They are all going to show the same thing as the tables. And I left the tables and numbers in because I think it is important for you know our numbers. They are not huge. They are bigger than whatever is out there. But as you can see, the numbers will whittle down as I go down and the outcomes change a little bit.

PH18: This is total cohort all-cause mortality. So, again, you could die for any reason, liver transplant at anytime during follow-up. So, just overall, again, comes out to about where Hy's Law would say, about 11 percent,

the positive predicted value. And you can look at the numbers there in the two-by-two.

PH#19: So what about no chronic liver disease? Here about pretty close, similar. Again, all-cause mortality, liver transplant anytime and this is 9.5 percent positive predicted value.

PH#20: When we restricted it to chronic liver disease patients, this is where we took a big jump. So, this would suggest that Hy's Law is of some worth. Again, all-cause mortality, anytime during follow-up and liver transplant. This was a positive predicted value of 24 percent. Of course, the numbers are smaller. We had a total, in this analysis, we had 79 that had preexisting chronic liver disease. But again, the positive predicted value of the total 25 was 24 percent.

PH#21: Then I put this as a final summary slide. Again, I wanted to show you the numbers but this is a summary of the last three slides I just showed you. All cohort, all-cause mortality, liver transplant at any time, 11 percent. But then

for chronic and non-chronic liver disease, it was 9 percent versus 24 percent for chronic liver disease.

PH#22: So, what about total cohort and liver-related death? So this is, again, this is a little different. We are restricting it on the other end of the scale. We are going to say that it is a death within six months that we feel is liver-related or a liver transplant within six months. And here the positive predicted value goes down a little bit. As I said, you might transplant somebody or have somebody die at six or eight months; they won't be in this outcome.

So, here 6.1 percent -- the numbers are pretty big because this is a total cohort -- positive predicted value.

PH#23: And here it is for no chronic liver disease. We had no fatty-liver, as we know. We had no viral hepatitis. Again, a liver-related outcome within -- bad liver-related outcome within

six months. Positive predicted value, again, is down to 5 percent.

PH#24: And then, of course, what about chronic liver disease patients? Well, it stayed up there. Again, the numbers are small or smaller, I should say. But again, the number was still up to 16 percent for a short-term bad liver-related outcome.

PH#25: So, again, summarizing that. This is, again, a liver-related outcome in a short-term interval. Bad outcome is 6.1 percent for the total, 5 percent for the non-chronic liver disease, and 16 percent for the chronic liver disease.

PH#26: So, a lot of people would be interested in what about viral hep versus fatty-liver. I did break it out for, again, the six-month outcomes. And it was 15.4 percent positive predicted value for patients with either hep C or hep B.

PH#27: And then NAFLD or unexplained elevated liver enzymes, the positive predicted value is 8.3 percent.

PH#28: This is the summary, again, for those two groups. As you can see for the biohepatitis group, it is a little bit higher. Well, a fair amount higher but the numbers are even small.

PH#29: In summary, patients with a fatal outcome in the U.S. DILIN cohort tended to have more baseline chronic liver disease and have more cases fitting Hy's Law. That is in Bob Fontana's paper that came out last year.

So, with those with chronic liver disease, so it is Hy's Law has a positive predicted value of 24 percent for all-cause anytime fatality or transplant. Hy's Law had a positive predicted value of 16 percent for liver-related deaths or transplant within six months. And both of these positive predicted values were higher compared to those without chronic liver disease.

The positive predicted value for viral hepatitis patients may be higher than that, but I caution you that the numbers will get pretty darn small there.

PH#30: The conclusions from this very preliminary data say that Hy's Law may have a predictive value for fatality or transplant patients with chronic liver disease than those without. Whether or how this translates into overall incidence and risk for acute liver failure in a drug trial using chronic liver disease subjects is unclear, but suggests a continuing role for Hy's Law.

Further research should focus on validations of these findings in other cohorts and maybe adjusting Hy's Laws parameters. Because if it is even more predictive, then maybe the parameters need to be dialed in a little differently. The caveats here, this is preliminary data. We were just looking at this data. I have not looked. For example, there is hep B. What does that mean? Were they hep B carriers? Were they active. We have not broken that data out. The hep C, were they treated? Probably not. Most of these were the

pre-oral agent era. But again, we haven't broken all that out.

And the last thing is this death causality. I will mention that. I think we are looking at some cases and it is another parameter. I have heard a lot about how we have to set standards up but how do you tribute the death to the drug, when you have a liver go down? So, I will give you an example. For example, we have had a case of DRESS. The patient died but at the time of death, the liver was sort of on the mend. Now, is that a liver-related death or not? Things like that are a little more nuanced and we are taking that on to look at it that more closely. And that may change what I have shown here for positive predicted values but I don't think greatly.

I want to thank everybody from the DILIN group, and especially Sherry Gu, who is in the upper right-hand corner of the picture. She is our statistician who put all this together for me today. Thank you.

Discussion Session IVA-2

DR. WATKINS: All right, we are a little bit in danger of going over here. Is there a burning question in the audience? One thing I will say is that, obviously, this is an extraordinarily rich dataset. We not only have an ancillary study's process that industry can participate in but we also still have, through the Foundation of the NIH a way for companies to contribute to the DILIN effort and give us money to do further analyses like this. And if you have any questions, you can contact me or Jose. Where is Jose? There he is, over there. I'm sure he would be happy to take your money.

Any other thoughts here? Arie, why don't you go to the mike. It is a very interesting issue with viral hepatitis studies and NASH studies where all of a sudden somebody develops ALT greater than three times, bilirubin greater than two times and the party line has been we don't know what to make of that because Hy's Law only applies to healthy

livers. This isn't an identical situation because you are not curing things. You are not moving inflammatory cells in and out of the liver, et cetera, but it is a sobering message that, in fact, the significance of a Hy's Law case may not be less than in a healthy liver. In fact, it may be worse. Arie.

DR. REGEV: So, to expand on what you started to say, I think there is a potential issue with using Hy's Law in patients with preexisting liver disease, since the problem with the definition of Hy's Law is no other cause for the abnormality in ALT. And this, especially, I think, is important when you try to use it as a predictor in datasets of term development. I think it potentially may create absurd situations. If you use Hy's Law on the UNOUS database transplant list, it will have 100 percent success. There is no problem. It is very predictive of mortality in liver transplant. So, I think we should be careful when use Hy's Law

in patients that have another reason for the ALT and bilirubin increase.

DR. HAYASHI: Sure, and that goes back to causality. And you are absolutely right. The cases, I hope the cases we have in there are reasonably clean for causality. But you are absolutely right.

DR. WATKINS: Dr. Kirby has the last question.

DR. KIRBY: I may have missed it, but did you provide some information about the severity of liver disease in terms of MELD score?

DR. HAYASHI: Well, we do have that data. We haven't crunched that out, as I alluded to. It is sort of a mixed bag of what the chronic hep C patients were doing. My general impression is we didn't have a lot of patients that were having MELD scores and things like that. But yes, we have to look at that.

Discussion Session IVA-3

DR. WATKINS: Round of applause. (Applause) All right, our next presenter is Tom Urban, who is an assistant professor, joint appointment between our institute and the University of North Carolina, and has really been a leader in the last seven years or so in ferreting out the genetics of susceptibility of drug-induced injury certainly in the DILIN network but also in the first and now the ongoing collaboration with the Severity Adverse Event Consortium. And he is going to give us the latest on what has been found. Tom.

Urban photo, biosketch, abstract

TU#1: Thanks, Paul, a*nd* I want to thank John Senior for giving me the opportunity to talk here today. This is a meeting that has been on my calendar every March for the past five years. In my previous post at Duke University, I had a course that I taught in the spring that basically kept me homebound every March. So, this is actually the

first time I have been able to attend, since the last time I talked here. And good timing because we actually have, I think, some new and very exciting data to present that probably would not have been available over the past five years.

So, I added a couple of words to the title of the talk, "…in humans." And that is becauselater in the session we are going to hear from others talking about different types of approaches using animal models or cell culture models of DILI that will complement what we find in humans.

TU#2: I am going to focus here on what are the susceptibility factors that we can identify in living, breathing patients that have experienced drug-induced liver injury. And I will start by saying that none of what I am about to present would be possible at all without the tireless and educated efforts of a lot of clinician scientists across the U.S. and across the world. We have heard about the Drug-induced Liver Injury Network here in the U.S., sponsored by the NIDDK. In

addition, the International DILI Consortium headed up by Ann Daly and Guru Aithal in the UK and lots of contributors, some of whom are here today, putting together these large patient cohorts of DILI cases that really are necessary to do the type of genome-wide work that we like to do.

TU#3: I think that many of you are familiar with the idea of an genome-wide association study but just to briefly get everybody on the same page, a GWAS, a genome-wide association study, is an attempt to find common genetic variants in the genome that associate with whatever trait of interest you are looking at and typically, these require fairly large sample sizes or fairly large affect sizes or both. And there are a number of examples of successful genome-wide association studies in the field of drug-induced liver injury. I think most famously in 2009 with the publication by Ann Daly and colleagues, showing the very strong association between the HLA-B*5701 and risk for liver injury due to flucloxacillin.

We heard a little bit yesterday about lumiracoxib and I will try to talk a little bit about that and how that is kind of a unique example of genetic susceptibility for DILI and amoxicillin, clavulanic acid, and others. And a lot of these studies are only possible, again, because we have been able to put together large cohorts of patients that have had injury due to not just drugs but collections of patients with liver injury due to the same drug.

TU#4: And so HLA seems to be a sort of common factor that we see associated with risk for DILI with different drugs and often with different HLA risk alleles associated with DILI due to different drugs. And there is some overlap in terms of the risk alleles associated with, for example, amoxicillin, clavulanic acid, and lumiracoxib or between the lapatimib and ximelogatran. But largely, what we see is when we find a new HLA association, it is specific to a particular drug. And the effect the size, the odds ratio associated

with carriage of a particular HLA type is often very different between drugs.

TU#5: What I am going to present today are the results of what we called the Phase 2 Meta-GWAS. This is a collaboration between, as Paul mentioned, the DILIN and the International Serious Adverse Events Consortium and iDILIC, where we were able to put together a cohort of over 1500 patients with drug-induced liver injury due to a variety of drugs. And the first thing that we try to do is say okay, are there any genetic variants in the genome that predisposed to risk for DILI, regardless of what drug the patient took. Are there sort of intrinsic DILI risk factors? And performed the same experiment back in 2012 and the answer was no, we can't find any such common -- any such variants that predisposed to risk across different drugs, different classes of drugs.

TU#6: Recently, what we found is that in fact there is a particular HLA association that shows a genome-wide significant association with what we

called all-cause or omnibus DILI. So, this is after excluding DILI cases due to flucloxacillin and amoxicillin, clavulanic acid, where we know there are HLA risk alleles with very strong effects. Yet, we still see the signal in the HLA region. In particular, HLA-A*3301 seems to show a very strong association with DILI, regardless of drug. And this is an allele that has not previously been associated with drug-induced liver injury nor any drug-related hypersensitivity reaction.

Of course, the next obvious thing to do is to ask well, what have we done here. We have pooled a bunch of patients with injury due to lots of different drugs. Might there be one or two or three drugs that are really driving the association and maybe the rest of the cases might actually be diluting that signal? And it turns out, at least for one drug that we are pretty sure about and a couple other drugs where we are less convinced that

terbinafine, in particular, does seem to be driving the majority of that association.

TU#7: If you look at just the 14 cases of DILI due to terbinafine that we have on hand, we see an even stronger association with this HLA-A*3301 with an odds ratio now of around 40 compared to around 2.5 for all-cause DILI. So, we have what looks like mostly a drug-specific risk allele, a new HLA risk factor that hadn't previously been associated with any adverse drug reaction.

TU#8: And then the next question we might want to ask is is it all just terbinafine. So, we found the association when we lumped all of the cases together. We found that terbinafine seemed to be contributing the most to that association but is there still a residual signal once we remove the terbinafine cases. And the answer is yes. So, we see for the same HLA-A*3301 allele, an odds ratio of only around 2.3 but clearly, statistically significant association with any drug.

And the question then, one that we haven't answered and that I don't have any slides to support is whether there is some cryptic combination of drugs that might explain that residual association. Is it truly the case that all individuals that carry 3301 are at high risk of DILI, regardless of drug or is it that there are certain drugs where this is a risk factor and we just don't have the power to identify them individually? So, that work is ongoing.

TU#9: Another exciting result from these recent studies is that we have found some relatively rare HLA types that appear to be risk factors for DILI due to individual drugs. And I will focus mostly on minocycline, where we find that the HLA-B*3502 allele, which has a population frequency of less than one percent is enriched to around eight percent individuals that have experienced DILI due to minocycline. So, the odds ratio there is around 30. We have virtually no doubt that this is a true association. What we

don't know is -- well, we don't know the mechanism, clearly. All we have right now is an association. We actually aren't really clear whether it is HLA-B*3502 that is responsible for the association. So, all of the stuff that I talked about with HLA associations actually are based on impugning or estimating HLA carrier status, based on SNP genotype data.

TU#10: So, we have genotyped patients for common SNPs across the genome, including lots and lots of SNPs in the region around these HLA genes. And then based on what we know from reference populations, where we have both HLA sequence-based types, and SNP genotypes, tried to assign HLA types our cases, based on SNP genotype data. So, that is different from actually sequencing the HLA genes in each of these individuals, which would be the sort of gold standard for HLA typing. So, that is the very next thing that we plan to do is to make ourselves certain that it is, in this example, HLA-B*3502 that is actually enriched in the

minocycline cases and not some other HLA type or combination of HLA types.

TU#11: We heard from Mark Avigan earlier about the HLA genes and their role in drug-induced hypersensitivity reactions. To remind everyone what these genes do, there are basically two classes of HLA genes: Class I which comprise HLA-A, B, and C are expressed on virtually all cell types; and class II genes, the DR, DQ, and DP genes expressed primarily on antigen-presenting cells. In both cases, their role is to present small peptides, usually 9 to 12 amino acid peptides, to T cells for immune recognition. And the thought is that a lot of these associations are probably explained by an inappropriate presentation of a drug peptide complex or the drug itself may change the repertoire of peptides that are presented by these HLA genes. And there has been a lot of really exciting work that has been done recently, primarily Dean Nesbitt at Liverpool and David Ostrov at the University of Florida. And what has

been seen for one of the most famous pharmacogenetic associations, the association between HLA-B*5701 and abacavir hypersensitivity reactions is that the drug can enter the binding cleft of the HLA protein so that the part of the HLA molecule that is responsible for presenting antigens for immune recognition by the drug binding in that cleft, that can change the types of self-peptides that are also bound and presented by those HLA proteins. What you have is a system where peptides that previously would not be presented as antigens on the cell surface now become "neoantigens" and, at least for abacavir, that is thought to be the direct mechanism for these immune-mediated hypersensitivity reactions.

For drug-induced liver injury we actually don't know how this works and the one example where there has been similar work done, flucloxicillin and the same HLA type, HLA-B*5701, it looks like the mechanism is not the same as what we see for abacavir, that there probably is actually a drug

peptide complex that presented. And that is the neoantigen. But if you think about it, what we don't know is how to generalize the information that we have about HLA associations with adverse drug reactions. We have abacavir, B*5701; allopurinol, B*5801; carbamazepine, Stevens-Johnson Syndrome and B*1502. On their own, these are anecdotes, but if you start to collect information across different adverse drug reactions, different HLA types, different drugs, you may be able to construct a model or a set of rules that would tell you, okay, among patients taking drugs with this type of structure that carry this HLA allele, the risk for some kind of immune-mediated adverse event is likely to be higher than others. And I think that is probably the ultimate goal or what hopefully will come out of all of this is a general sort of model for understanding the relationship between HLA and adverse drug reactions.

TU#12: Beyond HLA, we have also found some interesting genetic associations that are less

easy to interpret but are also exciting in their novelty. So, when we look at cases of DILI due to the combination sulfamethoxazole trimethoprim, we see a very strong signal association on the short arm of chromosome 9. And this is, to my knowledge, the first example of a genome-wide association study showing a result outside of the HLA genes. The difficulty here is that this is a common SNP that is intergenic and that is probably is an understatement. This is a SNP that is probably about half a million base pairs away from any known protein coding gene. So, how this actually works, we don't quite know. But we are looking forward to performing some studies to follow-up on that.

TU#13: To wrap up, there are some ongoing studies using Next-Gen sequencing whole genome, whole exome sequencing to try to identify rare variants that may be predictive of drug-induced liver injury. As a transition to the next few talks,

TU#14: I think understanding the mechanisms of drug-induced liver injury will help us to better

interpret the genetic data that we have in humans, to try to find clinical predictors of DILI. And that can then feed back into mechanistic studies of those genes. And so I see this as kind of a cycle of increasing our knowledge of DILI mechanisms.

TU#15: So, thanks.

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Discussion Session IVA-4

DR. WATKINS: Great, thanks. I realize that I have been a horrible moderator and Merrie has like 60 seconds to give her talk. How many people in the audience right now are willing to stay until 4:30 instead of 4:00?

Okay, I am afraid we can't take questions for Tom, but I know there are some people he would love to come to the mike. I think if you can stick around or email him, we will have to just deal with it that way.

Our next speaker, is Merrie Mosedale. She is a research investigator at our Institute. She heads up our mouse genetic program but she is also really the major coordinator and director of a very large research project we have that involve scientists not just at our Institute but at other academic center and particularly Otsuka, with Sharin Roth and Bill Brock, who are in the audience today. So, Merrie, tell us about it.

Mosedale photo, biosketch, abstract

MM#1: It is bad to be starting your talk when the session is supposed to be ending here but I will try to go through it quickly.

I am going to tell you today about the Tolvaptan Initiative, which is an effort to identify a personalized DILI risk management strategy.

MM#2: Tolvaptan is a vasopressin antagonist developed by Otsuka, already approved for the treatment of hyponatremia. It is a candidate as well for the treatment of autosomal dominate polycystic kidney disease. Unfortunately, liver injury was associated with tolvaptan during clinical trials, and about 4% of patients taking the drug developed ALT elevations greater than three times upper limit of normal, and there were three Hy's Law cases. So, FDA approval for this indication has not yet been received. I show this figure here, which is LFT plots that I know a lot of you are familiar with. So, I won't describe it

in detail. I just want to draw your attention to the ALT values in black. And the gray shading indicates where this particular patient was on drug. This is the time course of a liver response for an actual tolvaptan-treated patient. I want to point out that this patient was on drug for several months before there were any elevations in ALT. Then after the drug was stopped and the ALT values returned to normal, when the patient was re-challenged with the drug, ALT elevations occurred much faster during the second exposure. This kind of profile is suggestive of an involvement of an adaptive immune attack as sort of the critical event promoting the liver injury. There is quite a bit of evidence to support the role of the adaptive immune system in these liver injury profiles, including, as we had just heard from Dr. Urban, really strong genetic associations between susceptibility to these kinds of liver injuries and the HLA region of the genome. Demonstrated HLA risk allele associations have not been clinically

useful in risk management. We believe this is because there are actually unaccounted for susceptibility factors and a risk that occurs at the level of the liver.

MM#3: Illustrating the steps here, where drug elicits some hepatocyte stress. This results in an innate immune response and release of danger signals that, in combination with the adaptive immune attack, are actually responsible for the liver injury. But non-HLA risk alleles have not been clinically useful in DILI risk management. We believe there is need for both genetic and non-genetic biomarkers in order to develop a personalized medicine strategy. While it was unfortunate that liver injury was observed in the clinical trials for tolvaptan, one really positive thing to come out of this was that Otsuka was really diligent in collecting samples from patients in the trials, including genomic DNA. Plasma and urine were collected at baseline, at three weeks, and then annually for up to three years on drug

treatment, from both controls and cases in people that experienced the liver injury.

MM#4: Examples of sample collection from the cases are illustrated in the figure on this slide. And you can see there was plasma and urine collected at baseline, on three weeks on drug but before there was any sort of liver injury, and then also at the time of event. And then for all the cases, there is a DILI causality assessment by five hepatologists. Given this really rich sample set and the kind of tools and approaches, we realized this would be a great opportunity for us at the IDSS to collaborate with Otsuka, as well as their other partners, to identify a personalized medicine strategy for tolvaptan.

MM#5: Objectives of the Tolvaptan Initiative are to manage the risk of DILI in tolvaptan-treated patients through the identification of both genetic and non-genetic risk factors for tolvaptan-induced liver injury and to provide a mechanistic understanding of the tolvaptan

toxicity, in order to further direct discovery efforts and to provide biological plausibility for any empirically-derived biomarkers.

MM#6: The integrative approaches that we are using to develop this strategy really begin with the clinical data and samples collected from the patients in the clinical trials, where more unbiased approaches have been taken, such as metabolomics and genetic analyses to identify risk factors associated with susceptibility to the liver response. We are also coupling these unbiased approaches with more targeted approaches. For instance, using in vitro models to identify the activation of stress response pathways in primary human hepatocytes exposed to tolvaptan. We are also using some cutting edge genetically diverse mouse population models. And then we are taking data from all of these different approaches, including some others, and using it to guide the development of a computational model for tolvaptan-induced liver injury, using the DILIsym software. But what is

really cool about this approach is that we are taking data from all of these different studies and actually then using it to guide a targeted hypothesis base approach to biomarker discovery in the clinical data and samples collected from the patients in these trials.

I don't have time to tell you about all the different studies today. In fact, I feel like I barely have time to tell you about the mouse population-based approach we are using but that is what I am going to talk about mostly. Some of you may know that at the Hamner we have been working for a while with genetically diverse populations, which have allowed us better to model adverse responses observed in humans, even when there is no toxicity observed in traditional non-clinical models, as was the case for tolvaptan.

MM#7: But recently, we have transitioned to working with the next generation of these genetically diverse mouse populations, a genetic reference population called the Collaborative

Cross. The Collaborative Cross is a superior resource for this kind of work because of the rationally designed breeding scheme that has been used to develop this population. It has resulted into just a really extremely diverse population of mice and this allows us to not only model these kinds of toxicities that are observed in humans but also do high resolution genetic mapping to identify risk factors and to study mechanisms that are associated with the toxicity susceptibility. We have been fortunate to work with this population that is currently only available through UNC. And we have hypothesized for this work that evaluating the liver response to tolvaptan in a genetically diverse population like the Collaborative Cross could allow us to identify sensitive strains, which could be used to both study mechanisms and identify risk factors for tolvaptan DILI.

MM#8: One other point I want to make here before showing data from this study, is just going back to this figure I showed earlier. As you heard

this morning from Dr. Uetrecht, it is difficult to model the adaptive immune response in non-clinical models. So, we are actually focusing on evaluating these very early events, the hepatocyte stress and potentially innate immune response. But we believe these initial events may not actually involve cell death or hepatocyte death. So, we may not see a response by measuring traditional non-clinical markers alone, markers like ALT. What we have learned that liver gene expression profiling, after an acute high-dose exposure of a drug can actually be able to be used to identify these very early events, even in the absence of overt toxicity. For this study here, we are actually combining a mouse population-based approach with toxicogenomics to identify mechanisms and risk factors associated with the toxicity.

MM#9: This is the study design here. We treated 45 Collaborative Cross strains, eight male mice per strain; four getting vehicle and four

getting tolvaptan, with just a single dose. And then 24 hours later, we necropsy the animals. I want to make the point that the dose of tolvaptan that we are using is 100 mgs per kg. The human equivalent dose in AUC for this dose in a mouse is actually not that different from the dose used in the clinical studies. At necropsy, just 24 hours after this single dose, these are the endpoints that we are measuring. So, after the single dose of tolvaptan, we weren't expecting to see liver injury by measuring traditional biomarkers like ALT alone. But I think as you can appreciate here, we did see elevations in ALT in three of these 45 strains. We also did histology. Not surprisingly, we didn't see any changes after just 24 hours.

MM#10: We did find that these ALT elevations were well-correlated with AST and miR-122. We did a global gene expression profiling in the liver of all of these animals. First we looked at were gene expression changes that were associated with treatment across all of the strains, independent

of a liver response. And you can see here in those genes we found enrichment of pathways that were suggestive of mitochondrial dysfunction. We also looked for gene expression changes that were associated or correlated actually with the ALT fold change. And here we found enrichment of pathways suggesting some alterations in bile acide homeostasis.

MM#11: And then we looked for gene expression changes that were not only associated with treatment but that would differentiate our resistant and sensitive genes. And the most significant gene to come out of this analysis was actually a gene that is involved in the loss of immune tolerance. The really cool thing about this gene here is that the protein product produced from this gene gets secreted in the liver. It goes into circulation and it may be a serum biomarker.

MM#12: We also did QTL mapping, using ALT fold change. And I know you have seen a bunch of these Manhattan plots in the last talk, so I won't

describe what this is here. I just want to point out that the strongest genetic association we saw was on chromosome 14. We looked at the genes within the interval on chromosome 14 and narrowed it down to about six high priority candidates, some of which have a biological relevance in showing some association with apoptosis and innate immune response.

MM#13: I know I went through this quickly. I will just summarize the major findings from this work. A tolvaptan-induced liver response was observed in three of the Collaborate Cross strains. So, now we have animal models for additional mechanistic experiments. Our toxicogenomics work identified some treatment-induced stress response pathways that occurred across all strains in response to the treatment and some that were specific just to the sensitive strains.

We did QTL mapping and were able to identify some genetic associations with the susceptibility.And all of this was discovered with

just this single dose of tolvaptan that is comparable to that used in the clinical trials.

Going back to this figure one last time here, I just wanted to point out that we saw some evidence for mitochondrial toxicity and bile acid toxicity, apoptosis, and loss of immune tolerance. We have identified both genetic and non-genetic biomarkers and these will now go on to guide a hypothesis-based approach to biomarker discovery in the samples collected from the clinical studies.

MM#14: This illustrates that point here. I told you about the cutting edge preclinical models. But we are generating this kind of data from all of the approaches that we are including in this initiative. And all of this data is coming together and is being used to guide a really hypothesis-based approach to biomarker discovery

in the clinical data and samples from the tolvaptan studies. I think I have shown you that we have really transitioned from using these approaches to explain problems to now, hopefully, solving them. And we have learned a lot about how to do this work now and we believe that we can do this kind of study, a Collaborative Cross study, as well as some of the other approaches that I wasn't able to tell you about today, in as little as six months.

MM#15: There are a lot of people to thank that are part of this effort. And before Paul cuts me off here, I will just thank a few people that are in the audience today: Paul, who directs our Institute; some other folks like Dr. Urban, who is heading up the genetics work; Brett Howard, head of the DILIsym team; and then our partners from Otsuka, mostly Dr. Bill Brock and Sharin Roth, who have been extremely helpful in doing this work.

MM#16: So, thank you very much.

Moderators: Session IVB

DR. WATKINS: Great, thanks, Merrie. Yes, I know I have been a horrible moderator here letting things get so far over time. How many people here feel they absolutely need a break right now, versus just charging into the next session and staying on time? I will check to make sure the refreshments are going to stay out there but I think we should just head on to the next one.

(Refreshment break deleted)

DR. SZABO: Okay, I think we saved some of the most interesting things for last. So, I would like to invite Dr. Dan Antoine from University of Liverpool to talk about HMGB1 variations that determine DILI, whether it is benign or dangerous.

Antoine photo, biosketch, abstract

DA#1: Thank you very much and thanks to the organizing committee for the opportunity to come and present some of the work here to you today. And thank everyone for sticking around this afternoon to listen to the talks that we have to present.

As you know, I am based at the MRC Centre for Drug Safety Science at the University of Liverpool. I work with Kevin Park. We have a great interest in the development of biomarkers that we can utilize to understand the mechanistic basis of drug-induced liver injury, and to provide tools that we can use to assist our understanding of drug-induced liver injury, alongside the currently used standards.

DA#2: When I think about the development of biomarkers from my point of view, I am looking at some of the challenges and unmet needs that we have. We need to develop biomarkers with improved hepatic specificity, about which we have already seen some excellent work presented by Dr. Szabo, looking at miR-122. We need to develop biomarkers for an enhanced mechanistic understanding, particularly in that translational space, so that we can work between animals and humans to try to understand DILI better; and earlier identification of

drug-induced liver injury. I discussed that last year, so I am not going to touch on that today.

The focus of my talk today is really going to be biomarkers that are linked to mechanisms that we can really utilize to understand patient responses a lot better. And by that, I mean looking at patient outcomes and prognosis but also differentiating between benign changes and ALT activity and serious drug-induced liver injury. From my mind, to try and really understand that a lot better, to develop biomarkers associated with that, you have to really understand the mechanistic basis a lot better of drug-induced liver injury.

DA#3: I want to introduce you to one of my personal favorite biomarkers. I know you are not supposed to have favorites but I do. And this is High Mobility Group Box-1. I have an interest in HMGB1 because it acts as a dominant associated molecular patent protein. It links necrotic cell death to the activation of the immune response. And it does that by acting as a chemokine or as a

cytokine for toll-like receptors, in particular TLR4, and CXCR4, and also the receptor for advanced glycation end products.

DA#4: With respect to understanding its utility as a biomarker, we know that it can come out from the cell in a number of different ways. There is a passive release during a necrotic response. It also can be actively secreted from cells, particularly immune cells. And that requests a set of key lysine residues within its nuclear localization sequence. And I have just highlighted some of those on that schematic across the bottom of the screen of the various structural domains of HMGB1.

DA#5: Very interestingly, HMGB1 has three sustained residues, only three sustained residues and each is very important for its function. They are modulated by post-translational redox dependent modifications and it has a profound impact on its function as an inflammatory mediator and I am going to discuss that a bit alter during

the course of the presentation. We looked at HMGB1 as a biomarker in the paracetamol and overdose model in a mouse. And what we did is we initially tracked its progression from the loss and the release from the centrilobular region following necrosis, following paracetamol treatment, to its appearance in blood.

All this sounds quite a straightforward and an easy concept but it has not been actually presented in the literature, tracking the biomarker from the tissue to the periphery. We also looked at identifying the two different molecular forms in our animal model of paracetamol overdose. If you remember, I told you that two distinct molecule forms, which correlate with the mechanism of release is the hypo-acetylated form, which is shown in green, which is indicative of a necrotic response and the hyper-acetylated version of HMGB1, which gives us an indication of an active immune response. We were able to develop and validate a mouse-based approach to identify and

quantify these different isoforms of HMGB1 in blood. And what you can see from the data on the bottom right-hand side of the screen shown in green is the necrotic version of HMGB1, followed by a release of the inflammatory version of HMGB1. And essentially, what we see in mice is we see by indication of these two biomarkers, a biphasic response. We see necrosis, followed by inflammation.

DA#6: Of course, we are very interested to see if these observations hold true in man. And of course what you can see there on the left-hand side it he data from the mice. We further developed this assay to quantify HMGB1 in the blood of humans from acetaminophen overdose. And what you can see there is essentially we see the same pattern and response. We see the release of the necrotic version of HMGB1, followed the inflammatory version. So, the mechanisms hold true from both mouse to man.

DA#7: Of course we want to know if this is important. We know that inflammation plays an important deleterious role in animal models, following paracetamol overdose but can we use this biomarker to try and predict patient responses better? And that was the hypothesis. The acetylated version of HMGB1 would be upregulated in the blood of patients that had a worse outcome.

So, what you can see there on the data on the left-hand side is data from 78 patients that have taken paracetamol overdose and we have grouped them according to their outcomes. So, those that have spontaneously survived are shown in purple and those that died or required a liver transplant are indicated in red.

And what you can see from the data, this is old data now but what you can see that the patients that spontaneously survived, their levels of acetylated HMGB1 circulated in blood was not significantly different than healthy volunteers. Both the guys that required a liver transplant or

in fact died, their level of acetylated HMGB1 was significantly increased in blood.

DA#8: So, we show the HMGB1 can act as a biomarker but, of course, we are very keen to know that it is not just a -- it doesn't just act as a biomarker. We want to know if it plays a key role in the mechanism of the pathology and the mechanism of the drug-induced liver injury.

DA#9: One strategy we adopted was to see if by neutralizing circulating HMGB1 in blood we could reduce the adverse effects associated with the drug in a mouse model of drug-induced liver injury. So, what we did is we treated mice with acetaminophen and you can see the profile and the time course of the lethality over time. And what you can see there on that data on the top left-hand side of the screen is that coadministration of HMGB1 neutralized an antibody in fact has a positive outcome on outcome in these mice. And what we have done now is we have gone on to develop that a lot further and developed a humanized version of that

antibody. We could also see a positive outcome on ALT activity and then when we looked in detail at the livers, the histological sections of the livers from these mice, in the mice treated with paracetamol in a control antibody, we saw both necrosis and inflammation, characterized by an infiltration of neutrophils within the liver. But if we co-treated those animals with a neutralized antibody for HMBG1, we saw necrosis and knocked out, essential the infiltration of inflammatory cells into the liver. So, we essentially broke that cycle between necrosis and inflammation by knocking out HMGB1.

DA#10: But of course, these are antibodies and to really confirm the important role that HMGB1 might play in the pathogenesis of drug-induced liver injury in these mouse models, we had to create an HMGB1 knockout mouse. But, unfortunately, if you knockout HMGB1 from the whole body, it is embryonic lethal. So, we had to design a strategy to produce a conditional knockout approach.

DA#10: What we did is we blocked exosomes two to four and essentially cut out the HMGB1 gene and combined that with an albumin-based approach and this is some of the validation data from the bottom of the screen. You can see on the left-hand side that the wild type mice with HMGB1 immunohistochemical staining, shown up nice and bright in the nucleus of the hepatocytes. But in the HMGB1 specific knockout in the hepatocytes in the right-hand side, you can see that HMGB1 is completely knocked out from the hepatocyte and only expressed in the non-parenchymal cells. So, we had the tools to test the hypothesis even further.

We challenged these mice with acetaminophen and on the top left-hand side, you can see the ALT/AST data. And as you can expect from our antibody study, the mice that had HMGB1 knocked out from hepatocytes had a significantly reduced rise in ALT activity compared to the wild type. They also performed better, with respect to survival.

DA#11: We looked at the livers of those mice histologically. We could also see that the HMGB1 knockout mouse had a significantly lower score for necrosis in the liver, compared to the wild type mouse. Of course, if you utilize acetaminophen as you model hepatatoxicity, you have to look at metabolism. So, we looked at 2E1 expression, gultathione depletion, and the formation of paracetamol protein. And what you can see from the data here that 2E1 expression was comparable between both strains. The ability for the acetaminophen reactive metabolite to reduce glutathione was the same between both strains and also reacting metabolite to hepatic protein was the same across both strains.

We looked at the mechanism in a bit more detail and I will just briefly give an overview of these sections. I know they are quite detailed. But essentially what we saw by knockout HMGB1 from the hepatocyte, we prevented neutrophil infiltration into the liver but not macrophage

infiltration. And that was what also supported our previous studies, using the neutralizing antibody to HMGB1. But of course we wanted to really push this model and test this hypothesis further and really confirm whether or not HMGB1 played a significant role in the development of drug-induced liver injury following an initial hepatic necrotic response.

DA#12: To test that hypothesis, we expressed HMGB1 in hepatocytes that were normally not expressed in HMGB1, so a conditional mouse model, using an adenoviral gene delivery system. So, by restoring hepatocyte HMGB1 expression, we could restore the toxic effects that we saw with paracetamol shown by ALT activity on the top right-hand side of the screen. We have restored the neutrophil infiltration response into the livers and also the increased necrotic response we saw in the livers by re-expressing HMGB1 back into the hepatocyte. So, that is all from paracetamol overdoes and it is all from a mouse model.

DA#13: But recently, we have begun to show the utility and the importance of HMGB1 in other forms of liver disease. We published on HMGB1 in obstructive cholestasis with Helmut Jaschke. We published on the role that HMBG1 plays in alcoholic liver disease both in humans and also in mouse models. I was very fortunate to present that as a Webex at the AASLD and a hepatoxicity special interest group in January earlier this year. And also we have got HMGB1 and its role in ischemia reperfusion.

DA#14: But of course, we want to know if we can utilize HMGB1 to explore the concept of the development of serious drug-induced liver injury. And these are the concepts that have been widely discussed over the course of this meeting. The role of Hy's Law and its potential to identify and predict serious drug-induced liver injury. So, I won't talk about that in too much detail but we know that is really what we have at the moment and it

is our best assessment, according to the current standards.

So, of course for the development of new drugs, the increase in ALT activity is an important problem and one that we don't really fully understand, whether ALT is just a benign change or indicates a serious drug-induced liver injury.

DA#15: I am sure most people in the audience would recognize this paper published by Paul in 2006 in JAMA. He showed that about a third of those patients in that study developed a transient change in ALT activity. We have applied the mechanistic biomarker panel to those individuals in that study and we have shown a predominant increase in the M30 fragment of keratin 18, the apoptopic component. So, we concluded that the major form of cell death in this particular patient cohort in this particular setting was apoptosis.

DA#16: But if we look at quantifying HMGB1 levels in the blood of these individuals, we also see an increase in total levels of HMGB1 in blood.

So, these patients or these volunteers have quite significant value of HMGB1 circulated in blood had quite a potent dominant associated molecular pattern but they don't develop a serious drug-induced liver injury. They recover and they are okay. So, why don't they develop that serious reaction, despite having a high level of that potent inflammatory mediator in blood?

So, to understand that in a bit more detail, we need to understand HMGB1 biology itself. So, if you remember, I mentioned that HMGB2 has three cysteine messages and I have a biochemistry background. So, when I think about that, I start to get a little bit excited. Maybe some of you guys won't. But what I thought is put this schematic on the screen here, just to show you the importance really of cysteine residues and how they play in biological systems.

DA#17: If you think back to your biochemistry days, you know that cysteine can form disulphide bonds and that is quite important for structural

integrity of proteins and thiol residues are particularly important for protein-protein communication. But also, if you oxidize cysteine residues on proteins, that actually makes proteins targets for degradation and can actually inactivate proteins.

DA#18: This slide summarizes quite a significant amount of work led by my laboratory with some collaborators across the globe, where we pooled resources and we have all of an interest in HMGB1. And what we did is we utilized mouse-based technologies, coupled with cell biology and molecular biology to determine what post-translational modifications with respect to redox status impact on HMGB1 function.

What we showed is that the functions of HMGB1 are mutually exclusive with respect to cytokine induction and chemotaxis. For HMGB1 to act as a chemoattractant agent, all those cysteine residues must be reduced in a thiol state. If there is a disulfide bond present between cysteines 23 and 45

and cysteine 106 is reduced, then HMGB1 can act as a cytokine inducing agent as a lead-in for thiol receptor 4, in fact MD2 associated with thiol receptor 4. But if you continually oxidize all those cysteine residues to sulphonates, then HMGB1 has not function at all with respect to a cytokine and also a chemoattractant. We also know that these oxidation modifications of HMGB1 appear to be cell death mode-dependent and specific as well.

Previous to this work, another group showed that mitrochondrial cleavage -- a caspase-mediated cleavage in mitrochondrial complex one can induce ROS production and join apoptosis and can inactivate HMGB1 through terminal oxidation. Sort of an innate response to prevent the control and spread and damage associated with molecular patterns in and around secondary necrotic response. We tested the hypothesis that during apoptosis HMGB1 is oxidized and that could potentially one reason why you don't see a necrotic response.

DA#19: So, we simply tested that head to head in our murine model of acetaminophen overdose, where we see a mix of apoptotic response with necrosis and also necrosis only.

What we saw in the animals where we saw apoptosis and necrosis wsw oxidation of HMGB1. But in our mouse models, where we only saw necrosis, we saw the two perinflammatory isoforms of HMGB2 circulating in blood. To confirm the caspase dependency of those observations, we treated the animals where was saw apoptosis with a caspase inhibitor and then switched the phenotype to an necrotic inflammatory phenotype with the potent inflammatory isoforms of H and G we want to circulate in blood.

We know that those different isoforms of HMGB1 are cell death mode dependent. So, the next obvious question we asked ourselves is could, through looking at HMGB1 isoforms, can we explain why we see one cohort of patients develop serious drug-induced liver injury and those develop a

benign change in ALT activity by really understanding the mechanistic basis.

DA#20: If we divide our cohorts of patients into those that have a serious injury or the large overdose group could host the transient injury from Paul's study. And when we look at the mechanistic biomarkers, we know that the serious overdose guys have a really small portion of apoptosis, whereas the guys with the transient changes in ALT activity have a significant proportion of apoptosis. We looked at the HMGB1 isoforms in blood. If we first focus our attention on the serious injury, we see when we have isolated H and G, we want to characterize that by electrospray ionization mass spectrometry. We see many different isoforms of HMGB1 in blood.

If we isolate the H and G from the blood from those with benign changes in ALT, we only see one isoform of HMGB1 in blood. And if we characterize those a lot further using tons of mass spectrometry, we can start to put

post-translational modifications on top of those isoforms.

And essentially what we see in the patients with the serious overdose, we see all the bad players, the bad H and G isoforms, the cytokine-induced form, the chemoattractant, plus its acetylated derivatives from active release mechanisms.

But if we characterize the cysteine residues in more detail for the benign changes in ALT group, we only see the terminally oxidized form of HMGB1 or the form that has no inflammatory function, according to current theory. This led us to believe that HMGB1 isoforms could potentially not only act as a biomarker for serious overdose of serious liver injury versus benign changes in ALT but also could be a key mediator in these processes.

DA# 21: we took that a little bit further with pharmacologists at the University of Liverpool. So, we like to put a number on everything and quantitate things as much as we can. We quantified

those different isoforms of HMGB1 across those different cohorts. And what you can see by looking at that graph there, you can see that the patients with the therapeutic indication of paracetamol only had the terminally oxidized form of HMGB1. The guys that spontaneously survived, they had a mixed bag of HMGB1 isoforms but the guys that died or required a liver transplant, their redox balance was shifted towards the reduced form or the proinflammatory active forms of HMGB1.

DA#22: Lessons that we learned from these cohorts, these retrospective cohort analysis is that functionally distinct HMGB1 isoforms can determine if paracetamol liver injury is serious or benign. And of course, we can add an extra mechanistic understanding to that and link that back to the form of cell death.

And in this figure we have taken those three different groups of patients, the spontaneous survivors, the guys that died or required a liver transplant, or the guys with benign changes in ALT

and we have correlated the redox ratio so that the values associated with the inactive form of HMGB1 over the proinflammatory from of HMGB1 and we correlated that against the so-called apoptotic index using the M30, M65 ratio.

You can see from these data that those patients quite nicely separate. And what we see is that those HMGB1 isoforms are linked to cell death mode dynamics as well.

DA#23: I summarize there that we have shown that HMGB1 can be a key mechanistic biomarker in experimental and also clinical drug-induced liver injury. We have shown that in paracetamol overdose, and in other forms of liver injury. We have developed conditional knockout mouse models to explore the mechanism of pathology. We have looked at different HMGB1 isoforms to inform patient outcome and prognosis and also try and differentiate between benign changes in ALT to serious liver injury.

And now we believe that HMGB1 is not one protein, but it is a number of different proteins and isoforms.

DA#24: I would like to thank some of these people that here in the audience, particularly Kevin Park from the University of Liverpool and, of course, the external mentorship from Paul Watkins and his lot at the Hamner. Thank you.

Discussion Session IVB-1

DR. SZABO: Thank you for this really beautiful presentation. I think we have time for one or two questions. Linda -- Dr. Greenbaum.

DR. GREENBAUM: Hi. Linda Greenbaum. What would be the predicted effect of N-acetyl-L-cysteine, which we know is affective in apop injury on the redox ratio of the HMGB1?

DR. ANTOINE: Obviously, that could have a huge impact, as you said but all these patients had NAC treatment, actually. So and we still see a difference post-cell death mode dynamics with those patients. So, we really need to test that head to head, actually, in an experimental model.

PARTICIPANT: I have two questions. What is the turnover of each one of these different forms of HMGB1? Because if you measure them at different times because of the attack then they may be missing certain data.

The other question is are these different forms by a different receptor that you mentioned

or they are all have the same targets? Because you mentioned like three of them, like TLR4, receptor 4 and another one.

DR. ANTOINE: Your first question was with respect to turnover. We know that these isoforms have a shorter half-life than ALT activity and we know that their terminally oxidized form has an even shorter half-life. That is one of its mechanisms, actually, to grade the proteins to terminally oxidize it and switch it off as an inflammatory mediator. With respect to the receptors, we know that the disulphide form will only interact with MD2 as part of the TLR4 complex and not RAGE the CXCR4 receptor. And of course, the opposite is true. The reduced from will only interact with CXCR4 and RAGE but not TLR4. So, they are completely mutually exclusive isoforms and have independent cell singling pathways.

DR. SZABO: Last quick question.

DR. WATKINS: It is fantastic work. It is very hard from me to imagine ALT elevations

observed in a Phase 1 study anywhere without measuring these kind of markers. Are you open for business? In other words for people wanting to collaborate with you who may have issues like this?

DR. ANTOINE: We are open for business. Anyone that wants to collaborate, we are very keen on, and we are really hoping that the development of this new Liver Safety Research Consortium can bring a sample base to us to be able to do that in a precompetitive way.

DR. SZABO: Thank you. Fantastic. We are going to move on. The next presentation is by Dr. Brett Howell from USC on serum cytokeratin 18 as a biomarker for liver injury.

Howell photo, biosketch, abstract

BH#1: Thank you for the introduction and thanks to the organizers for allowing me to give this talk, and for you all for skipping your coffee break so that we can get our talks in.

I am going to be discussing serum cytokeratin-18 and its role in the clinic as a biomarker, as an example. So, I will get to the questions that I want to raise towards the end. And unfortunately, I am going to be raising more questions than providing answers but really just starting the conversation on this.

BH#2: This example comes out of the DILIsym Initiative, which is an effort by the pharmaceutical industry to support us in developing a tool for predicting, understanding, and decision-making with respect to DILI. So the goals are here on the right-hand side.

BH#3: The problem I will discuss today, is just one of the many different applications that to which we have tried to apply DILIsym, such as extrapolating from in vitro data to get early clinical predictions, understanding variability and response across individuals, and so on.

Today I want to discuss a DILI dose response scenario where the question of whether there is or

isn't DILI is not the question. The question is whether there is a risk mitigation strategy that can be taken forward.

BH#4: And this is an example for a drug that is in development. I will be referring to Compound X. But just so you know, it is an actual example we are working on. The clinical concern with this novel compound is that is in development to address an important unmet medical need. Importantly, this is for the inpatient setting, patients in the ICU, more than likely, treated with the compound.

BH#5: The concern is that ALT and other markers including cytokeratin 18 were elevated in some subjects in these studies. The question was whether there is any way forward for this. Some of the data that the company has given to us is shown here on the bottom left. You see ALT elevations in some of the subjects in one of the cohorts. The ALT time course showed three times and two times the upper limit of normal with no explanatiojn. In this case, 4 out of 8 or so, 4 out of 7 were above

three times the upper limit of normal and some well above. It has hard to see the green curve there at the bottom but that was actually the control.

But if we look at the data in a tabular format, you can see on the left-hand side in this table some numbers and words. So the numbers really refer to the dosing level, so of blinded the actual dose here but just think of 1x as the target dose, target daily dosing level. They did a number of small clinical studies with daily dosing levels below and above the targeted dose. This drug happens to be infused intravenously. So, they varied from long infusions to shorter infusions and in-between. You can see the DILI dose response on the right, with the ALT elevations they saw in the clinical study.

In general, their problem wasn't correlated with infusion length but it was quite correlated with dose. So, as the dose went up, they saw more problems and more severity.

In addition, they also assessed, at our suggestion, some model biomarkers. For example,

they assessed miR-122 or allowed us to measure. And miR-122 correlated on an individual patient level quite nicely with ALT and showed clearly for specificity. Cleaved cytokeratin 18 was also elevated and showed that this was a mode of cell death that was seen with both apoptosis and in some necrosis but predominately apoptosis. I will come back to these biomarkers at the end of the talk.

BH#6: What were the goals for us with DILIsym? What were we trying to accomplish? First of all, to help understand what the potential mechanisms for this problem could be, in combination with some in vitro studies, and then also to help optimize the dose and monitoring protocols to find, if possible, an adequate liver safety margin for the compound.

BH#7: To give you a very brief snapshot of DILIsym, it is a computational tool made up of ordinary differential equations and parameters that represent several species and humans, but they are focused on humans.

The liver in this model is represented by three distinct zones, rather than continuously. They are lumped and assumptions are made, but you can see some of the key processes that we have been working on, including PK, oxidative stress, intracellular bile acids, and their homeostasis throughout the body, as well as mitrochondrial dysfunction and disruption. For this particular project, we focused on a few areas within DILIsym: pharmacokinetics, and of course oxidative stress were key mechanisms, and of course the turnover and potential death of cells and the relationship to biomarkers that would come out. To do this project, we went through different steps that are not atypical for a DILIsym application.

BH#8: First, was gathering of laboratory data and experiments to understand the mechanisms. In this case, the key mechanisms that came out of that data were electron transport chain inhibition and oxidative stress being caused by the compound. And those endpoints were assessed in hepG2 cells.

We built a compound profile for this compound in DILIsym and simulated some of their early clinical studies. So, these were studies they had already run. We ran the simulations and we got, for the most part, very good qualitative agreement with their studies. We had issues at the higher dose levels in the simulations, but no issues at the lower levels. But the simulations didn't correlate spot on. As we typically do, if we have clinical outcomes data, we combine that with our in vitro data to get the dose response as close as possible to what they saw in the clinic. And then we move forward to look at what might be safe for future studies to extrapolate to unanswered questions, really. So, we went through this process. In addition to that, we also wanted to apply this to a number of different simulated individuals, not just sort of an average person, which we know doesn't truly exist. And do to this we used what we call our SimPops or our populations.

BH#9: There are a number of different parameters that are varied in the population we used. They include areas such as oxidative stress production and how the body handles that stress, apoptosis, mitochondrial dysfunction pathways, and others. For each of these parameters, imagine there is a distribution, based on the literature. And when we pull that parameter out from these distributions and put them altogether, you have a simulated virtual human.

BH#9: We have 300 distinct simulated humans for this project and we actually ran each simulated human at three dosing levels or three exposure levels to incorporate sort of PK variability in sort of an estimated way. So, we ended up with 900 distinct simulations for what I am going to show.

BH#8: First we looked at seven or so subjects per group in these phase 1 studies, and we had 900 simulations per group. So, as you can imagine, our tails are a little larger. So, just keep that in mind.

BH#10: What you will see in this table here across the top, to the right-hand side of the table are our simulated ALT elevations. And then the overall minimum percent of hepatocytes that were viable. To interpret that, it is the worst case scenario that we saw out of the 900 people we simulated. The lower that number, the more liver that was lost in that worst case person. The little circle in blue denotes that we incorporated, if you like, an in-silico physician. A component in these simulations was that when we hit stopping criteria that they had defined in their clinical studies, we stopped dosing just like they did in their clinical studies. What you see here are the results that I showed before on the left for the left two columns, which is their data. But then on the right you see our simulated dose response. And so we see, by and large, fairly good agreement between the simulations and the data. We saw increasing ALT elevations as dose went up and increasing severity. And we predicted a severe

liver injury event at the highest dose level, if they had dosed out to 900 people. In addition to this, we did see within the simulations apoptosis and necrosis present based no oxidative stress as a mechanism. This fit well with the cleaved cytokeratin 18 levels that were measured before.

BH#11: In terms of dynamics for the time course we were predicting, we saw changes that were very similar to what they saw in patients. This is one example of a particular infusion length and dosing time. And you can see the black arrow at the bottom shows when they had to stop dosing, and then we had to stop dosing in the simulated study. So, the dynamics were fairly similar as well.

BH#12: The first question they asked was what was the margin safety above their predicted efficacy level of a predicted dosing level. So, the part of the table highlighted in black shows their target dosing level, which was 1x and the medium infusion length. And in their early clinical study, they saw no ALT elevations, no

issues. We saw a very few number of ALT elevations and no significant DILI events. Within the simulations increased that and looked for the margin. We saw serious liver injury at three times the dosing level. So, it seemed like the simulations would at least suggest that there was a three-fold margin of safety for the compound. However, without monitoring, there was a lower margin of safety. So, that was one key component of this is that we sort of reinforced or quantified, I guess you would say, the importance of monitoring in this scenario.

BH#13: We then went on to look at these individuals and to isolate the effects of why some simulated humans were responding and some weren't to this treatment. And some of the things that fell out of that were their ability to respond to oxidative stress, their propensity for caspase activation but also body weight or exposure. And so that is pretty intuitive. You have a dose

response or a dose-dependent DILI event exposure would be an important component.

And so one of the things that we then went on to do for this simulation project was to help them assess, quantify the importance of potentially dosing on a body weight basis. In the same patient setting, you could imagine that you could give smaller individuals less drug and larger individuals more drug, and actually adjust your dose for the individuals. And because this is infused, it is certainly not as complicated as if it was in oral form.

BH#14: So, we went on to do those simulations prior to them having conduct the clinical study. So, we first suggested the weight, the dosing for the weights of the individuals that we were simulating. We normalized it at a 78 kilogram individual and then we extrapolated out with that weight-adjusted strategy. So, again, smaller individuals getting less, larger individuals

getting more, and the margin of safety went up to 4.5-fold.

So, it shows that perhaps this strategy combined with monitoring could help, given a little bit more safety margin and a little more comfortable. The things that we did really here were help identify the mechanism for injury, which we think is oxidative stress, or at least that is what we would suggest, and also help optimize the right dosing level with the right monitoring strategy and dosing strategy, in this case, a weight-adjusted dosing strategy.

But some of the things that came out along the way for this project really relate back to the biomarker issue. In this project and some others as well, we are seeing really early assessments of some of these novel biomarkers in phase 1 studies. So these cleaved cytokeratin 18 and full-length keratin 18, miR-122, HMGB1, the things that have been discussed today. And you can see our simulated values for these biomarkers here.

BH#15: One interesting thing, first of all, as I pointed out, the cleaved cytokeratin 18 supported the mode of cell death, which was important, I think, for the company to understand the mechanism. But also you may have noticed that there were scenarios in our simulations where hepatocytes were lost but no ALT elevations were predicted. And this is because the mode of cell death at those low levels of hepatocyte loss were primarily apoptotic.

The hypothesis is that perhaps there are levels of cell death that are so low with apoptosis that you wouldn't see ALT rise, and cleaved cytokeratin 18 might be more sensitive in that scenario. We found ourselves addressing questions and asking questions, such as how should markers like cleaved cytokeratin 18 be applied clinically. First of all, is apoptosis a good thing or a bad thing? I think these have presented some interesting data that suggest that at least in low dose acetaminophen scenario apoptosis is a better

outcome than necrosis. But by and large, there are arguments or discussions you could have on both sides of that coin.

Are there any stop-rule applications to be implemented for some of these new biomarkers? There was a question earlier about special populations in miR-122. And then also what might be the clinically relevant levels of these markers? We know with ALT and AST there is a lot of empirical clinical experience that is brought to the table for those questions but not with these newer markers. And sometimes in these early phase 1 studies, decisions are being made and these questions are on the table.

The only point here I am going to address today briefly is the last one, and put forth a strategy to think about for how we are trying to perhaps address this issue of clinically relevant levels.

BH#16: To do that, I am going to show this schematic here, where we have on the top a number

of different gray shapes, representing hepatocytes. And just imagine that the baseline ALT in an individual, at least in our model, is 30 U/L. If we induce the process in a simulated environment to raise the ALT from 30 to 60, a two-fold change, we can then count the exact number of hepatocytes in the simulation that it took to get that change. And then we can go and kill the same number of hepatocytes via apoptosis and determine how much cleaved cytokeratin 18 was released in that scenario. By doing that, we can assess a number of different cell death levels and determine sort of "equivalent" fold changes for cleaved cytokeratin 18 on the right in the blue table here, as a corollary to the ALT fold changes on the left. You can take the exact numbers with a grain of salt, because we are still working through this cytokeratine-18 model within DILIsym and pulling together datasets like this from clinical studies where we can get really nice datasets. But the concept is that we can use this

simulation tool to help draw parallels between what an ALT level might look like and what at least a cell death-relevant level of cleaved cytokeratin 18 might look like.

With the understanding the ALT is an imperfect marker, should we correlate with ALT? That is another question. But at least it is a starting place for how a group developing a drug, a physician might think about an ALT or cK18 level and what it means for cell death and for the liver. Of course, fold-changes aren't going to correlaate properly because the baseline levels are totally different for these markers.

BH#17: Some of the questions that we have been left with in several of these projects is should emerging biomarkers be assessed in a clinical trial setting as early as phase 1 and how should data be interpreted when considering the different modes of cell death; and the inactivation with respect to the patients in these studies and at these study sites; and then what levels of cK18 should be

flagged as significant. And we have tried to address this within the DILIsym Consortium early on but we are still just starting out.

BH#18: I want to thank the conference organizers for the chance to give this talk, the sponsor here who graciously let us present this while they are still working through this problem, and our members who continue to support our work. So, thanks a lot.

Discussion Session IVB-2

DR. SZABO: Thank you for the great presentation. Any questions? Let me ask a very naive question. How stable is the cytokeratin 18 level in the blood?

DR. HOWELL: My understanding from people such as Dan, with whom I have had conversation, is that it is very stable. I believe the half-life, in terms of its natural clearance, is similar to ALT. And I think it is pretty stable in storage samples but if any of the experts out here disagree with me, speak up on that.

DR. SZABO: Okay, Dr. Urban.

DR. URBAN: Hi, Tom Urban at UNC. Thanks, Brett, for a very interesting talk. I wondered if you probably know Fischer-Amari and published or not, have published extensively on genetic polymorphisms in cytokeratin 18 that seem to be increased frequency in patients with acute liver failure or other types of liver disease, not for DILI. But I wondered, do you have DNA from these

patients in this program that could be sequenced for mutations in keratin 18. And what is your guess as to whether that might explain some of what you are seeing?

DR. HOWELL: That is a good point. They do have samples from the studies. I'm not sure if they have samples from all of the studies. I know they have samples from one of the early -- one of the time course studies that I have shown. So, that is a good idea, something that we could ask them about and maybe open to sort of a genetic analysis. That's a good point.

DR. SZABO: Last question, Dr. Regev.

DR. REGEV: Thank you. Excellent talk. As we know, NAFLD is not really the most common liver disease in western countries. And as we know in the UK we have this very strong association with NASH. And I was wondering how does that play, how do you reconcile that in your assessment?

DR. HOWELL: That is a good point. That we haven't addressed it yet is really the short

answer. But it is something that, as we start building special populations, we are going to have to address for all these biomarkers, namely what is a relevant level? And it is relevant to all of the conversations that have gone on today. But what are the relevant levels and the fluctuations in those markers for those populations? So, it is something that is definitely on our radar that we have to take into consideration.

DR. SZABO: Thank you Dr. Howell The next talk is Dr. Minjun Chen and he is going to talk about the Rule of 2: Do drug properties predict DILI?

Minjun Chen photo, biosketch, abstract

MC#1: Good afternoon, everyone. First, thanks for inviting me here to introduce our work. I will talk today a little about the LDKB work. I am a toxicologist or I can say bioinformaticist, not a clinician. So, I will give you from my perspective whether drug properties can predict drug-induced liver injury.

MC#2: We have talked many times in the DILI field. One big challenge, I think is the lack of a reliable predictive model. Especially today, we don't have a good animal model predict to predict human effects.

MC#3: As we know, FDA still relies on the high-dosing healthy animal study. This study still can only identify 50 percent of DILI problems. This technology was developed more than 50 years ago, so we need some new technology to improve predictions today.

MC#4: So, we developed a project called the liver toxicity knowledge base. And this database provides a better predictive model. We have put some of the collected data in the particular model to a public domain. We can either use a LTKB such as Google to find that.

MC#5: This slide gives you some more idea what data we have in our database. And basically, we have collected about 3,000 drugs. And basically these drugs, including almost all the academia

drug, drugs that were pulled by the other agencies. Basically this we started to collect the human data and the non-human data or we collect part of the data. For the human data, we tried to collect all kinds of the DILI-related information, especially we have it noted as a DILI risk associated with the drug. For the drug property data, we also collected each drug from the chemistry property. DILI markedly related individual assay or some whole special biology risk poles using microRNA data or this other data.

MC#6: At the end of the day, we tried to correlate these drug properties with human data, build a particular model. This is our goal to do the project. To develop a particular model, we need to list the drug have known DILI positive and DILI negative. The amount of the DILI drug in this model is to develop all kinds of translational biomarkers.

We tried all kinds of approaches. Finally we found that drug labels are good enough to serve our

purpose. The drug label, basically, is an information tool. It provides certain data to the doctor and the patient. By the way, the FDA should inform the patients about the drug label.

MC#7: We agree that the drug label is not perfect but it might be the most consistent, best information we can have to help us codify the drug.

MC#8: We published a paper several years ago, describing our approach uinge drug labels to identify DILI drugs. The drug label has three sections to disclose a DILI risk: Box Warning, Warnings & Precautions, and Adverse Reactions. Dr. Temple discussed drug label a bit yesterday, so I don't want to repeat today. If you are interested, go to our 2011 paper (Drug Discov Today 16:697-703, and get more details.Tthis approach, classified each drug into most concern, less concern, and a non-DILI concern.

After we had risk classification by labeling and we know the drug is a DILI drug or a non-DILI drug, we then go to our LTKB data.

MC#9: We tried to develop some predictive model based on our drug property data. The data we thought about was the daily dose, because most of the DILI drug we know was given -- but the daily dose alone basically is not predicting now because we know many signature, also given the 100 milligram.

We thought about whether we could we find some other way to help. The LTKB database finally found that lipophilicity can also help for this purpose. If you could use, the DILI we are marking here, we found if the drug dose was more than 10 mg, then there was toxicity. Most non-DILI drugs got kicked out. Because of the rule of 2 there is a significant association with DILI risk.

MC#10: I show you some more examples to demonstrate the Ro2, using drug pairs. Drug pairs are basically two drugs capable of causing the same or similar effect and have similar structures, but show toxicity differences. For example, alpidem and zolpidem, two drugs with high logP, greater than 3. but alpidem had a much higher dose. Now

look at troglitazone and two other glitazones: troglitazone, has a larger logP greater than 3 but only troglitazone had a much higher dose than the pioglitazone or rosiglitazone. Another example is bosentan. Dr. Temple mentioned yesterday this drug was also a RO2-positive drug. Its daily dose is 400 milligram, and AlogP also greater than 3.

MC#11: We also show that logP helps in other cases, for example, tolcapone and entacapone. Those are drugs that have high doses but only tolocapone has the much higher logP. The same applies to nefazondone and trazondone.

But we don't say that RO2 always works. The RO2 only has limited sensitivity, about 30 to 35 percent. We have some false negatives, and false positives, for example, trovafloxacin, a drug we know was withdrawn. The daily dose is about 200 mg but logP is very low.

MC#12: We wanted to know how to work on all FDA-approved oral drugs. So we collected all drugs approved by FDA before 2010, 748 oral drugs.

And of these we had 168 drugs with most DILI-concern in labeling, but Ro2 identified only 72, about 43% sensitivity. Next, 193 drugs with no DILI-concern, of which only 11 drugs were ALT positive. That means that specificity was about 95%. There were 387 drugs of less DILI concern, but we only identified 13% as ALT positive.

MC#13: We also wanted to know whether the Ro2 could help us identify drug failures in clinical trials or in drug development. Interestingly, in this model, Dr. Regev presented a drug with daily dose of 225 mg and AlogP of 3 to 4, a RO2-positive drug, a drug we discussed this afternoon. In this other drug, they had a daily dose of 120 mg and logP is 4.1, another RO2-positive drug. So, both drugs discussed today were RO2-positive.

You can see some more examples here, collected from the literature. but some are RO2 positive, some negative. But anyway, it shows that RO2 can identify some of the hepatotoxic drugs during drug development. We also want to call

industry to study the failing drugs more, to learn if they can help give us a better predictive model. We know RO2 has limited sensitivity and we are trying to incorporate some more related data.

MC#14: And finally, in this paper we use a high-content screen assay to improve sensitivity from 30 percent to 50 percent.

MC#15: Going to the question John asked me: Are drug properties or host factors predictive? I think this cartoon is a very good answer to the question. In this cartoon, there are blind people who want to know what an elephant looks like. The first time, they don't agree because they are concentrating on a different part of the elephant. But very interesting, at the end of the story, original story, these blind men stopped talking and they started listening and collaborating. And then they envisioned the whole elephant.

So, we have some blind people discussing our chemistry. If we were to figure out what the data looked like, at least addressed, we proposed DILI

basically an interaction between the drug property and the host factor. Drug properties and host factors work together to initiate cellular injury. In the individual patient, the host factors will contribute to the individual response and then finally determine the final outcome. So, I suggest considering in a DILI case not only the host factors but maybe also the drug properties, to help you understand what DILI is.

MC#16: Overall, we believe that drug properties and host factors together contribute to DILI prediction, DILI development.

Although LTKB has collected diverse DILI-related drug property data, it can be helpful for understanding. We have developed a predictive model. A comment from Dr. Kaplowitz was that RO2 has added value to predict idiosyncratic DILI. We also believe if we incorporate more data. It can be improved. We still have a long way to go to make a better predictive model.

MC#17: Finally, I want to thank the many people who helped me on the LTKB project, and especially the LTKB interest group. And also we thanke many people in this room. Especially I want to thank our collaborator Dr. Jurgen Borlak from Germany and my colleagues at NCTR. Thank you so much.

Session Discussion IVB-3

DR. SZABO: Thank you, Dr. Chen. Any questions from the audience?

PARTICIPANT: I was wondering. Did you also incorporate it all in assessment of basicity, most basic PKA? We have done a similar analysis at Lilly and found that you also need to look at how basic the molecule is, especially when you are talking about phospholipidosis risk and DILI associated with properties leading to accumulation in tissues and high volume of distribution is the other thing that we have noticed is correlated with toxicity.

DR. CHEN: Yes. Our LTKB we also collect all the PD/PK that you mentioned about and we tried to also correlate this the PD/PK pattern with the DILI, the DILI drug and non-DILI drug which one can accomplish it. The company is still working with that. Our database is still in development. We know the drug properties and put it in our database. And finally, we correlate not only work on the whole

population DILI risk maybe overall, maybe correlate other people didn't have, for example, it is come today that immune-related DILI, you know we basically hepatitis is the drug property can contribute this DILI.

DR. SZABO: Thank you, very much. Thank you. Okay, moving on to the last talk and the topic is transforming monocytes into hepatocyte surrogates. It is a very exciting topic and Doctors Gerbes and Benesic will present it.

Gerbes photo, biosketch, abstract

AG#1: Thank you very much. First of all, I would like to thank the organizers, in particular, Drs. Senior and Dr. Watkins, for inviting us to this exciting conference and for the challenge of giving the final presentation.

AG#2: I will just give a short background about the rationale for our cell model, in order to set the stage for Dr. Benesic then to provide

what we think are the very interesting data from our clinical pilot study.

AG#3: Why start with monocytes? Monocytes seem to be important for hepatic repair in the rodent models of acute liver injury due to paracetamol. Moreover, monocytes may be capable to transform into hepatocytes, as shown from previous data suggesting that cells with hepatocyte-like functions can be generated form peripheral monocytes.

AG#4: We used EDTA-plasma and separated monocytes by gradient centrifugation and adherence separation. These cells then underwent a 10-day culture with a proprietary protocol, as shown on the slide. The resulting cells, which we called monocyte-derived hepatocyte-like cells, MH cells, were characterized in particular in view of hepatocyte properties. Interestingly, these cells can synthesize urea and coagulation factors. They have metabolic properties such as cytochrome P450.

For the sake of time, I am not going into detail here but I just would like to show you interesting results that we obtained when we had the opportunity to obtain primary human hepatocytes from three subjects.

AG#5: We compared properties of these primary human hepatocytes with monocytes of the same subjects and with MH cells generated from monocytes. I show you here two interesting sets of research. This is a gene expression profile, of 270 mostly ethnic genes. Not surprisingly, as you can see, on the left illustration, the gene expression profile of monocytes was similar to the primary human hepatocytes. However, following the cultivation process, the MH cell gene expression profile resembles much more closely that of primary human hepatocytes in this same individual.

AG#6: Possibly more important are the metabolic properties. We also found similarities in cytochrome P450 activities. Here is an example, the highly variable CYP2C9 and, again, the

left part of the illustration shows the basal activities and rifampicin-reduced activities in these three donors. And as you can see, the profiles of the MH cells resemble those of the primary hepatocytes. These and other exciting findings suggested to us that possibly these MH cells could reflect individual hepatocyte properties of these subjects. This prompted us to investigate if this could be a model to reflect individual DILI.

AG#7: The next figure shows you a typical spider web, as we illustrate the data. We exposed these MH cells for 48 hours to various drugs in different concentrations. The circle shows the upper limit of normal; any signal outside reflects toxicity. The readout is LDH release. You see a negative control, just medium, and a positive control with cell lysis, and paracetamol in different concentrations as functional positive controls. Exposure to different drugs revealed no

signal for diclofenac or pantoprazole, but a clear signal for the higher dose of omeprazole.

AG#8: As all of you know, DILI is a rare event. So for any test, you need very high specificity. We typically compare the toxicity signal in the index patient with the signal obtained in numerous healthy subjects. We have data from almost 100 drugs, tested in cells from more than 150 subjects. So, we thought it was about time to look for a real world test, so we set up a clinical trial that will be presented to you by Dr. Benesic.

Benesic photo, biosketch

AB#9: Thank you, Professor Gerbes. The aim of this study was to investigate, if we generate these cells from patients with drug-induced liver injury or other acute liver injuries, if these cells might be able to help with the diagnosis and more importantly, to make causality assessment. In this study, we had patients that were treated with at least one drug and had acute liver injury that was defined as ALT at least five times upper limit of

normal, or AP two times upper limit of normal, or the combination of ALT three times and bilirubin two times upper limit of normal. The patients underwent diagnostic workup, laboratory testing, biochemistry, virology, immunology, imaging, and histology where available. For all these patients and the drugs involved, we calculated a RUCAM score and made a clinical assessment using drug signature and the history. From patients, MH cells were generated and toxicity testing was performed with all the involved agents, done independently of causality assessment.

AB#10: This slide just shows how the diagnosis of DILI was made in the study. You all know that diagnosis can be very challenging. We used a combination of the exclusion of other causes for drug-induced liver injury and, where available, typical drug signatures, for example, using the LiverTox website. We came up with a classification that is quite similar to the one used by DILIN.

AB#11 These are the results. We had 31 patients with iDILI and 23 with other causes for acute liver injury. This slide shows that the two groups did not differ significantly for demographic characteristics, and the predominant pattern of liver injury was hepatocellular.

AB#12: Drugs with the highest causality likelihood in the iDILI group were NSAIDs, oral anticoagulants, anti-thyroid and anti-infective drugs, immuneodulators, and antipsychotics. Well, the diagnosis was either unequivocal DILI or unequivocal liver injury from another cause. And MH toxicity was present in 10 of 11 iDILI patients with unequivocal diagnoses and we have no signal in 12 non-DILI patients.

AB#14: Then we looked at the total study population. And in the total study population, the drug with the highest causality likelihood in each patient was tested. On the right-hand side, MH toxicity was seen in 29 of the 31 DILI patients showed positive results with MH toxicity; two were

missed. In the non-DILI cases, there were no positive results.

On the left-hand side, the RUCAM score; 29 were identified by the RUCAM score; 2 cases were missed but these were not the same two cases as in the MH cells. But the RUCAM scores showed a relevant number of false positive results.

AB#15: Then we did the litmus test. You probably know it can be very challenging to make causality assessment in patients taking several drugs.

AB#16: We analyzed in this busy slide all drugs that were taken in the total population of our patients. So, these were altogether 103 different drugs in the iDILI group and 68 drugs in the non-DILI group. On the left-hand side, the RUCAM score, as you see, we had 11 cases that are definite DILI that are all identified by RUCAM. And in the unlikely case or the non-DILI case, RUCAM performs quite well. It gives mostly correct results. But the more ambiguous the diagnosis is, the worse the

performance of the RUCAM scores, which was quite expected.

On the right-hand side, the results from the MH toxicity showed mostly correct results. Only 2 false negatives. I showed these in the slide before. And 4 patients showed false positive results.

This suggests to us that maybe this model could help in causality assessment for DILI in cases that are not so clear.

AB#17: To summarize, our data suggests that monocytes can acquire some hepatocyte properties in vitor and it seemed to reflect donor-specific characteristics.

In this pilot study, there was higher MH cell toxicity when the cells were derived from iDILI patients, compared to patients with non-DILI acute liver injury or healthy donors.

Thus, MH cells might offer the possibility to assist with a diagnosis of iDILI and causality assessment, especially in more ambiguous cases.

Ongoing research further characterizes the model using omics technologies and for sure, we need further data from more patients and especially those who tolerate the potential iDILI drugs. Thank you very much for you attention.

DR. SZABO: Thank you for this provocative and really exciting story. Have you tested the effect of drugs on monocytes of these individuals without pushing them towards hepatocytes?

DR. BENESIC: Yes, this was the beginning of this work and we did many experiments with paracetamol. And usually with paracetamol, you don't get any effects. And we also have tested in some cases the monocytes of the patients and there was no reaction.

DR. SZABO: Questions from the audience?

PARTICIPANT: Were there any gender differences?

DR. BENESIC: No. No, so the gender distribution was quite equal.

DR. SZABO: Other questions? Yes.

PARTICIPANT: Have you been able to test for cells that are normally found in the liver when you have had these liver samples to see whether the Kupffer cells, which are the monocytes that are actually normally there, were comparable to the cells that you are making with the MH cells?

DR. BENESIC: No, actually, not because the hepatocytes we got already isolated so there were no Kupffer cells.

PARTICIPANT: When you took the cells from the DILI patients, when was that in the course of the illness, and did that matter, and how reproducible was that on sequential within the same subject?

DR. BENESIC: Yes, thank you. Usually the test was done or the blood sampling was done about two or three weeks of the DILI event, after the diagnosed event. We have some cases in which we have sequential blood samples and the cell generation

for up to six months after the DILI event and we could reproduce these data.

DR. SZABO: Last question from John Senior.

DR. SENIOR: Forgive me for not getting up. I have a question for you but it may apply also to what we have just heard from Doctors Gerbes and Benesic. When the liver is injured by drugs, some but not all of the hepatocytes are injured, release enzymes and all that, and lose function but there are cells that remain. You have heard talk about exosomes, and we asked Jack about that this morning. Do you think exosomes have a role in adaptation, by sending messages from the injured cells to the uninjured cells to change their behavior and adapt, or even more to go out and send a message to a monocyte telling it behave like a liver cell, as a recruitment to reserves when you are in trouble?

DR. SZABO: Very likely. There are data from other fields suggesting that yes, indeed, injured cells send out messages in about every

package in exosomes to activate immune cells or to induce regeneration or suppress immune responses. So, that is very plausible.

DR. SENIOR: And then do you have any idea how that message is communicated?

DR. SZABO: Well, I think that probably depends on the biological situation. Some of the messengers could be HMGB1, microRNAs or other kind of molecules that are packaged in the exosomes or in the microvesicles. And that way, they can just enter the cell in a receptor-independent manner and express a functional activity on the target cell.

DR. WATKINS: As I recall, you need fresh blood. Right?

DR. BENESIC: Yes.

DR. WATKINS: And how long does it take from when I gave blood of a patient to when you have an answer?

DR. BENESIC: Okay, so the generation of the cells takes ten days. And if we do the test as

performed in the study, we incubate for 48 hours. So, about two weeks.

DR. WATKINS: And again, you are looking at standard toxicity endpoints in these cells. Correct?

DR. BENESIC: Yes.

DR. WATKINS: So, the assumption is that there is different machinery in those cells in the susceptible cells than in the nonsusceptibles, presumably mimicking differences in the hepatocyte. Which is interesting in GWAS we are not coming up with very few exceptions with anything actually in ADMI machinery and sort of genes that have hepatocyte function is it is epigenetic change over time that makes the ACTG code less relevant. But I guess the assumption would be that monocytes have the same epigenetic changes as an hepatocyte.

DR. BENESIC: Well, we don't know this yet because we have to look. We don't have the explanations right now. What we think is that in

the course of drug-induced liver injury, perhaps an initial trigger corresponds to hepatocyte injury in these cells. And as I recall, it has been described, for example in diclofenac, that there are different changes in different phase 1 and phase 2 enzyme activities that can result in damage. So, this could be an explanation why genotyping for metabolic genes wasn't effective in identifying DILI patients.

DR. SZABO: Okay, thank you very much. I really would like to congratulate Doctors Gerbes and Benesic on this nice paper. Thank you. I believe that with this we come to the end of the conference. On behalf of the audience, I would like to extend congratulation and sincerest thanks to our organizers, Dr. Senior, Dr. Watkins, Dr. Avigan, and Lana Pauls. I also would like to thank the speakers and the audience for their active participation. And I suppose we shall look forward to having the meeting next year. Thank you. (3:56 p.m.)