by reaction with glutathione. So we depleted the glutathione. And there is actually a published study where they induced acute liver injury with amodiaquine. But they only got liver injury if they depleted glutathione.

3.3#12 JU: In this delayed onset injury, with a much lower dose, instead of increasing the toxicity it totally prevented the toxicity. So, a very different response, and I think that indicates a different mechanism.

3.3#13 JU: And so if immune tolerance is the issue, then maybe if we immunized animals with amodiaquine-modified hepatic proteins we could induce a stronger immune response and get more severe liver injury.

3.3#14 JU: And this is sort of a complicated slide, but in fact what we see, this is in unimmunized animals. And you see the mild liver injury. In the immunized animals there was no liver injury. And in fact, what we saw was an increase in myeloid-derived suppressor cells and T-regs.

3.3#15 JU: So, even when we immunized animals with drug-modified hepatic protein, what we induced was

immune tolerance. Even though we used Janeway's dirty little secret of an adjuvant we still got immune tolerance. It's really difficult to break immune tolerance in the liver.

3.3#16 JU: So, if that doesn't work, this was the time when checkpoint inhibitors were coming along, maybe we could block immune tolerance with checkpoint inhibitors. And the major ones are CTLA4 and PD1. And in fact, I've shown this slide before.

3.3#17 JU: In the wild type animals, adding antibodies against CTLA4 increased the injury, but it still resolves by continued treatment.

But in a PD1 knockout, when we add anti-CTLA4, we get sustained liver injury. In these animals, the histology looks almost normal, but in these animals we get piecemeal necrosis that looks very much like what happens in humans.

3.3#18 JU: And even though we've blocked immune tolerance, at least partially, the animals are trying to respond. In the PD1 knockouts there's a marked increase in T regulatory cells. And there's also an increase in cytotoxic T cells producing granzymes, et cetera.

3.3#19 JU: And we can block the liver injury by depleting CD8 T cells. So, this is what we would normally see. If we deplete CD8 cytotoxic T cells, we prevent the liver injury.

3.3#20 JU: And so we tried it with other drugs, with isoniazid. We don't get as much injury with isoniazid as we did with amodiaquine. Again, in wild type animals we see nothing. In the PD1 knockout alone, we see some injury. And if we add anti-CTLA4, we get more injury. But it does still resolve.

3.3#21 JU: The same is true with nevirapine. In the PD1 knockout, we get some injury. If we add anti-CTLA4, we get even more injury. But it resolves despite continued treatment with the drug.

3.3#22 JU: We can separate troglitazone from pioglitazone. This is in the PD1 knockout. This is with an anti-PD1 antibody, which is not nearly as effective. With pioglitazone we see no injury whatsoever. So we can differentiate troglitazone from pioglitazone.

3.3#23 JU: And we've tried other methods to decrease immune tolerance. We tried the anti-Gr1

antibodies. If you remember, as presented yesterday, this markedly increases the halothane-induced liver injury. And we found that it did increase amodiaquine-induced liver injury, but when we combined it with the PD1 and CTLA4, it was actually antagonistic. So these things are complicated. Gr1 is expressed on many other cells besides myeloid-derived suppressor cells. So it's complicated. We've also tried 1-methyltryptophan, which is an inhibitor of IDO, which Amy talked about. We tried anti-CD137. So, anti-CD137 directly activates T cells, and it's being developed, and we'll hear more about this, I think, this afternoon, to treat cancer. And the anti-CD137 alone causes liver injury. When we add amodiaquine, it doesn't further increase the liver injury.

3.3#24 JU: So, blocking immune tolerance, again, works, but stimulating the immune system does not potentiate the small molecule-induced liver injury. So, now that we have a model that I think represents, although somewhat artificial, a very similar mechanism of liver injury that we see with idiosyncratic DILI, we can test hypotheses. And

two other hypotheses that are very attractive are mitochondrial injury and BSEP inhibition. And so we should be able to, in the case of troglitazone, since now we see liver injury in this animal model, we can add another BSEP inhibitor and see if that increases the injury. That is what we would predict, if, in fact, BSEP inhibition is important. It's been shown in vitro that rotenone, an inhibitor of complex 1 of the mitochondria, increases isoniazid injury. Well, we can do that in vivo and see if, in fact, what was seen in vitro also occurs in vivo. And given the effect of metformin and other things I suspect it won't, but we'll see. We're interested in inflammasome activation and we've shown that some drugs that cause idiosyncratic reactions activate inflammasomes. But it would be very nice to be able to do this in vivo. And another I think important issue that we'll be talking about in a subsequent session is interactions with other drugs like vemurafinib. Again, there should be an interaction there, there is clinically, but not with the anti-CD137.

3.3#25 JU: Going back to what Mark Avigan asked

yesterday what are the risk factors in human? Obviously genetic factors are important. There are some reactions that have a very strict HLA association, especially with abacavir. But abacavir is fairly unique because with other HLA associations even if the patient has the appropriate HLA and are given the drug it's unlikely that they'll have an idiosyncratic reaction. And I suspect with drugs like isoniazid there won't be a strong HLA association at all. So, genetic factors are certainly important but they're not the whole issue. T cell receptors are different in even identical twins because they're produced by random recombination. But there are an awful lot of T cell receptors. Again, one would expect that activation of the immune system with inflammatory conditions would be a factor, but clinically and in models it doesn't appear to be. I think immune tolerance is important, but patients that have idiosyncratic reactions don't have the degree of impaired immune tolerance as our model. If they did, as Mark said, they would have reactions to all sorts of drugs and they don't. So although polymorphisms in IL10 and some other things are

weak predictors of increased risk I don't think that is the major issue.

3.3#26 JU: In fact, the immune system is a product of everything it's ever been exposed to. And something that I was not aware of until fairly recently was heterologous T cell immunity. So although there's an almost limitless number of T cell receptors, there are not a limitless number of T cells. You have a fixed number of T cells, and if you develop a new memory T cell to some new pathogen other T cells have to die. And so how can this limited number of T cells respond to all the potential pathogens out there? Well, if you think about it there are many ways that a peptide can interact with a T cell receptor. And so the same T cell receptor can recognize different immunogens, even if they're structurally unrelated. So this is how the immune system gets around the limited number of T cells, but it also means that if you're exposed to some pathogen that produces a strong immune response, and there are memory T cells, and you happen -- and those T cells happen to recognize a drug-modified peptide, even though it's structurally unrelated, you're in big

trouble. Now, the problem is I think hypotheses are only as good as you can test them. And I don't know how to test this hypothesis. I can't come up with a better explanation of why these reactions are idiosyncratic, what the major risk factor is, but I don't know how to test this hypothesis and that's a problem. And I should mention that there is certainly animal evidence that infection with one virus will affect the animal's response to another virus that's totally unrelated. So there's certainly experimental evidence to support this idea.

3.3#27 JU: The other thing, and Bob Temple did not like this idea, but if these reactions are really immune-mediated we should be able to treat them. And if we could treat serious idiosyncratic DILI it would markedly decrease its significance. I demonstrated that if you depleted CD8 T cells at least in our model that was protective. And I think most of the most severe idiosyncratic reactions, whether they be idiosyncratic DILI, or toxic epidermal necrolysis, are mediated by CD8 T cells. However, with halothane it looks like CD4 T cells are more important. So, even though I

think CD8 cells are most important they may not be the whole story. Certainly the usual care for toxic epidermal necrolysis is IVIG. Steroids are often given, but they do not appear to affect mortality.

Liver injury is often treated with steroids, but with the exception of autoimmune hepatitis I don't think they're very effective. Will Lee did a study, it's not a controlled study, but I don't think steroids are all that effective in treatment. With aplastic anemia the treatment is a combination of antithymocyte globulin and cyclosporine. And whether it be drug-induced and immune-mediated in that way or idiopathic this treatment almost always works. And so if I was going to pick a treatment to start with it would be antithymocyte globulin and cyclosporine. Again, I think the most severe cases are mostly mediated by cytotoxic T cells, but there are probably exceptions to that.

3.3#28 JU: So, in conclusion I think, although still I don't think everyone agrees, that there's compelling evidence that most idiosyncratic DILI is immune-mediated. Mild idiosyncratic DILI is more common than serious idiosyncratic if the mild

injury, and certainly the same HLA association where there is an HLA association is involved in the mild injury as the severe injury. So I think the mechanism is related. If it's immune-mediated then the adaptation must involve immune tolerance. I think in the past most animal models have not represented the same mechanism as the idiosyncratic reaction in humans. I think the amodiaquine example is a good one. Impairment of immune tolerance leads to models of idiosyncratic DILI that are similar to what happens in humans, and I think we can now use this model to test hypotheses that we weren't able to test as rigorously before. In terms of risk factors, genetic factors are certainly important, but I think heterologous immunity may play a very important role in determining who's at risk. And again, I would plead for a controlled trial to treat serious DILI. I think it has a good potential to work and would really help a significant number of patients.

3.3#29 JU: And with that I will end, thank the people who actually do the work and thank you for your attention.

(Applause)

DR. REGEV: Thank you very much. This is fascinating stuff.

We have about 30 minutes for discussion and questions. And as people make their way to the microphone I have a question for you, Jack, regarding this model. Actually it's two questions. Now, we have seen quite convincingly that using those checkpoint inhibitors just on their own with nothing else may cause significant immune-mediated liver injury. So, using that as an addition to another drug, or another disruption of the cell cycle, and in an attempt to uncover another response how would you differentiate which one is due to the other drug, and which one is due to the checkpoint inhibitor?

DR. UETRECHT: Well, I think it's the combination. And a speaker in the next half will talk about the interaction with vemurafenib. And I think such interactions can be anticipated. And dosing schedules will have to be developed so that they're not given simultaneously. Hopefully that will ameliorate the problem, but we'll have to see.

PARTICIPANT: Hi, Jack. So, my question came after you showed the levels of anti-P450 antibodies in certain patients. It occurred to me, I mean those are patients that are showing liver injury. But might a contributing factor be patients who already have levels of those antibodies preexisting through some other mechanism? Because I mean there is evidence out there that some people have those.

DR. UETRECHT: Well, certainly some forms of autoimmune hepatitis have antibodies against P450. In the control samples that we did we didn't find them, but of course we didn't -- we couldn't possibly check the patients before they were treated.

PARTICIPANT: Right.

DR. UETRECHT: I think that's unlikely, but I have no evidence so I can't be sure.

PARTICIPANT: It's a tough question to answer. Well, thanks.

DR. NORCROSS: Hi, it's Mike Norcross, FDA. So I've got a couple of criticisms and also some comments. The first is if you don't find a specific HLA link to a drug reaction it doesn't mean

really that HLA is mediating those responses in a non-linked fashion. In other words, other HLAs can present epitopes that are either haptenated, in this case probably after metabolism. So I'm making a point that most of these are probably HLA-mediated antigen presentation, and linkage is only in the specific ones where in some ways maybe a drug fits into a specific pocket like we found with abacavir.

The second thing I want to follow up with what Arie said. And I think that's something that I noticed in your papers that you publish. Most of it doesn't have anti-CTLA4 alone without the drug. I know you have maybe one figure out of a number of papers, but I think we'll hear this afternoon that the checkpoint inhibitors can actually induce an autoreactivity. And I noticed in the ones you showed today actually you always put the drug, and then drug plus antibody, and for some reason you leave out the checkpoint antibody by itself. And I'm not clear why you do that. I mean, I assume one time it doesn't work and then you don't have to repeat that. But in our experience, especially in mice we always do the control with whatever

antibody you're doing, even if it's just immunoglobulin.

The second point, or third point is the heterologous immunity. That's been proposed by others obviously with different drugs, that you have an antiviral response, it cross-reacts. But I make the point that I don't think you need memory -- I mean, you need memory cells. I'm not saying that I'm against that concept. It sounds great. You can get rapid responses. But as far as can a naive cell do this, yes, obviously, a naive cell can respond to neoantigens. For instance, in what we look at with abacavir, or even flucloxacillin, and it's been published, that you can develop in vitro responses in a normal person that has the same HLA. Those responses in general are coming from naive cells. You can have both naive and memory, but we're talking about an anti-drug response generated from a naive cell. So, it's not really clear you need memory. What the key element is that tips the balance in the liver is another story. We haven't really figured that out. But I'm just making a point that it's not all just memory cells. When you rechallenge, that's a different story.

You've got memory cells. For some reason or another they're not suppressed and they can respond very quickly.

So, do I have any other -- no.

DR. UETRECHT: But again in the liver the dominant immune response as Amy said is immune tolerance. And so if you haven't somehow primed the immune system before the immune response is going to be tolerance, and that's what we see. And so, yes, especially if it was in the skin naive T cells might work very well, but not in the liver. Because if the first exposure is in the liver which is what will happen with a reactor metabolite you get immune tolerance. Yes, we did do controlled experiments with antibody alone and you don't see anything. We have a problem in that the anti-PD1 antibodies don't work nearly as well as the PD1 knockout. And breeding up enough PD1 knockout mice is an issue in keeping up with the experiments.

DR. NORCROSS: But I think the key issue is when you use checkpoint inhibitors, even in your experiments, how do you know that's directed against drug? The drug may just accelerate the self-reactivity of this checkpoint

inhibition. So I'm asking even a broader question. You use drug to initiate this, but how do you know what you're seeing has anything to do with drug? How do you know they're directed against the drug itself, rather than self?

DR. UETRECHT: Well, certainly it's conceivable, although I would propose unlikely that the drug is somehow -- especially when we're talking about multiple drugs, that the drug is somehow initiating an immune response unrelated to the fact that it forms a reactive metabolite. I can't say that that's impossible, but it seems like a much less likely hypothesis.

DR. REGEV: Thank you. Terry?

DR. WRIGHT: Terry Wright, Genentech. A question for Dr. Bonkovsky. I was interested in your observation of autoimmune hepatitis associated with interferon. And my question relates to -- first of all, how you define that in the setting of a patient who almost certainly has hepatitis B and hepatitis C, number one.

Number two: is there a different in risk depending on whether patients are hep C positive or hep B positive?

And number three, if there is a liver disease associated with the drug itself do you think that could have a role in the non-response or the disease associated with non-response to interferon? Because most of our trials you know have been done uncontrolled -- we haven't had uncontrolled comparators. We've basically treated patients. We've looked at the response and non-response. We haven't actually looked at the never-treated patients in terms of their liver disease.

DR. BONKOVSKY: Well, fortunately it's relatively rare, and of course we're not generally using interferon for treating viral hepatitis in the new era of direct-acting antivirals. But it certainly, you know -- it's hepatitis with autoimmune features. Now, of course autoimmune features occur in a minority of patients with hepatitis C as well, and yet some of these older case reports from the annals and so on in the early years of interferon were pretty convincing that, yes, in a few patients adding the interferon really did seem to trigger an autoimmune hepatitis response with all of the trimmings, with positive smooth muscle, and lots of plasma cells in the

inflammatory infiltrates and so on. With respect to the second question I think most of the evidence for this has come from the hepatitis C patients. I myself, I mean you know, many of us have treated more hepatitis C with interferon than hepatitis B so our experience is greater. I can't think of a single case that I've treated years ago with hep B who developed this. Maybe you've had that experience because I think you used to treat more Asians who have the higher prevalence of hepatitis B. I've sort of forgotten your third question.

DR. WRIGHT: Well, the second part of course is interferon is still being used for the treatment of hepatitis B. I gather that it's not being used for hepatitis C, but it is still being used for hepatitis B.

DR. BONKOVSKY: Right, right.

DR. WRIGHT: But, now the third is whether there could be a component, I don't know the mechanism of -- the sort of cytokine mechanism of disease, but could there be a contribution of that mechanism to liver disease in non-responders to interferon? Because our trials have looked at sustained responders and non-responders. Our

trials have not compared untreated patients and the disease associated with treatment in patients who don't respond. That's my question.

DR. BONKOVSKY: Yes, I think that's quite possible, but I just don't have sufficient data to be able to answer the question. If I could ask Jack a question. You know, we've talked a lot about adaptation. Have you studied that from an immune standpoint? What's your current view of what is actually going on when there's adaptation? You've shown all these things. Oh, they got better even though the drug was continued. What are the key factors there? Is this a reestablishment of immune tolerance somehow?

DR. UETRECHT: Well, certainly like with the amodiaquine what we see is an increase in T regulatory cells and myeloid-derived suppressor cells. And there's so many things in the immune system that you can investigate. We could do an almost limitless number of experiments. But certainly we see an increase in T regs and myeloid-derived suppressor cells. In the patients getting isoniazid we saw an increase in T cells making IL10. They weren't classic T-regs. And

unfortunately, unlike the animal experiments where we can look in the liver we're limited in humans to looking at peripheral blood. I'm sure there's a lot more going on in those humans if we could look in their liver. But there's clearly an up regulation. And even more so when we do things like use the checkpoint inhibitors. There's a huge increase in -- the immune system is trying to get back to that balance that Amy was talking about.

DR. REGEV: Can I catch right on this question just to follow up? I know you check mostly things that are related to immune tolerance. But do you also check anything that has to do with regeneration in your studies to explain adaptation?

DR. UETRECHT: There's certainly with amodiaquine some increase in turnover of cells, but that's sort of a different issue. We haven't studied changes in metabolism in the -- when we depleted glutathione we did not see any increase in covalent binding as you might expect. And I think what happens is when we depleted glutathione through different mechanisms we up-regulated other protective mechanisms like reductases that reduced

that oxidized reactor metabolite back to the parent drug. So overall we didn't see any change in covalent binding. The more surprising thing is that it actually prevented, and there's evidence that NK cells both -- there's evidence if we deplete NK cells in the mild injury we prevent the injury. And there's evidence that NK cells require glutathione to be active. And so I think what we've done is just hobbled the NK cells so they can't do what they would normally do. But that's speculation.

DR. SISTARE: Hi, Frank Sistare from Merck. I want to pick up on a line of investigation that Mike started. So Jack, it does seem like your fundamental hypothesis is that there's this covalent binding neo-antigen creation. But the one experiment you showed to sort of test that, the BSO experiment, came out the opposite way that you expected it to. With all the other experiments you've done now in these immune checkpoint type models, you've got troglitazone, you've got isoniazid, you've got amodiaquine, there's other things you could do to sort of regulate the level of that covalent adduct formation, inducers, other inhibitors of other pathways, that kind of thing.

Have you done any of those additional sort of tests to manipulate? You would expect the more you form or the less you form you would get a different reaction in the liver. I think that is fundamental to your hypothesis.

DR. UETRECHT: Yes. And as I mentioned when we looked at covalent binding, when we depleted glutathione it didn't go up as you might expect. And I think it was because of --

DR. SISTARE: Didn't go up, but you suppressed, if I understood it correctly, you actually suppressed the level of effect that you did have.

DR. UETRECHT: Yes. So we got basically the same amount of covalent binding, but it was protective. So you would expect increased covalent binding. We didn't see increased --

DR. SISTARE: You didn't see increased, but you at least preserved the same amount of reactivity to it. But it went the other way. And that's a complicated experiment, understood, yes.

DR. UETRECHT: It's hard to prove that a chemically reactive metabolite is responsible

for an idiosyncratic reaction. The one place where we were able to do that was not DILI. It was with nevirapine-induced skin rash. And the final pathway for covalent binding is making a reactive benzilic sulfate in the skin. And where we apply a topical sulfotransferase inhibitor we block the covalent binding and we block the rash where we apply that sulfotransferase inhibitor. And certainly now that we have an animal model it will be a little more difficult to manipulate the metabolism in the liver than it was with a very specific pathway in the skin. But again, I think the major benefit of this model is going to be to test these hypotheses.

DR. SISTARE: And I think also to Mike's point, to bring a lymphocyte transformation test into that model too, to show that is the antigen that's being created. It's not some other indirect mechanism.

DR. UETRECHT: And it's surprising, you know, the studies have been done and in general the lymphocyte transformation test is falsely negative in about 50 percent. It's surprising that it's positive at all because it's presumably a

drug-modified protein. And with isoniazid, not my work. With the mild injury it was only positive if you used a drug-modified protein. And if you don't know what the reactor metabolite is you can't modify the protein in the appropriate way. So as a general test --

DR. SISTARE: But even if you're successful 50 percent of the time that's still, that would be exciting I think. And also TNF alpha manipulation. Is that on your plan as well? To sort of manipulate to see if you can --

DR. UETRECHT: It wasn't. There's so many things that we can do I don't know sometimes where to start.

DR. REGEV: On the other side.

DR. OMOKARO: Stephanie Omokaro, FDA. I wanted to know is there anything known about patients with multiple drug allergies that could be applicable here? Are they more at risk for DILI? Could there be chronic or subacute forms of DILI going on?

DR. UETRECHT: There are such patients, but they're rare. In the drug safety clinic that I spent quite a few years at when a

patient came in and said they were allergic to all drugs red flags immediately went up. And I never found one that was. So, again, I think such patients do exist and they may have some genetic defect in their immune system that does, in fact, make them susceptible to multiple drugs. But that's not very common in my experience.

DR. BONKOVSKY: If I can add to that. In the Drug-Induced Liver Injury Network we now have about 1500 total patients enrolled in the prospective study. And there may be about 10, maybe not even 10 that had pretty good history of a prior drug-induced liver injury and they had it again from a different drug. There have also been a few that had it again from the same drug because they were reexposed to the drug. So it is rare. But about 50 percent of our patients give a history, oh you know, how good is a history and so on, of having drug allergy when -- at the time that they're enrolled. So it does seem as though maybe that's higher than if we just took a poll of everybody in this room how many of us have a history of drug allergy.

DR. REGEV: You did try to do that,

compare it to just a similar population? How high is the drug allergy history?

DR. BONKOVSKY: We do not have a control population that didn't develop DILI. That would be great, of course, to have.

DR. REGEV: Right.

DR. UETRECHT: And certainly there are polymorphisms in things like IL10 that can affect risk.

DR. REGEV: Neil?

DR. KAPLOWITZ: Jack, of course you're always very provocative. Gradually over the years you sort of wear me out. (Laughter)

DR. REGEV: Because he's usually right.

DR. KAPLOWITZ: Well, I don't know about that. But you know, I'm kind of intrigued by something you showed which is basically that the inhibition of the immune checkpoints seem to actually increase the initial injury. So that raises a question in my mind regarding the kinetics. Because the conversation has been sort of focused on adaptation, but it seems to me that it's very likely that this is sort of a dynamic

process that is concurrently both pro-immune and immune tolerance are battling each other from the get-go. So one could envision the outcome of that being absolutely no clinical phenotype, or a mild phenotype, or a severe phenotype, let alone the question of adaptation. So, I'm kind of curious about the kinetics of the development of tolerance. Is this something that starts right away, or does it follow a sequence?

DR. UETRECHT: You know, there's a limited number of times we could sample the isoniazid patients because we would just get blood with their monthly routine. And there's even a limited number of times we can sample the same animal. We certainly need to look at the evolution. Because it isn't just a specific immune response. I think that immune response evolves over time, and it's something that needs to be much more clearly defined than we've done to date. I think there's a lot to be learned there.

PARTICIPANT: Hi. I come from China. I have two questions to Jack.

DR. REGEV: If you came all the way from China you can have three questions.

PARTICIPANT: Thank you. I have two questions. One of the slides shows that the patients with milder DILI has increased in TH17 cells. Is there some difference between the frequencies of TH17 cells between mild DILI and the serious DILI?

And question two is: what's your opinion about the T helper cells in the development of DILI.

DR. UETRECHT: The first one, you know, I don't even know in those six patients that had a small increase in ALT, I can't remember their sex. And six is small enough, I'm not sure that it would be that meaningful. Some DILI is more common in females, other isn't. I can't remember with isoniazid. Will, is there much of a difference? I didn't think so. So, I haven't really looked at the sex differences. What was your second question?

PARTICIPANT: What's your opinion about the role of T helper cells in the development of DILI?

DR. UETRECHT: There is such a large number of cells. I mean, even CD4 T cells. There's so many different CD4 T cells, they don't

have to follow our rules. So that the same apparent phenotype can do very different things depending on what molecules they're expressing, what cytokines they're producing. Certainly helper T cells are involved in immune responses, but there are so many cells, as Amy was suggesting, it's really complicated. The more I try to understand what's going on, the more I almost feel like I'll never really understand what's going on.

DR. ROSENBERG: Just to add to that a little bit. The phenotype and the cytokine profile of T helper cells is not fixed, it's plastic. So even if you have regulatory T cells that have suppressive activity, if given enough stimulation and activation they can be converted back into T effector cells. So it's not -- it's plastic. It's not rigid.

PARTICIPANT: I came all the way from Silver Spring, so.

DR. REGEV: Half a question.

PARTICIPANT: Half a question. It's not for Jack, but for Herb, actually. So, I have a patient that I referred to NIH that had a little bit of alcohol exposure let's say in the past, but

was sort of misdiagnosed for having multiple sclerosis because he had a lytic lesion in his spine and was put on interferon beta basically for 10 years while he was on statins. So, I wonder -- just asking whether you've seen -- and then now has kind of developing cirrhosis. And I'm wondering whether in your, what you talked about with these adverse effects with interferon beta in this case, have you seen anything along the lines of other co-factors, sort of a statin-interferon beta, and then this unusual I think, unless you've seen other cases like that, development into cirrhosis. Because I think the alcohol is a confusing factor and there's been no exposure in 20 years. So I'm just asking actually for everyone else if they've seen any cases like that.

DR. BONKOVSKY: Well, that specific question I need a consult from Dr. Fontana who was the first author of the paper. Do you remember that level of detail, Bob?

DR. FONTANA: So, we published a paper in American Journal of Gastro I think just this past year looking at two-year outcomes of patients with DILI. So, the protocol is everyone who gets

referred we plan to see them back at six months. And so if at six months after DILI onset you still have evidence of liver disease, like abnormal LFTs and so on, then we followed everyone out to two years just to see what happens. And basically we had about 120 patients who had quote unquote "evidence" of ongoing disease at 6 months. When you follow them out to 24 about three-quarters of them still have some mild evidence of liver disease for the most part. One quarter were just sort of slow resolvers. In other words their enzymes normalized by month 12 or 24, and it appears that they didn't have liver disease. But we don't have liver biopsies. Because as we saw yesterday, and as I mentioned and Arun mentioned, you can evolve into cirrhosis with normal LFTs. So, what we're planning to do going forward is to do a little more sensitive testing of these patients prospectively like the FibroScan which is non-invasive to see is the liver getting stiffer over time just during pure observation. So all that being said within the 110 patients that we followed out to 2 years there clearly were patients who had evidence of cirrhosis. It was oftentimes a bland cirrhosis, but

they had, you know, a big spleen, and clinical features that were consistent with cirrhosis. So it does happen from DILI. The frequency is probably pretty low. But that's the best we can say. And it wasn't just one drug. And Herb is in the process of writing up the vanishing bile duct. I don't know if you want to comment on that also in terms of the evolution of that.

DR. BONKOVSKY: We have a total of 26 patients who on the first biopsy, or at a biopsy at a certain point in the course had just a lack of bile ducts, moderate or severe. And those that had severe loss, so at least 50 percent of the portal tracts on an adequate biopsy that didn't show bile duct had a very poor outcome. They died of liver failure. They needed liver transplants. They had ongoing disease a year and two years later. Bob, in that -- but in the interferon for multiple sclerosis do you remember any patients that were on statins?

DR. FONTANA: Yes, that was complex. We did have, I remember there was one patient who was on it for 10 years, sort of similar to what you're describing, and then got DILI which is very

strange. How does that happen? And these patients, again, if they had strong autoimmune features oftentimes got treated with steroids. And then this becomes even more convoluted in that, well, how do you know it's not drug-induced autoimmune liver disease versus sporadic autoimmune disease in someone who has an autoimmune disease that you're starting with. And the clinical thinking behind that is, well, if you taper the steroids off and they do okay it probably was drug-induced. But you know, that's a bit empiric. And so in the 11 cases that we had I think half of them got treated with steroids. And we tried to taper them as best we could, but it's incomplete information. So, your particular patient, were they having sort of a subclinical injury for a hile and then they ended up with cirrhosis from the interferon beta? It’s certainly possible. Or did they have preexisting disease like we talked about yesterday that they may have already been halfway there and then they got accelerated with the drug.

DR. REGEV: Bob, before you go I think I asked you that before. And these 110 patients, I mean they're obviously very disturbing for many

people including drug makers. Because all of a sudden we have this new entity of chronic, prolonged DILI which Hy Zimmerman never thought of. And the question is: are we actually dealing with a real entity given the fact that you don't have baseline levels in those patients? This is not a typical prospective study. It's likely that some of your patients before they were presenting with this dramatic drug-induced liver injury they might have had some ongoing process that was not documented anywhere because nobody was following, +or even documented and they had mild elevations of ALT.

DR. FONTANA: So we, by definition when we did the analysis we excluded anyone who had preexisting hep C to actually, for example, avoid that issue, was it hep C that just progressed. And then when we looked for sort of clinical features suggestive of metabolic syndrome, or potentially risks to having NAFLD to start with we didn't see an over-representation. So I really do think it's real. Like you said, we don't have a baseline liver biopsy before they started the drug, but for all intents and purposes there was no medical

history or laboratory evidence that they had overt, chronic, preexisting liver disease before they got the DILI.

DR. REGEV: And how far do you go as far as excluding metabolic diseases, such as -- I'm sure you do that. Just hemochromatosis and --

DR. FONTANA: Pretty thoroughly. I mean, everyone gets worked up for every chronic liver disease in every case prospectively.

DR. REGEV: Alpha-1 antitripsin.

DR. FONTANA: Yes.

DR. REGEV: And Wilson. You go over everything.

DR. FONTANA: Yes. I do think it's a real entity. And it is disturbing because it was 18 percent of all the DILI cases had some evidence of chronicity at six months. And then 75 percent of them at two years were still going on. So you do the math, that's 15 percent overall of the consecutive DILI cases have evidence of some injury two years after they had the DILI episode. And the features when they got the DILI that were predictive of them going there were the cholestatic patients and those who were a little bit older.

DR. REGEV: Thank you.

PARTICIPANT: I just want to say in the case I was talking about that a neurologist -- sorry --

DR. REGEV: One last question.

PARTICIPANT: -- was taking care of this. And basically the patient was told, oh, your liver enzyme elevation is because of your statins, and we all know that the statins increase that, while they were getting interferon beta. And that went on for 10 years.

DR. BONKOVSKY: I mean, I'm not aware that statins increase injury, or increase abnormal liver test from beta interferon. I don't know where that comes from.

DR. REGEV: I'm afraid we are at the top of the hour so we'll probably have a lot more to talk about, but we are going out for a break until 10 a.m.

(Applause)

(Whereupon, the above-entitled matter went off the record at 9:45 a.m. and resumed at 10:00 a.m.)

MS. PAULS: Okay, we are going to get

started. And just while I'm waiting for a couple of people here please note that we are already in the process of planning the meeting for 2017. So pencil in your calendars March 22 and 23. Again, we avoided all spring breaks to our human possibility as well as some of the major meetings. So please pencil that in. I talked to John yesterday. He already has a vision for the 2017 meeting so there will be more to come.

DR. ROSENBERG: So, welcome back to the second session of the morning. And again this is focused on checkpoint inhibitors, immunotherapy and the unleashing of autoimmune DILI in this setting. And our first speaker is Cyril Konto who will be speaking about DILI caused by checkpoint inhibitors and other anti-cancer antibodies. Looking forward to this.

3.4#1 CK: DR. KONTO: Thank you. I'd like to start by thanking the organizers for your kind invitation. And to introduce myself I'm Cyril Konto. I'm a medical oncologist and clinical lead at Pfizer for the immuno-oncology portfolio responsible for the early phase of the clinical development. I'm going to talk about the DILI

caused by immune checkpoint modulators used to treat cancer. The original topic was pretty broad so I had to make arbitrary choice and I focused on what is doing the buzz today, immune checkpoint modulators. But there are also ADCs by specifics that could be covered at a later meeting.

3.4#2 CK: I'm a current employee and shareholder of Pfizer. As a disclaimer the opinions that I'm going to express in these presentation are my own and do not reflect the view of Pfizer, Incorporation.

3.4#3 CK: What are we talking about? We're talking about those modulators that could either boost the -- inhibit the blocking, the inhibitory receptors. Those are blocking antibodies on the left. And they're mainly CTLA4 and PD1 at this stage. Both got approval in melanoma. PD1 also got approved in non-small cell lung cancer and renal cell carcinoma. There are monoclonal antibodies, either -- mainly IgG4 monoclonal antibodies targeting the receptor expressed as a surface of activated T cells. And those CTLA4 and PD1 receptor were mainly put in place to shut down an immune response. By inhibiting the inhibitory

receptors we hope to boost the activation of those T cells. And I'm going to show you that we succeeded. On the right-hand side of this slide are the activating receptors on the surface of activated T cells. And I'm going to talk about the experience with CD137. I had to pick one. But we could also talk about OX40 receptor at the surface of activated CD4 T cells, in a shorter time on activated CD8 T cells. Indeed our receptor which is expressed on the T-regs.

3.4#4 CK: Why is there so much enthusiasm about immune checkpoint modulators as an introductory? I picked up one of the overall survival figure. And this is the survival outcome of the experience with nivolumab in untreated advanced melanoma patients who are BRAF wild type. The yellow curve here shows the survival outcome of patients who receive nivolumab compared to those who received the standard of care, dacarbazine. Those curves speak by themselves. There was a statistically significant risk reduction in death with a hazard ratio of 0.42. With those immune checkpoint modulators we've also learned a new type of safety profile.

3.4#5 CK: I show you on this slide the safety profile of nivolumab in pre-treated advanced melanoma patients. Nivolumab is a fully human IgG4 anti-PD1 immune checkpoint inhibitor. And this could also be the same slide for pembrolizumab, the PD1 from Merck. You can see here that the hepatic drug-related adverse events is in the range of the fourth or fifth most common drug-related adverse events. In that study any grade liver, drug-related adverse events occurred in 5 percent of the treated patients and severe hepatic adverse events occurred in 1 percent of them.

3.4#6 CK: What are the DILI due to immune checkpoint modulators? There are mainly and I should say exclusively immune-mediated hepatitis. Most frequently they are diagnosed clinically. There were a few patients who had liver biopsies and those patients were mainly treated with ipilimumab at the early stage of immuno-oncology when we were learning this safety profile with immune checkpoint inhibitors. Those hepatitis are defined as LFT elevation after ruling out other etiologies. And you can imagine that those are cancer patients. Some of them have liver

metastases. Some of them receive -- come with potential hepatotoxicity. Those hepatitis case require corticosteroid therapy. The patient had variable imaging findings according to the clinical severity. Interestingly, those cases of hepatitis are mainly asymptomatic, and in the rare case where symptoms are present they are general weakness, nausea, vomiting, or dizziness.

3.4#7 CK: If we look at the increased incidence of liver test abnormalities compared to baseline in patients who were treated with nivolumab we see that for AST, ALT and total -- and alkaline phosphatase they're almost double compared to the chemotherapy control arm. Looking at the definition of hepatitis I gave you earlier we observed 2.5 percent of any grade hepatitis. And CVR grade being grade 3 and grade 4 hepatitis were diagnosed in almost two percent of the patients receiving nivolumab. And pardon me, here I'm using the CTCAE grading systems. I wish everyone is aware of that system. Grade 1 for AST/ALT elevation range up to 2.5, grade 2 up to 5, grade 3 up to 10, and grade 4 above 20. With regards to presentations, the median time to onset was pretty

long for anti-PD1 checkpoint inhibitor, compared to experience with anti-CTLA4 inhibitors, 3.7 months ranging from 6 to 9 months. The way we treated those patients, if the LFTs were greater than -- the transaminase elevation was greater than 8x the upper limit of normal we would discontinue patients. And this was the case in 5 patients. Otherwise we would withhold therapy until recovery. There was complete resolution in almost three-quarters of the patients. Resolution in that case again is a clinical definition where we expect the LFTs to recover to baseline number with the completion of the corticosteroid therapy. We talked yesterday about rechallenge. Four patients were rechallenged and one had a positive rechallenge with a recurrence of immune-mediated hepatitis with nivolumab.

3.4#8 CK: Switching backward to anti-CTLA4. Ipilimumab was the first approved anti-CTLA4 IgG4 in monoclonal antibodies, the first immune checkpoint inhibitor approved if we disregard the cytokines previously approved. Interestingly, for ipilimumab there are two doses currently approved: a dose of 3 mg/kg given every 3 weeks for a total

of 4 doses in advanced melanoma patients, and a dose of 10 mg/kg with an induction regimen, given every 3 weeks for 4 doses, and a maintenance regimen given every 3 months to up to 3 years. Very few patients continue therapy after 3 years. What we observed with ipilimumab is a dose- proportional incidence of hepatitis cases. At the 3 mg/kg dose severe cases of hepatitis were diagnosed in 2% of the patients. Some of them were fatalities. A quarter of them required hospitalization. And again, these were the very first cases of hepatitis due to immune checkpoint modulators we diagnosed here. And moderate hepatitis, so-called grade 2 hepatitis was diagnosed in 2.5%. Onset of liver injury in patients treated with ipilimumab was earlier than for patients treated with nivolumab, usually after 2 to 4 cycles ranging from 3 to 9 weeks.Eenzyme elevation was most frequently hepatocellular. Also we noted cholestatic pattern at the onset of injury. With regard to the 10 mg/kg dose, you can check yourself. The incidence of severe hepatitis was significantly greater: 10% of the patients experienced severe hepatitis; 5% had moderate hepatitis. The median time to onset is pretty

similar to the 3 mg/kg dose. There was a complete resolution in most of the cases, testifying to the high level of education of our medical community with immune checkpoint inhibitors. The 10 mg/kg dose was approved last year, very recently, after a long experience with this immune checkpoint inhibitor. Median duration of corticosteroids ranged from 2.6 to 4.5 months.

3.4#9 CK: This is a case of a 63-year-old patient with melanoma treated with ipilimumab. And you can see on the upper left corner the pattern of the LFT elevation where at the onset we had both hepatocellular and cholestatic patterns. And you can also note a relapse during the taper off of the corticosteroids. The CT scan shows here edema in the periportal space, and also here you can see enlarged lymph nodes in the periportal space. If you look at here you can see at the ultra sonography hyperechogenicity of the portal area and the periportal space. Here is the gallbladder with a multilayered thickening that likely represents an inflammation of the gallbladder. We had similar T2 hyperintensity on the MRI. With the histopathological findings in this patient showed

here, hepatitis with an infiltrate of lymphocytes, with very few fields depicted on this slide, also with signs of endotheliitis on the lower right. The patient was treated with corticosteroids.

3.4#10 CK: Having those two breakthrough therapies in the field of melanoma with two different immune mechanisms, a CTLA4 blockade boosting the T cell in the peripheral blood, and pouring them, and inducing proliferation of cytotoxic T cells, and having PD1 inhibitor blocking the mechanism of immunoresistance within the tumor environment we logically tried to combine those two compounds.

3.4#11 CK: The combination of nivolumab and ipilimumab achieved interesting results in terms of survival of the patients compared to our previous experience in advanced melanoma, but also was a cause for concern with regards to the severe safety profile.

3.4#12 CK: More than half of the patients had severe side effects with this combination. And here it's represented, the increased ALT and AST in the range of 10 percent of subjects treated with the combination of nivolumab and ipilimumab. We

also tried to combine immune checkpoint inhibitors with TKI. And this was a failed experience that we had with Roche. At the time I was at Bristol-Myers Squibb when we combined ipilimumab and vemurafenib. They were two recent drugs approved in the field of advanced melanoma. And we wondered whether we could achieve better disease control and also longer survival with this combination. Unfortunately there were synergistic liver toxicities that resulted in the phase I study termination. We published the results in the New England Journal of Medicine.

3.4#13 CK: This study was a phase I, a phase I study with a primary objective of finding the utility of this combination. We had originally a lead-in -- we had a lead-in phase with vemurafenib for 28 days. And then we did combine ipilimumab and vemurafenib. The DILI observation occurred was during the combination obviously. We had three dose levels planned originally but were only able to treat patients at the starting dose and the -1 dose reduction. Vemurafenib was given at the approved dose, 960 mg BID, and ipilimumab at 3 mg/kg. And we tried to reduce the dose of vemurafenib,

maintaining ipilimumab at 3 mg/kg but failed.

3.4#14 CK: The outcomes were published. Among the 12 patients who were enrolled into those two dose levels only 10 received the combination of ipilimumab and vemurafenib. Six out of those ten patients had a severe transaminase elevations including two patients who had grade 2 to 3 bilirubin increase. Those patients were asymptomatic. We couldn't get any liver biopsy in oncology center in the U.S. unfortunately. This hepatotoxicity finding was higher than what we could expect. It was even higher than the sum of the two hepatotoxicities reported for either single agent at that time. The USPI of ipilimumab showed 2 percent of severe hepatitis, and the USPI of vemurafenib had 3 percent of LFT elevation. The worst case was a grade 3 case, and hopefully all the cases were reversible with the established management decisions.

3.4#15 CK: I'd like to finish with the unfortunate case of 4-1BB receptor and urelumab. 4-1BB is also named CD-137. It's a TNF super family receptor. It's expressed on the surface of activated T cells, CD4, but mainly CD8 T cells, activated NK cell and

NKT cells. Its ligand, CD-137 ligand is expressed on macrophages, monocytes, dendritic cells and B cells. And this ligand enhances the ability of the CD-137 positive dendritic cells to -- for T cell responsiveness to alloantigens. Also the CD-137 ligand has been shown to augment T cell trafficking of those activated lymphocyte from the peripheral blood to the tumor environment. Urelumab is a drug that is being developed by Bristol-Myers Squibb. It's a first agonistic anti-CD137 fully human IgG4 monoclonal antibody. It's a non-blocking CD-137 ligand monoclonal antibody. The non-clinical toxicology studies performed with a mouse surrogate antibody of urelumab at multiple doses have shown skin and liver toxicity in mice, but no such toxicity in monkeys. In the clinic we observed hepatotoxicity was the most frequent toxicity with urelumab at doses above 1 mg/kg. And two patients actually died from severe hepatitis at 1 and 5 mg/kg in the early phase I trial that ended up on clinical hold. After BMS did further research on the cases of hepatitis among the entire program, it was realized that the entity was certainly below the 1 mg/kg dose given every three

weeks. And the current clinical trials are run at this dose, at the flood dose that is actually even smaller than 1 mg/kg. The most frequent grade 2 level abnormalities were ALT and AST elevation. And the anti-tumor of urelumab was observed across the different doses. There is no impairment of the efficacy expected by selection of this lower dose.

3.4#16 CK: Pfizer is also developing an anti 4-1BB. And I'm showing you on this slide the current safety profile of this asset that is in phase I clinical development. And you are certainly searching for the liver toxicity on this slide. It does not appear. There was one controversial cases of severe LFT elevation that was at the end adjudicated by the investigator related to the disease progression and not to the drug.

3.4#17 CK: The difference between those two assets is striking. For the same indication with doses that range, it was the same drug exposure in patients, so we tried to understand the possible differences between urelumab and utilimumab, the new generic name given to this Pfizer 4-1BB.

3.4#18 CK: I'm showing you on this slide our two main hypotheses. The first, I think is the same

between the two monoclonal antibodies. The first is the epitopes. The epitopes targeted by urelumab are on the cysteine rich domain 1 and 2. And it's actually outside the human 4-1BB ligand binding region which explains that urelumab is a non-blocking monoclonal antibody. On the other hand, utilimumab is binding on the human 4-1BB binding -- 4-1BB ligand binding region, cysteine rich domain 3 and block the binding of the natural 4-1BB ligand.

The second hypothesis, which we don't know yet how it can translate, is the difference in the isotope of the monoclonal antibody, IgG2 versus IgG4, just 1 ml of IgG2 in the micro-environment. There are more macrophages than NK cells, and IgG2 actually in human beings triggers ADCP, an antigen-dependent phagocytosis by the macrophages that are present in the tumor micro-environment.

3.4#19 CK: This is my last slide. Those are the take-home messages I'd like you to take from this presentation. And I would like to start with this statement that the benefit of the immune checkpoint modulators outweigh the risks has been shown by the multiple recent approvals. We do observe mild to

moderate serum LFT elevations during treatment with anti-CTLA4 and anti-PD1. Those DILI are asymptomatic, detected by LFT monitoring. And we recommend testing LFTs before starting therapy and regularly on treatment prior to each dose. The biochemistry findings were hepatocellular patterns of enzyme elevation, but they can be mixed, as I said earlier. Severe hepatitis was diagnosed in 2 to 10 percent of the patients treated with immune checkpoint modulators. The range of the incidence of the hepatitis depended on the type of checkpoints used, on the dose and on the combination. The DILI with those immune checkpoint modulators have an early onset, usually 3 to 15 weeks after the initiation of treatment, and on the pathological findings this time we observe an immune-mediated hepatitis with focal or confluent necrosis and preeminent infiltration of activated T cells. And as Arie is going to tell you in a second, corticosteroids are the main treatment, and as I showed you they are often successful.

Thank you very much for your attention.

(Applause.)

DR. UETRECHT: The next speaker is Arie Regev, who really needs no introduction, but he was an academic hepatologist at the University of Miami and then moved to Eli Lilly to be in charge of liver safety there, and also appointed at the University of Indiana. Arie?

3.5#1 AR: DR. REGEV: Thank you very much. And thanks again to John and Lana for inviting me. This is a great opportunity to discuss this topic, mostly because we get to discuss it with oncologists and hepatologists at the same time. I think it's a great opportunity. At the very least we should end up with at least one name for this entity rather than four different names.

3.5#2 AR: And a little bit of a background information. So, DILI due to cancer immunotherapy targeting immune checkpoints is one name for this entity. We could call it immune-related hepatitis. We could call it immune-mediated hepatitis. The jury is still out. But this is one of the leading, as you've heard, immune-related adverse events occurring in patients receiving checkpoint inhibitors. And you've all heard the huge advantage of these molecules. They basically unleash the

immune system to cure cancer. This is something that has achieved results that we've never seen before with cancer patients. And the severity of liver injury in those patients may range all the way from mild increases in ALT, which is completely asymptomatic, to all the way to fulminant hepatitis, what we would call acute liver failure and death. The percentage of what is called grade 3 to 5, I'm not sure I'm very comfortable with this terminology, but grade 3 to 5 injury occurred in 1 to 7 percent of the patients. And unfortunately, like we've seen in other cases of idiosyncratic drug-induced liver injury, presently there is no dependable way to identify which patient will develop severe liver injury or liver failure out of hundreds or thousands receiving this treatment, and we have no diagnostic biomarkers. So if there's an abnormal liver test, we have no way to diagnose that this is actually what is going on. By the way, the numbers I'm showing here, as just noted by Cyril, they could be much higher in combinations therapy. We could get as high as in the twenties and sometimes even in the thirties as far as percentage of severe adverse events.

3.5#3 AR: So, a little background about treatment of immune-related liver injury. So first of all, as we know, in general the immune-related adverse events due to checkpoint inhibitors are attributed to unopposed T cell activation. So logically, the overall strategy for treating those side effects or adverse events is directed towards reducing T cell activation. Specifically in adverse reaction due to checkpoint inhibitors, we know that in many cases high dose steroids actually get that result. But not in all cases. As in many other cases of severe liver injury, it is extremely important to assess for other possible causes because just the fact that we are having a patient receiving checkpoint inhibitor does not mean that this is the cause for his liver disease. There are other several causes and we will discuss this in a minute. Two very exciting areas that we may want to discuss in the -- during the discussion break, the role of flare-ups of chronic viral hepatitis in these patients and DILI due to concomitant drugs. Both are matters of active debate. Remember, we are decreasing the threshold for immune response to maybe a statin that the patient

is receiving at the same time, or other drugs. So these are interesting discussions.

3.5#4 AR: A little lesson learned regarding what we call autoimmune hepatitis, or some would call now idiopathic autoimmune hepatitis, and idiosyncratic DILI. So there are interesting similarities and differences between those and the entity that we are discussing right now. First of all, autoimmune hepatitis, as we know, involves loss of tolerance to self-antigens. But the actual pathogenesis is incompletely understood, even today. Interestingly, autoimmune hepatitis, the classic autoimmune hepatitis, is a steroid-responsive disease. And standard therapy is quite successful with corticosteroids and with azathioprine. For those who are what we call steroid refractory, there's a list of other drugs that have been used with various levels of success, such as mycophenolate mofetil, cyclosporine, tacrolimus, rapamycin, and even infliximab with some success. On the other hand when we talk about idiosyncratic drug-induced liver injury, the current consensus is that in most cases this is a steroid non-responsive disease. And there's no

good evidence to suggest that steroids are the way to treat even the most severe cases of idiosyncratic hepatotoxicity.

3.5#5 AR: So, related specifically to the treatment of checkpoint inhibitor-related liver injury, first of all, we have very limited experience. We are now at the process of gaining data and learning. And as expected, there's no consensus regarding, for instance, when do we start treating those patients? So if a patient has an ALT that doubled from baseline, or ALT of 2.5 times upper limit of normal, should we treat this patient? So when to initiate treatment is unclear and is being done differently by different companies. In most cases, with ALT levels of more than five times the upper limit of normal, patients generally respond rapidly to corticosteroid therapy. It could be various speeds of response. It could take a few weeks. But this is very similar to what we see with autoimmune hepatitis. Sometimes it takes even months. There have been a few cases of fatal hepatic failure. At least one was confounded by delayed initiation of steroids and medical care based on the literature which

gives us pause regarding how much we can delay such treatments under certain circumstances. In some cases, additional immunosuppressives, as I mentioned, for autoimmune hepatitis, similar immunosuppressives have been used in these patients that seem to be not responding to steroids. And those included mycophenolate mofetil, infliximab and antithymocyte globulin with varying success.

3.5#6 AR: And I will just show you two published case reports just to illustrate the amount of confusion and disagreement regarding how to approach these patients.

This is a case that was published in 2011 in Journal of Clinical Oncology describing a 60 year old man with metastatic melanoma that received two infusions of ipilimumab three weeks apart, and then one month after starting this treatment presented with fever, rash, nausea, and vomiting with ALT of 2,500. He was started on methyl prednisolone IV for nine days, and showed rapid improvement. And then unfortunately, what happens sometimes, he developed severe steroid-induced psychosis, and steroid doses had to be reduced leading to a rapid

recurrence of symptoms and increased ALT.

3.5#7 AR: And this is what the changes in ALT/AST look like. Unfortunately I do not have a pointer, but you can see the blue arrows showing where the -- I don't have anything that would hit this screen but I'll basically point out the important points. So we can see the initial doses of ipilimumab with the blue arrows. Then there is, by the way, an interesting star that says alcohol binge, which I'm not sure is relevant to the course of this patient at all. But somebody thought it might be. And then on day 30 there is a sharp increase in ALT and AST that was met on day 40 with those red/orange columns which are the steroids therapy. And as you can see, as soon as steroids were given ALT and AST started responding. However, they had to stop it because of the side effect of psychosis. And you can see, it's very difficult to see that initially they started with those little green columns which are mycophenolate mofetil. And at that point, even though the green columns are there, meaning the patient is receiving mycophenolate mofetil, ALT is gradually going up and going to a level that is even higher than the previous case. We're now talking

DR. ROSENBERG: So, I'd like to ask a quick question. You made a -- Dr. Suzman, you made a very interesting point which was that the maximum tolerated dose was actually independent of the targeting of the monoclonal antibody, which then suggests that, you know, if you use the maximum tolerated dose you're way overdosing the patient. Because in point of fact, the exquisite specificity of monoclonals is what you're relying on to deliver an effect. And so if you're getting non-specific toxicity it means that you're really using too much.

DR. SUZMAN: Well, that was specifically with regards to antibody drug conjugates and sort of an empiric analysis that our toxicologist did. But certainly it seems that it's more the payload than the antibody with toxicity in general with these agents.

DR. ROSENBERG: But I thought you mentioned that this conjugate did not -- had good stability, the linker was good.

DR. SUZMAN: I think the linker can be an issue and the linker varies between different -- it tends to be the linker that has more to do with the toxicity when you're comparing across these

agents as opposed to the target. So it tends to be sort of the linker and the payload, and as you keep those constant the toxicity seems to be fairly constant. So the stability of the linker becomes critical.

DR. WRIGHT: So, a follow-up question -- again, Terry Wright, Genentech -- relates to the VEGF inhibition. And we are now -- you have experienced obviously at Lilly using VEGF inhibitors in patients with hepatocellular carcinoma. There are potentially multiple effects of VEGF inhibition on the liver, which I think we haven't perhaps fully explored clinically. One is to reduce portal pressures. And actually if you look at GI bleeding rates across the phase III programs they tend to actually be quite low, and if anything a little lower in the subarachnoid treated versus placebo. So that's one potential good. There are also potential effects on fibrosis and fibrogenesis, both. So, fibrogenesis and fibrolysis. So maybe both a positive and a negative effect in fibrosis. But there are also potential effects on collateral circulation in the liver, negative effects, and also potential

negative effects in hepatic regeneration. So, I only bring this up because as we are going more into -- there have obviously been significant problems with adverse drug reactions in HCC trials. That's why many of those trials have failed. And just a heads up for all of us, perhaps think about what these drugs may be doing to the underlying liver disease, not just to the tumor as we set up clinical trials.

DR. REGEV: I heard your comment yesterday and I think it's a very good point. I don't think any of us have any hard data about this, but these are very important questions. If anybody else has any data I would love to hear it, but I don't --

DR. ROSENBERG: Next question.

DR. VIERLING: John Vierling, Baylor College of Medicine, Houston. I was struck by these emerging stopping rules, by their lack of inclusion of measures of excretory hepatic function such as total and direct reacting bilirubin, or PTINR perhaps as a measure of synthetic function. They're relying on aminotransferases, which of course are not necessarily liver-specific. There

are many other potential sources as well as ALT and AST that would need investigation. But I'm very struck by the fact that with these stopping rules and this emerging concern, especially the concern that's also been raised of whether treatment with the immunosuppression might actually be contraindicated for the person in whom you're trying desperately to control and to modify their immune response regarding a tumor. So, what I would urge is two things. One, I would think that these cases, just as we saw for the cases that Herb Bonkovsky carefully described to us this morning, that we have liver biopsy. We do not know without a liver biopsy what is going on and how to interpret actually, the meaning of aminotransferases for sure. And part of that question is that if this is an acute hepatitis presentation of an autoimmune hepatitis, then we would anticipate that the majority would show the lesion of that acute onset, which is a central zonal perivenulitis rather than a portal-based hepatitis with interface hepatitis. That can actually be seen in acute hepatitis due to any etiology. But were it the latter then that would portend the perhaps worse outcome, as shown by the

acute liver failure study group in their biopsy series of the autoimmune hepatitis candidates. Now, the other thing is that this would be, I believe, the perfect opportunity to study quantitative function before, as baseline, and after. And these are non-invasive tests. They are available and one is FDA approved as I mentioned yesterday in my talk. Because I'd like to know the correlation between hepatic dysfunction and the aminotransferases before we throw the baby out with the bath water.

PARTICIPANT: I've got a couple of questions. One is I guess the comment would be I would expect the activity, the cancer-related activity, to parallel this autoimmune activity in the liver, and I was wondering whether you also saw that effect. And the second one is I had a question about the distribution of the antibody. The anti-PD1, is that actually going to the liver? Did you guys quantitate how much -- what is it binding to? Are you generating the T cells in the liver, or are they being activated in the lymph node and then going to the liver? What's the pathway that gets these going? And I think if it's the RES system that captures the antibody then maybe you would

focus all the PD1 positive T cells. You would capture them back into the liver to sort of make this worse. And then the last thing is your 4-1BB, I think you're getting reverse signaling on the antibodies that don't block the 4-1BB. When you cross-link it with the first antibody I think maybe you're getting signaling backwards that give you the toxicity.

DR. KONTO: So, I'm going to start with the correlation of immune-mediated or related adverse events. And by the way, immune-mediated adverse reaction is a term that the FDA previously raised during the discussion with the label of ipilimumab, that's the reason why I feel that this is the official term, just to close that debate. There was a correlation between severe immune-mediated adverse reactions and the outcome in terms of overall survival. And this was shown by Dr. Weber at Moffitt in Tampa in the early days of ipilimumab. There are caveats to those type of analysis because those studies are not powered to rigorously assess this type of analysis. And I don't feel there is any need to make any promotion with that regard. About your question on the liver distribution of nivolumab, we haven't assessed the

liver distribution of the monoclonal antibodies. The only work that has been done is the characterization of cytotoxic CD8 T cells infiltration in the liver parenchyma. With regards to 4-1BB difference I think you're right, if I capture what you say correctly, that the blocking feature on the 4-1BB receptor is important to avoid a dual agonistic effect on the 4-1BB receptor. So having a 4-1BB monoclonal antibody binding to the epitope of the 4-1BB ligand and blocking the binding of the natural ligand to the receptor may avoid an increased burden in toxicity.

DR. REGEV: Just to go a step back to John's comments, which I think are excellent, and I think just enhance the points of how much we need to get to some kind of a consensus regarding guidelines on how to treat these patients. One of the things that I think is illustrated here, and again, is something that we see when we work with our oncology colleagues, the classification of severity of liver injury is not even similar because we -- DILI people don't use the staging, the CTCAE system, which differentiates between 3 to 5, 5 to 20, and more than 20 ALT. We don't think that

having more than 20 ALT should be stage 4 because we think that having a bilirubin of two times the upper limit of normal is much more dangerous than having an ALT of 20. So, there's this ongoing discussion on how those classifications need to be harmonized and after that how we should have common guidelines to stopping rules both for discontinuation of the drug and initiation of steroids. I think this is a work in progress because right now there are very different approaches by different companies that go about developing these drugs.

DR. KONTO: I just want to add one thing, and I appreciate your comment. I think this type of meeting is really necessary to create harmonization. Within the grade 3 of LFT transaminases elevation ranging from 5 to 20, the discontinuation rule is not the same depending if the elevation is below eight times the upper limit normal, or above eight times the upper limit normal. So there is a clear disconnect on the rules and the CTCAE classification that should be harmonized indeed.

DR. REGEV: I agree.

DR. AVIGAN: Well, there are many points that come up with these immune therapies and so I just want to touch on a few. But one -- and I wanted to hear about the PD1 drugs, which is not just the liver, because we happen to be liver specialists here, but in reality if you look at what is also in the post market being reported in terms of extraordinary bad reactions, not unexpected is a variety of different targets, organ targets, including the enteritis which is problematic where there are perforations, for example, as complications. Skin, polyendocrinopathy, and so on. So, in the scheme of things, we're talking today about the liver, but in reality I wonder what your experience is across kind of the whole body. And then somebody mentioned, this is actually very important from an FDA perspective because we get the post marketing reports of people who have very bad outcomes, and in cases of liver failure, for example, many of the patients at autopsy when they have these drug reactions and die of liver failure, they have metastatic disease with melanoma in the liver. Not necessarily a big surprise, but then the question becomes patient selection for

appropriate treatment. And this is not a trivial question. If you come back in a hundred years from now if we're going to use these immunotherapies we need to get better at predicting which patients would have a positive rather than a negative result.

The final question, and again -- but one that I'd like to hear some discussion about is the recovery or adaptation phase. So in other words, these are monoclonal antibodies. They target T cells. And what we're asking for if we overshoot is a recovery for the patient's immune system to kind of get back into an equilibrium where we have too much autoreactivity. So the question is with these drugs, what is the recovery mechanism once the monoclonal is binding to its CTLA4 PD1 target? What are the steps of recovery to get back into equilibrium? Are we talking about very rapid recovery because of replacement cells that come back, suppressor cells that come back into the picture? Or are there other effects, steps that are necessary to get that recovery?

DR. KONTO: With regard to the safety profile of nivolumab, the anti-PD1 monoclonal antibody, I think you're completely right and that

was the purpose of my first slide was to put the hepatitis in the broad context of immune-mediated adverse events. With PD1 the range of side effects is lower than with ipilimumab. That's where we had these very severe enterocolitis cases in the range of 5 to 10 percent. Overall any grade of enterocolitis affected 20 percent of the patients. As you mentioned, among the severe cases of enterocolitis patients had colonic perforation, some of them even died of peritonitis at the early clinical development of ipilimumab. With regard to the specific toxicity of PD1, I think it's worth mentioning the skin reaction, the endocrine reaction, the pneumonitis cases, the nephritis cases observed. It's basically we break immune tolerance so any kind of autoimmunity against self-antigen is possible. That's how we warn our investigators. So that requires vigilant monitoring, and a prompt rule out of other etiologies, and rapid start of corticosteroids. With regard to the patient selection and specifically liver metastases the problem is that we would exclude a high range of patients for an immune-mediated side effect that we know how to

detect, we know how to manage, and we have pretty good confidence that we are able to reverse this side effect. So, in the case of melanoma it's up to 30 percent of the case series of melanoma have shown that patients may have liver metastases. So it's a significant proportion of the population we intend to treat. Finally, the recovery. And how can we expect the recovery? I think there are two things. First of all, we're dealing with monoclonal antibodies with a very long half-life. They have high affinity to the receptor. Some of them have slow off rate. And those monoclonal antibodies have half life up to 20 days. So it's -- a full clearance of the monoclonal antibody in the body system would take up to 100 days. On top of this we're dealing with binding to cells that are activated. And those cells, they may have even more than 100 years of activation. I'm considering here the memory T cells that are activated by ipilimumab. And those cells actually even provide further efficacy against relapse. So they are also interesting -- it's an interesting benefit of those monoclonal antibodies. So it's hard to tell you exactly how can we predict the recovery because of

those multifactorial issues.

DR. AVIGAN: Great, thank you.

DR. UETRECHT: One side comment. I thought it was interesting, there was a recent paper. The microbiome is sort of blamed for everything. But there was a recent paper that found that the presence of specific gut bacteria were protective with respect to ipilimumab-induced colitis. So, not surprising in that case that the microbiome would be very important.

DR. TILLMANN: Hans Tillmann, East Carolina University. Would prophylactic budesonide be an option? Prophylactic budesonide given the high frequency of DILI with some of the problems with enterocolitis, budesonide might be an option for both without compromising perhaps the rest of the immune system outside of the liver.

DR. REGEV: So, I'll try and then I'll let Cyril. I don't think that would be within the realm of possibilities. I think the -- as I mentioned, the occurrence of immune-related adverse events is actually considered a potentially beneficial sign. So that we would like to activate the immune system. Given immune suppressant up

front I'm not sure what would go along with that purpose. But I'm happy to hear Cyril's opinion.

DR. KONTO: So, it's been tested in the clinic for ipilimumab. We tested the emergence of severe colitis in patients treated with ipilimumab alone versus ipilimumab plus budesonide prophylaxis. And there were no difference in the incidents of severe colitis. This was a randomized phase II trial. I think we have the issue of also the activation of -- the depletion, sorry, of the T regulatory cells in the systems that is one of the main cause of those colitis cases which is far beyond the inflammation at the colonic level.

DR. ROSENBERG: I have one point to make -- to do with what Arie had said which was that one possibility for the cause of the autoimmune hepatitis one sees in this context might be the HCV and HBV. But if those are truly latent, I mean, they are latent because they've escaped immune pressure. So, when you have heightened immune pressure I don't see where they would then become targets. So, I think in the latent state it's unlikely that those would become targets for autoimmune types of responses. But perhaps more recent infection

possibly.

DR. REGEV: So actually there are a few case reports on hepatitis B, patients with hepatitis B, C and HIV that were treated. And generally the outcome is okay. The specific case of a hepatitis C patient that was treated with -- the patient refused to get interferon initially. He had a detectable HCV RNA level, refused to get interferon, and was treated with checkpoint inhibitors. His disease showed regression, and then he was treated with interferon.

DR. ROSENBERG: Yes. Well, that scenario doesn't surprise me. Poorly treated, ongoing, yes.

PARTICIPANT: Hi, I have two questions. One, I want to get comments on the combinations. So, what's the criteria to combine the immune checkpoint inhibitor with other anti-cancer treatment? Because from the epiblast inhibitor in the mouse system or in the subconscious system has worked perfect. And no toxicity, the tumor shrinks. But nearly 80 percent develop the liver toxicity. So my concern is that there are so many combinations PD1. I check the clinical trial

and nearly like 500 trials ongoing. So, when the company develop this kind of combination what's the rationale? First question.

DR. ROSENBERG: Can you repeat the question?

DR. KONTO: Yes. So, the question if I captured it right, and please feel free to correct me, is what are the rationale that a pharmaceutical company could put in place to prioritize the numerous potential combination between immune modulators, but also between immune modulators and other type of interventions, should it be TKI, chemotherapy, radiotherapy, or anything else. I think that's a critical question because as you mentioned there are many potential combinations and we cannot develop all those combinations. We don't have the patient first. So, what we're doing is we're assessing an immunogenic cell death score for the different types of modalities. Depending on the necroptosis, apoptosis, you have release of HMGB1, calreticulin which are immunoactive agents. So, those type of -- we are assessing the different immuno score for the different TKIs, chemotherapy but also radiotherapy. And we will privilege those

interventions with a higher immuno score, immunogenic cell death score with the assumption that those agents are going to prime the immune system, and that those immune checkpoint modulators will boost the original immune response against the tumor. So that's one strategy. On top of this what we do is we assess the immune system impairment induced by those potential combinatorial candidate, making sure to select those agents that are going to have less impairment to the immune system because we rely on the immune system to attack the tumor. So that's a second consideration we're having. And of course once we have selected this we have mice model which are pretty poor model to predict what's going to happen in the clinic. So we do our tumor growth inhibition model in different model, in the CD38, MC58, B16 melanoma mice models. But it is fair to say that those models do not necessarily reflect what's going to happen in the clinic. Sometimes we have to use mice surrogate monoclonal antibodies because the immune system of mice has differences from the immune system of human. And more and more what we are evolving toward is those humanized mice model. We have now a

transgenic mouse with complete human systems. That's also pretty expensive at this stage.

PARTICIPANT: Yes, just to follow your answer. So, the ideal combination, the tyrosine kinase inhibitor or other anti-cancer cell with PD1 is that you --

DR. ROSENBERG: No one can hear you.

PARTICIPANT: Okay. So you buy the time for the immune anti-PD1 because from the clinical trial this build anti-cancer immune response with time. And your hypothesis might be to kill the tumor cells, then increase immunogenicity for the PD1. But now from a lot of literature you can see actually a lot of the cancer cell, they actually have a profound effect on the immune system. So this combination for example in the epiblast inhibitor plus ipi, this two drugs combination we actually know the mechanism. Because the inhibitors have an effect. They can activate non-tumor cells and produce the TNF alpha and IL6 after that. So this is a comment.

So another question as to that. From the clinical trials, the anti 4-1BB. So like toxicity is dose dependent we know. But from the ipi trial

of PD1 we may not see this dose dependent. So my question is from these trials what we can learn.

DR. KONTO: I believe that with ipilimumab we observed dose dependent toxicity. And it's been shown in studies when at 3mg per kg and 10mg per kg dose. For 4-1BB the question was more for what is the cutoff for the higher risk of liver toxicity. And the retrospective analysis done in all the patients we did with urelumab at Bristol-Myers Squibb came to higher risk in patients that were treated at 1mg per kg and above, inducing those discussions with the FDA to restart the clinical development of urelumab at far below doses than 1 mg/kg.

DR. REGEV: So, two last questions on this side before we break.

DR. WRIGHT: Terry Wright, Genentech. Just to follow up on the hep B/hep C question which we haven't really fleshed out. I agree most of those patients have been excluded the oncology trials. Nevertheless there's overlapping epidemiology of hepatitis B and lung cancer, for example. So this will become an issue in clinical practice even if we don't sort it out in the registrational trials.

There's some data on treatment of hep C patients, actually a lot more data on the treatment of hep C patients in the literature than hep B patients in the literature. With that being said if we're going to try and reduce the risk to patients who have hep B and hep C, and there are of course half a billion people with hepatitis B and hepatitis C in the world who are also going to get cancer, one approach is to try and clear the hepatitis C virus preemptively. I don't know whether that will reduce risk, but it's one approach. Another approach for hep B is to put patients on antivirals and have them virally suppressed. I think that's what most people are doing. I'm not sure that really reduces -- it makes us feel better. But since most of the immune-mediated injury is based on proteins rather than intact viruses I'm not sure -- and there's plenty of protein around in patients who are virally suppressed. I'm not sure we're doing a whole lot, but I think we should nevertheless do it. So my question I guess to the panel is with where we sit now should these patients be studied preemptively, proactively, before or as these drugs are approved so we really understand the risk-benefit in these

patients? Or do you think this is something we can just monitor in the post-approval setting?

DR. REGEV: Well, my personal opinion, I think we should study preemptively. I'm sure FDA would support this. We wouldn't want to find out how these patients behave only after these drugs are on the market. And in our case and I think in your case as well, and in other companies, if you have hepatocellular carcinoma patients in your portfolio then you have no choice because those patients have hepatitis B and C regardless. You can't find HTC patients with no viruses. So, I think the approach should be to try to somehow find approaches to minimize complications such as prophylactic hepatitis B treatment, again, without really knowing what it does. And getting rid of the hepatitis C virus before we start treatment. This is a whole new chapter in the book. We'll have to find out through data how these patients behave, I agree.

PARTICIPANT: I wanted to follow up on something I was asking before. This is sort of thinking out of the box which we are paid to do in the FDA. If you don't believe that or not. So, I

asked about the PD1, or these antibodies being captured in the liver through this RES and the possibility that that actually could capture cells that are coded with antibody, or the PD1 positive cells. And so it's clear now that in the periphery in cancer patients from Rosenberg Lab that if you look at the PD1 positive cells in the circulation you find the tumor reactive T cells, they're actually the same T cell receptors as you find in the tumor. So, I'm bringing up this possibility that if those PD1 positive cells that could do some good get captured in an immunosuppressive environment that in fact could tolerize those same T cell reactivities. And I would suggest, not that that's possible or not, but that maybe you want to interfere with this capture, FC receptor capture, in this immunosuppressive liver environment. So the concept would be you're capturing T cells into this suppressive environment, turning those off. If there's an active suppression, in this case it would be CD8s, they could turn off what you're actually trying to stimulate. And are there ways to prevent that from happening. And these are models you could probably test, you know, just block FC

receptors and prevent the cells from localizing to the liver.

DR. REGEV: Any response?

DR. KONTO: No. Interesting comment.

DR. REGEV: Yes, I agree.

DR. UETRECHT: So, lunch is ready. I have one last plea that somebody needs to figure out how to do studies on how to treat these patients better because it's a mess right now. And I don't know what the mechanism is, but I really think it needs to be done. Thank you.

(Applause)

(Whereupon, the above-entitled matter went off the record at 11:42 a.m. and resumed at 12:44 p.m.)

DR. WATKINS: I'd like to take a moment to say what a successful session this is. This is the highest paid attendance in the almost two decades these meetings have been occurring. And I guarantee it's the highest attendance after lunch on the last day here for sure. And I think it shows not only the continued and maybe rising importance of drug-induced liver injury, but the real excitement around the science that's evolving here.

It's no longer about bromobenzene and acetaminophen anymore. So it's really exciting.

And I'd like to take another opportunity to thank John and Lana really for putting together yet another meeting program here. (Applause)

DR. WATKINS: Now, we've shortened the duration of presentations to 15 minutes which I'd like to say is not because at the end of two days your attention span is less, but it was everybody we asked said yes which was unanticipated. So at this point I'd like to hand over the podium to my co-chair Gyongyi Szabo to introduce the first part of the session.

DR. SZABO: Thank you. It's a great pleasure to introduce John Senior who is going to give the first talk in this session. And I think his visionary leadership shows even in the title of his talk that's going to be "Is the eDISH program the Long-sought and Best Current Biomarker for DILI?" So we all look forward to your presentation, Dr. Senior.

4.1#1 JS: DR. SENIOR: Thanks, Gyongyi. We are so pleased to have Gyongyi, who's the immediate past president of AASLD. And I guess I'm the oldest past

president who's here, but we have a total of 8 AASLD past presidents at this meeting, 5 speakers and 3 in the audience which is remarkable. We are delighted to have support of AASLD that publishes our proceedings on the internet. Wonderful!

So, is eDISH a biomarker? That's a question that's come up. When Ted Guo and I developed eDISH 13 or 14 years ago, we weren't thinking biomarker. We were thinking about what we could do to help make a diagnosis of DILI according to ideas that Hy Zimmerman had been talking and writing about, because what Hy was writing about worked. It told the truth. It said what was effective. So, we at that time were both in the same office, called the Office of Pharmacoepidemiology and Statistical Science, OPaSS. So, Ted, a statistician, and I, an internist, were able to work together in the same office. And Ted's a remarkable person. We come from different cultures, Ted from Shanghai and I from Philadelphia. I was an internist and he's a statistician. But somehow the chemistry worked.

4.1#2 JS: What is a biomarker? There was a excellent conference in 2001 when they tried to define what a biomarker was. It's a lot of things.

It's a test that makes a diagnosis; a test that says something about severity; it can be predictive. There're many things that a biomarker can do, and there are many kinds of biomarkers. If you just enter "biomarker" in the PubMed search window you get over 760,000 papers. And a lot of them just say, we really need a biomarker; wouldn't it be wonderful to have a biomarker . . . a biomarker that takes the thinking away. Just do the test and you have your answer. Well, it's not that easy.

4.1#3 JS: So, this is the usual disclaimer, but I recall when Hy Zimmerman and I were working together I was concerned about two drugs, bromfenac and troglitazone, that the FDA had approved in 1997 shortly after I had arrived there in June of 1995.

4.1#4 JS: In the year 1997 FDA approved eight drugs that later had to be taken off the market for fatal toxicities, four for liver (especially trogltazone and bromfenac), three for heart and one for muscle toxicity. I spoke to Hy that summer of 1998 and suggested that we should discuss this problem. He agreed, and offered to speak.

4.1#5 JS: But unfortunately he couldn't because he developed cancer of the tongue. When we had the

meeting in April of '99, he could no longer talk and Jim Lewis gave his talk for hi. But Hy came and was there when Bob Temple suggested that we should call his findings Hy's Law. So, what did Hy say? --- at this meeting in 1999 , the last public meeting that Hy ever attended. He modestly objected. But he died in July. And he gave everybody a copy of his book (second edition).

4.1#6 JS: Hy had said and written repeatedly that drug-induced (one), hepatocellular (two), jaundice (three), is a serious, sometimes fatal disorder. Drug-induced, hepatocellular, jaundice; three items. It is not just some chemistries; it's something very special. Hy stated it in 1968 when he gave the Kober Lecture at Georgetown; he talked about it at a NIH Fogarty meeting in 1978. And Bob Temple was there, heard him talk about his ideas and he said, “gee, that's interesting.” And he kept it in mind for 20 years, and he found that what Hy said always worked, was always true.

4.1#7 JS: So here was the first conference. We started the first conference for FDA reviewers in April 1999. And we had a tremendous turnout, 320. We had to turn people away. We had people sitting

on the steps in the auditorium. And they demanded that we do a rerun which Bob and I did for 75 or 80 more in November that year. So we reached 400 FDA reviewers.

4.1#8 JS: Well, what was so special about that? Well, the FDA reviewers recognized the problem, and they began to demand that companies give them liver data when they submitted new drugs for approval. Well, the companies want to get approvals so they did what they were asked to do. So, through the reviewers, the companies began to get the word. And look, we have a couple of hundred people from them here right now. And every company in the world is now paying attention to this problem. Am I right, Arie? Right. Okay. We didn't record that first program like we're recording this program, but we were just learning.

4.1#9 JS: So, in the years following that NIH had a conference the following year at the Lister Hill Center. Industry said, “we want to come.” So we had a conference in Chantilly in 2001, and 300 people came, which was Lana's first meeting, and we've been inviting them ever since.

4.1#10 JS: The major problem was making a

diagnosis. That's the hard part. How do you diagnose DILI? We're still struggling with that. How do you diagnose it in people who have no previous liver disease, or some previous liver disease, or they're getting some other treatment, this, that, or the other? Not an easy thing to do. So, making a diagnosis was the first challenge that we had.

4.1#11 JS: We started by looking at clinical trials. The idea was to ***prevent*** DILI rather than try to find it after it occurs. That was an interesting idea. But how do you prevent it? Easy: don't approve drugs that are going to kill people from liver failure. So we developed this eDISH program.

4.1#12 JS: And you've seen this two-dimensional plot, x-y plot. It plots on the abscissa the peak ALT, the highest value anytime during a clinical trial for each subject, and peak total bilirubin (TBL) on the ordinate. There are almost 4000 subjects here, a symbol for each of them. So, each point represents peak values for two variables. One indicates the rate of injury to hepatocytes, leakage of the enzyme into the plasma, and the other a measure of dysfunction, because one of the

principal functions of the liver is to clear the blood of bilirubin.

We heard yesterday from Greg Everson about clearance of cholic acid. But clearance of bilirubin is a little simpler. So everybody does TBLs and ALTs. We plot the two measures, but it is *not diagnostic*. And I want to emphasize that fact over and over again. A point in the upper right quadrant is not diagnostic of DILI, because nothing says what the cause might have been. It says only there's been some liver cell injury, and some loss of liver function. They've taken the drug, but we don't know what caused the abnormalities. It could be disease, it could be a lot of things. So, causality was not determined.

4.1#13 JS: So, how did we approach that? Well, we did another thing. We looked at a variable called ***time***, not in everybody, only in the few people who might have possibly significant liver injury and we had to find out why. So, just click in the right upper quadrant on one of those subjects, and now you're looking at changes in one person over time.

4.1#14 JS: And what we get was one of these figures that you've seen many times now. The time course

of all four liver tests (ALT, AST, ALP, TBL) done for that one patient over the entire period of time of study. What do we learn from that? Does it make a diagnosis? No. But it does help you to see what came first. Did the bilirubin rise precede the ALT rise? If so, it probably wasn't caused by the liver injury. On the other hand, if you got the liver injury first and then got bilirubin rise it might be. So you began to get some idea of causality. You also see if it's getting better or worse and how fast, other characteristics of a biomarker.

4.1#15 JS: But that wasn't enough. We got what was called a narrative. Ideally the narrative should reflect what the attending physician thought and did to make the diagnosis. Unfortunately we don't always get good narratives, often just a data dump of the case report because they're not prepared by medical doctors; they're prepared by clerks working for the company. That's not the same thing because we have to get the medical diagnostic reasoning involved. Chemists, pharmacologists, statisticians, and they're all very learned people and work on developing new drugs. They have PhDs; they're all called doctors. But there's one thing

that's different about medical doctors that the others don't do.

4.1#16 JS: What are they doing? A patient shows or complains of this, that, or the other thing. ***Why*** do they have it? What's ***causing*** it? That's important to know because it maybe tells you what you need to ***do*** about it. You have to take action. You're expected to know what's going on, or immediately find out and take action. Stop the bleeding; remove the tumor or the infected appendix; treat the infection; correct the deficiency. Whatever it may be, you have to take action. It's expected of you; you can't just walk away. You alone have that responsibility. So making the diagnosis is critical.

4.1#17 JS: There is no such thing as Hy's Law chemistries, just because the ALT and bilirubin are elevated. That's nonsense. That's not the end; it's only the beginning. It's the beginning of the process of thinking, thinking about what's ***causing*** it, what can you rule in and rule out. So we have to stop using that term. I don't know how we can get rid of it. I've said it till I'm jaundiced in the face -- to get rid of this bad term that's used

by statisticians often to say that's a Hy's Law case. It's not.

4.1#18 JS: So, the first graph, the x-y plot is a useful starting place, but it does ***not*** make a diagnosis. Now you need to start thinking about n of 1, a single person, using a different kind of thinking, starting with some information – person took drug, showed worrisome findings. It's only the beginning of a process of thinking. What is this medical differential diagnosis thought process? The time course and additional biomarkers then become very useful in ruling out or in the possible causes. It represents sequential logical reasoning described by Reverend Thomas Bayes in 1763. Get more information; think; make the diagnosis of what was probably **causing** the problem.

4.1#19 JS: So, eDISH does **not** look like just a biomarker. It uses biomarkers, but it uses medical thinking, logic. It's not using just counting numbers. You're beginning to think about what is going on in this individual person. But eDISH really does a lot of the tasks that biomarkers do.

4.1#20 JS: Bob Temple has pointed out that Hy's Law works, and I'm saying eDISH also works because eDISH

was developed to reflect the thinking that Hy used when he developed his concept.

All societies in the world have put responsibility on medical doctors to take care of patients, which physicians and surgeons have accepted -- responsibility for the patient's life. Anybody who's ever done that can no longer go back to the way they were; it's a life-changing experience. Regardless of whether they become administrators, businessmen, or even congressmen, they can never forget that they once took responsibility for their patients' lives.

4.1#21 JS: Now, I told you about these drugs. It was a bad year at the FDA in 1997. There was pressure from Congress to approve drugs more quickly and so they did, sometimes over some concerns or objections from the reviewers. And it was a disaster. Two we got interested in 1998 were troglitazone and bromfenac, that began to kill people with liver failure. Bromfenac, who needs another analgesic that kills people? Troglitazone was a new treatment for diabetes and there was a lot of pressure to keep it around and it hung around for about three years until alternatives called

rosiglitazone and pioglitazone came along. So, FDA has not approved a single drug in 18 years that had to be removed from the market for liver failure. I think we may be getting somewhere.

4.1#22 JS: There were also four other drugs approved that year that later were removed from the market because they caused heart failure, cardiac arrest, or muscle-kidney toxicity, and two more that caused liver failure.

4.1#23 JS: I hope that someday we can say that drug-induced liver injury is a preventable disease, that we can avoid it. We can learn how to not kill people with drugs. I don't know if we'll get there. The new monoclonals are really challenging.

4.1#24 JS: Here again is what biomarkers were defined to be. It looks like eDISH is something more.

There's something it doesn't do very well so far, and that is predict the future. So, maybe eDISH needs to be improved, Paul, and you are working on it.

4.1#25 JS: It is not just a biomarker, it's a thought process, a medical reasoning process looking for the cause, to make a diagnosis so you

know how to treat the problem to relieve it, make it better, or make it go away.

4.1#26 JS: Can eDISH be combined with other methods? Recently Gaby Danan in France has teamed up with Rolf Teschke in Germany to update the old RUCAM, the Roussel Uclaf Causality Assessment Method. It's no longer RU; Gaby's not working for Roussel Uclaf anymore, and his friend Christian Benichou has died. It's now a European update. So I proposed to Gaby that they call it **EU**CAM instead of RUCAM. Well, he didn't buy that. He wanted to honor the memory of Christian Benichou. So, okay, at the moment it's just a proposal that the revised RUCAM be considered the European standard. If it could be combined with eDISH, a good RUCAM or EUCAM might be used to look at the patients in the right upper quadrant that would then improve it.

4.1#27 JS: Now, can it be improved further? Can it be improved to be more predictable? And you will hear in a few moments about a new technique to ***predict*** the likelihood of death in patients with DILI. And Paul's group is working on that, with Dan Antoine who will be speaking to you momentarily. Thank you very much.

(Applause)

DR. SZABO: Thank you very much, John. So we will move onto the program and it is my pleasure to introduce Rachel Church who is a research assistant professor at the Eshelmann School of Pharmacy at UNC And Rachel is going to talk about transformative DILI biomarkers, DILIN/SAFE-T collaboration.

4.2#1 RC: DR. CHURCH: Great, thanks, everybody. I really appreciate this opportunity to talk to you guys about some really exciting findings that have come out of this collaboration between the DILIN network and SAFE-T.

As we've been discussing for the past day and a half DILI is a serious problem that can have some very dire consequences in individuals who experience it. And I think that's summarized nicely here in this table that was actually published by the DILIN. And what it shows is that of the patients almost 660 in this cohort of DILI patients 10 percent actually by 6 months needed a liver transplant or had actually died. Unfortunately, right now we don't have any great biomarkers to

predict who is going to go on to experience one of these really serious DILI events and that motivated this collaboration.

4.2#2 RC: So, as I mentioned this was a collaboration between the Drug-induced Liver Injury Network and they prospectively collect serum samples from patients with suspected DILI resulting from prescription use, herbs and dietary supplements. And it was in collaboration with the Safer and Faster Evidence-based Translation which is a biomarker consortium supported by the Innovative Medicine Initiative in Europe.

And the aim of this is to qualify some of these newer candidate biomarkers in drug-induced toxicities, and in this case in DILI. So as I said the aim of this project was really to determine whether some of these newer candidate biomarkers was more prognostic for an adverse outcome compared to some of the more conventional tests that we use currently. So, this project looked at many biomarkers and there was some very interesting data. But due to time limitations I'm only going to focus on two today, and that's total HMGB1 as well as acetylated HMGB1. And I'll give you a little bit

more information on how those are mechanistic biomarkers in a minute.

But before I get into that I just want to give you a few definitions for characteristics to define a biomarker's performance because I'll use them and I want to make sure that you understand what they are. So, sensitivity is the proportion of positives that are actually classified as positives. So the cases that are identified as cases by the biomarker. Specificity is the inverse. It's the proportion of negative cases, or controls that are correctly identified as controls. Positive predictive value is the percentage of positive tests that are cases. And the negative predictive value is the proportion of negative tests that are controls.

4.2#3 RC: Okay, so to give you a little bit of background on how HMGB1 is used as a mechanistic biomarker I will give you some background. So, HMGB1 is a ubiquitous protein that's primarily nuclear. However, it can shuttle back and forth from the nucleus to the cytoplasm. And so here it is in our hepatocyte. And the hepatocyte is going to undergo some form of injury, and then two of the main forms of cell death that it can undergo are necrosis and

apoptosis. Now, both forms of these cell death can passively release HMGB1 into circulation. But it's important to note that these two different forms of cells death actually result in different post-translational modifications that allow you to distinguish it in circulation. Now, I won't spend any time talking about that, but it is important to note that the form released from necrotic cells actually has a post-translational modification that marks it as a drug-associated molecular pattern, or a DAMP. And this DAMP in circulation can stimulate recruitment as well as activation of immune cells. And these immune cells, including Kupffer cells, actually can actively release HMGB1. And this requires acetylation of specific lysine residues on HMGB1, and then that can be released into circulation as well and identified by means of measuring these post-translational modifications.

4.2#4 RC: So with that being said I will get back to the study that we did. As I mentioned, we wanted to look for prognostic biomarkers so we only analyzed samples that had been collected within two weeks of DILI onset in cases with a causality assessment of probable or higher. And this turned

out to be 147 patient samples. So, within this cohort there was 131 who had not undergone an adverse outcome by 6 months, and 16 who had. And that's defined as somebody who either needed a liver transplant or had actually died as a result of their DILI.

4.2#5 RC: So, because this is a comparison to traditional biomarkers I wanted to first show you some of that data. This is ALT and AST. You can see ALT was not significantly elevated in those who had experienced an adverse outcome. AST actually was. But what I think is pretty obvious is that there's a great number of false positives there. ALP similar to ALT was not significant. When you look at INR and bilirubin you can see again there was a significant elevation in those who had an adverse outcome. But again there was quite a bit of false positive noise. So, the take-home message is that while several of these biomarkers had good sensitivity they really lacked specificity.

4.2#6 RC: Okay, so without further ado I'm going to show you the HMGB1 data. First we looked at total HMGB1. And you can see that there was a shift, there was a significant elevation in those who had

an adverse outcome. But again quite a few false positives. However, the really exciting finding was that when we measured acetylated HMGB1 you can see that there was a very nice shift in individuals who had an adverse outcome. But something that might be even more interesting to you is that the individuals who did not have an adverse outcome actually had this bimodal distribution. So obviously there's more to this story. You can take the people who did not have an adverse outcome and break them down further.

4.2#7 RC: So there were 112 patients who had recovered by the six-month visit, and there was 19 individuals who actually were still meeting the criteria to be considered DILI. And those are the chronic patients.

4.2#8 RC: So again, this is the data I just showed you, but when you separate the non-adverse outcome patients into recovered and chronic you can see that remarkably you can separate recovered from chronic from adverse pretty spectacularly.

4.2#9 RC: So I went ahead and did an AUROC analysis. And for anybody who's not familiar with this, basically the horizontal line across the

middle represents 0.5, and anything close to that is basically saying you can flip a coin and you would be just as accurate at identifying a case from a control. But what you can see is that the AUC of acetylated HMGB1 was almost 1. And when you look at the 95 percent confidence interval you see that it didn't overlap at all with the next best biomarker which was INR. So now when setting a threshold of I guess significance to determine who you should call a case and who you should call a control it's really important due to the severity of this adverse outcome that you identify every single person. So you really want to have a sensitivity of 1 and then you want to have the highest specificity as possible. So with the natural log value of 1.332, using that, anything above that, you have a sensitivity of 1 and a specificity of 0.96. So again, very, very good biomarker. So then I guess the next question is using this, is it better than some of the other criteria that we've established.

4.2#10 RC: So I looked at Hy's Law as well as MELD because of the talks yesterday. So this is the contingency table for Hy's Law. And when you look

at the performance you can see it was significant and it had pretty decent sensitivity and specificity. But the positive predictive value was pretty low.

MELD did much better. It was very significant. It had very good sensitivity and pretty good specificity. But again the positive predictive value was pretty low. So then when you look at acetylated HMGB1 you can see that the positive predictive value was now pretty high, and then it had as I mentioned perfect sensitivity and nearly perfect specificity.

4.2#11 RC: So then I wanted to look at whether you could get better measurement if you separated not recovered into -- to not recovered or recovered. So basically I grouped the chronic and adverse outcome patients. And you can see when you do it that way there's basically no contest. The other biomarkers really didn't do a very good job of identifying the chronic patients. But when you group chronic with adverse outcome you see you get a perfect AUC. And the best value for that was anything above -0.2078. And it gave you sensitivity and specificity of 1.

4.2#12 RC: So there's a little bit more to this story. SAFE-T also had several cohorts of patients that they looked at and they measured these biomarkers as well. So again, this is what the DILIN data looked like. And to everybody's surprise this is what the SAFE-T data looked like. Basically all of their patients recovered. They didn't have anybody who experienced an adverse outcome, yet you can see a good many of the patients had acetylated HMGB1 values that were in the same range as our not recovered patients.

4.2#13 RC: So, what we've thought about this is that one of the main differences between our data sets is that SAFE-T, many of their patients were enrolled in clinical trials. So they had these DILI samples collected very early after the onset on DILI. On average while DILIN were collected eight days after DILI onset SAFE-T samples were collected within one day. And even when you break it down and look at symptom onset to blood sample collection you can see the SAFE-T samples were collected earlier.

So our hypothesis now, our working hypothesis is that all of these DILI patients initially had a bump in acetylated HMGB1. And then

depending on whether they would go on to recover, have a chronic outcome, or an adverse outcome, their levels either went back to baseline which is in the same range as healthy volunteers, kind of stayed consistently lowly elevated which is the chronic patients, or continued to become elevated and those would be the individuals that go on to experience an adverse outcome.

4.2#14 RC: So, we're exploring that further right now. Several samples from the SAFE-T that were actually taken one week after onset are now being analyzed to determine if now, if you look at these one week samples the acetylated HMGB1 levels have fallen down to baseline. We're also looking at new and repeat DILIN samples to see if we can confirm these results that we've seen. And finally, given that HMGB1 is not a liver-specific biomarker we're also measuring miR-122 and hoping to normalize the data to that to get specifically at the proportion of this biomarker that's liver-specific.

So, in conclusion what I've shown is that acetylated HMGB1 levels measured within two weeks of DILI onset appear to be highly sensitive and specific prognostic biomarker for chronic as well

as adverse outcome. And that performance parameters for the identification of adverse outcome were superior when looking at acetylated HMGB1 compared to Hy's Law as well as MELD score.

4.2#15 RC: With that I'd like to thank everyone in DILIN and SAFE-T. Thanks a lot.

(Applause)

DR. SZABO: Thank you. It's my great pleasure to call Paul Watkins to the podium who will talk about application of novel biomarkers to assess liver safety in clinical trials.

4.3#1 PW: DR. WATKINS: Okay. That was, what Rachel presented was the first public presentation of that data. And obviously it's a work in progress. But if in fact the hypothesis is verified from my perspective that's the most exciting clinical finding in drug-induced liver injury ever because it will transform potentially safety monitoring, but also allow us in DILIN for the first time to identify patients that an IRB would allow them to intervene and do some sort of therapeutic intervention.

So, I used to be with the Hamner UNC Institute

for Drug Safety Science. The Hamner suddenly dissolved, but the school of pharmacy, Eshelmann School of Pharmacy, came in and hired everybody. They rented the space and Hamner gave them all the equipment so we were able to go on with no problem. The public-private partnership which was housed within the Hamner Institute moved over to the proprietary arm called DILIsym Services. There was no choice because companies weren't writing their checks at that moment that the Hamner made their announcement. And my only potential conflict is I own equity in that company.

4.3#2 PW: John showed you this slide, which shows treatment with the drug up until about day 65 of this individual. The drug was stopped when the ALT went over three times the upper limits of normal (3xULN). In spite of that, if you follow along you'll see the ALT, AST continued to rise for a few weeks, typical for these delayed idiosyncratic reactions. We think that can be explained by the adaptive immune attack being initiated and then turned around. The point that I'm making in this slide is the patient didn't qualify biochemically for a Hy's Law case until several weeks after the drug was stopped. And

this is not a unique situation in clinical trials. For instance, when this patient's ALT first rose above 2xULN, they were at risk for this occurring even though the drug was stopped before the ALT actually met the current 2009 guidance criteria. And this isn't just trivial liver chemistry abnormalities. We have an ALT of 30xULN, bilirubin 10xULN. This patient in the clinical trial had a greater than 10% chance of dying according to Hy Zimmerman.

So, there's a desperate need for new biomarkers that will be able to tell not only whether an individual patient is in trouble or not, but whether a drug has the capacity to cause progressive liver injury and acute liver failure at much earlier time points, where the green bar is.

4.3#3 PW: And one thing that's worth mentioning is that I think we can get more information out of our existing biomarkers. As shown here on the left, you have the same peak serum ALT. But since we know the AlT is being released from dying cells, so obviously the kinetics of the release, the duration, the AUC should have some correspondence to the actual percent of hepatocytes lost. And it turns out that

you can with the same peak ALT -- this was a publication from the DILIsym Initiative -- have threefold difference in the estimated percent of total hepatocytes that are lost. And that's just basic pharmacokinetics.

4.3#4 PW: In addition, what the modeling has done -- all part of the public-private partnership initiative -- is to look in the literature for the content of ALT per cell, and the variation in half-life of ALT, ranging from a day to almost three days in the literature. And actually create then a virtual patient population. And when you do that for any given drug. Drugs tend to have a characteristic profile in terms of rate of upstroke and downstroke of ALTs in clinical trials. For that given profile for a drug you can then estimate the variation in percent hepatocyte loss at any peak ALT.

4.3#5 PW: So what's shown here along the x axis are peak ALT ranges going from less to higher. And then the 95 percent confidence intervals for this virtual population and percent of hepatocyte loss is shown as the shaded bars. So you can see in this case someone with an ALT of 1,500 with this particular drug has a small chance of actually

losing enough hepatocytes to cause global liver dysfunction and a rise in serum bilirubin, which corresponds to about 70 percent loss of functioning hepatocytes. And as that peak ALT goes up again for this drug and this particular kinetic profile the percent chance goes up. And the question would come up if this is true, why would you have to wait till a patient actually satisfied Hy's Law if there was a 25 percent chance they would have anyway. Now, this is again drug-specific, profile-specific, and also assumes cells are bursting open and releasing their contents by necrosis. And we know though that this is, you know, showing part of what Rachel showed, that in necrosis we get a robust release of miR-122 and ALT, and also a full-length cytokeratin 18. But in apoptosis because the cell is digesting itself you get less release of ALT and miR-122, and you get then the cytokeratin, the caspase-cleaved fragment of cytokeratin 18.

4.3#6 PW: So, what Dan, Antoine and others do is they can measure this and come up with what is called the apoptotic index. So, these aren't liver-specific and you might need to normalize for miR-122, but in a phase I clinical trial where you

have serial measurements you can actually estimate the percent of cell death occurring by apoptosis or necrosis.

4.3#7 PW: So it's now possible to estimate from serum sampling the percent of hepatocyte death and the chance of encountering a Hy's Law case for any given profile for an individual. But I think this is just the beginning of the transformation that's going to occur in the next few years.

4.3#8 PW: And that's based on the current thinking, and this is consistent with what Neil Kaplowitz showed, that there's an adaptive immune attack that's the final mediator of at least serious liver damage.

4.3#9 PW: But it starts at the hepatocyte. And the drug is doing something to hepatocytes. Some drugs do it, some drugs don't, we're not smart enough yet to figure out what that is.

4.3#10 PW: It leads to some sort of stress, that leads to a neo-antigen and leads to release of damage-associated molecular patterns, creating an innate immune response, the right cocktail of cytokines and chemokines to set up the liver for an adaptive immune attack if you have the right

predisposition, presumably HLA risk alleles.

4.3#11 PW: And so it's now possible as Rachel showed you to measure all these things.

4.3#12 PW: So, for instance, you can see whether it's apoptosis or necrosis occurring in an individual. You can see whether the DAMP that's released is oxidized or reduced, that is, is it an active or inactive DAMP. And you can see whether there's been activation of innate immunity, whether there's acetylated HMGB1.

4.3#13 PW: So, for example, and this was shown by Dan Antoine who does all these measurements, with acetaminophen we know it's the most common cause of acute liver failure, yet it does not cause delayed idiosyncratic hepatocellular injury as we've discussed in this forum before. No one's ever described that. Clear dose-dependent toxicity. So it's stressing hepatocytes. We get protein adducts that we can measure in the circulation with therapeutic dosing. And it causes prominent ALT elevations in healthy volunteers getting recurrent therapeutic dosing, yet it does not cause this idiosyncratic DILI.

4.3#14 PW: So, in the samples that we collected in

our healthy volunteer study, sent them to Dan Antoine. And what he found was that it was apoptosis, not necrosis. It was the oxidized HMGB1 that was being released so there was not activation -- there were not active DAMPs that were released. There was no acetylated HMGB1 detectable. And that provided a plausible explanation for why the liver would not be set up for an adaptive immune attack. This is still unpublished.

4.3#15 PW: So, the absence of adaptive immune attack on the liver may be because active DAMPs are not released, and there is not activation of an innate immune response which is a prerequisite for an adaptive immune attack. And already I'm aware of three regulatory submissions or communications -- and there's about to be a fourth by a group in the audience here -- who have used this in early clinical trial data to argue that their drug has less of a chance of an adaptive immune attack. So, are these going to be -- that is, they showed it was apoptosis, not necrosis. There was no activation of innate immunity as measured by acetylated HMGB1, in spite of ALT elevations.

4.3#16 PW: So, can we move this earlier so that we

look at these biomarkers during early ALT elevations? Could perhaps they even be useful prior to ALT elevations in a subset of population? And the only way we're going to find this out is really for the industry to begin archiving serum from clinical trials and having uniform data management tools with the right phenotypic information that are directly linked to the biospecimens. Because regulatory acceptance will require I think a lot of data, a lot of different patients, a lot of different drugs.

4.3#17 PW: So, pharmacokinetic approaches to current DILI biomarkers and incorporation of these novel biomarkers should transform the assessment of liver safety of new drug candidates.And I think while improving subject safety, it may not be necessary to do 8,000 patients per year to rule out idiosyncratic DILI when you see some ALT elevations due to your drug. Troglitazone was approved with 1,000 patients getting the drug for six months. The FDA has not approved drugs that they haven't known about serious liver liabilities, but the cost has been much larger, longer clinical trials. And these biomarkers hold the promise of being able to

tell a good drug from a bad drug very early on, perhaps even in phase I.

4.3#18 PW: So, thanks to our own toxicity biomarker core, the DILIsym Initiative and Scott are here.

4.3#19 PW: And then the Liverpool group -- we collaborate with closely and closely with Dan Antoine, who is next going to show us that acetylated HMGB1 may not just be a marker, but actually an etiologic agent and a target for therapy.

Thank you.

(Applause.)

DR. SZABO: Thank you. So, as indicated the next speaker is Dan Antoine. He is a Wellcome Trust fellow and a senior staff scientist at the University of Liverpool. And we look forward to hearing about blocking HMGB1 in monoclonal antibodies and inhibitory peptides in his talk.

4.4#1 DA: DR. ANTOINE: Thank you, Dr. Szabo, for that introduction, and of course thank you to John and Lana of course as always for the invitation to

come over from the UK to present our data to you today. And of course I just wanted to extend a thank you to Paul, who has also noted some of the mechanistic work that we're doing underneath the layer of the biomarker projects that we're doing, and again the chance to present this data to you today.

You've already heard from Paul and Rachel about some of the aspects of HMGB1 as a biomarker, potentially a mechanistic driver of drug-induced liver injury, something that can provide added information to the tests that we already have to try and prove our predictive knowledge of drug-induced liver injury. So, I'm not going to spend too much time talking about that really. But over the years I really have developed a real special interest in this particular protein much to my wife's disdain. But what I'd like to do today is fill in some of the gaps actually really and the back story of how we got to this particular talk today. And Rachel's talked about the work that we've done in idiosyncratic drug-induced liver injury. Paul's mentioned the work in novel phase I studies.

4.4#2 DA: But really where we started this as with

most people in acetaminophen overdose. And you can see the translation of those findings in idiosyncratic drug-induced liver injury. We really developed them on the concept of patients needing a liver transplant following acetaminophen overdose. We've also showed that total HMGB1 itself when measured very, very early on can be a mechanistic biomarker of early detection of the potential to form acute liver injury as well in patients with paracetamol overdose in this pilot study that we published back in 2013 of about 130 people. And we've also replicated that now in 1,200 individuals in a multi-center study in the UK. But HMGB1 is not just a biomarker. We really believe it's a key mechanistic driver of the process and the response during drug-induced liver injury.

4.4#3 DA: And to define that, we had to produce an animal model. Unfortunately HMGB1 knockout is embryonic-lethal, so we designed a strategy in collaboration with the Schwab Club at Columbia in New York to produce this conditional hepatocyte-specific knockout mouse. The hypothesis was that if we could knock out HMGB1 from the hepatocyte that initial DAMP signal that kickstarts the cascade of

inflammation and potentiation of toxicity, we could define the mechanistic basis of those events.

4.4#4 DA: So we produced the conditional knockouts with the albumin-Cre driving system. You can see the validation data there on the bottom lefthand side with HMGB1 in the nucleus of the wild type mice, and HMGB1 absent from the hepatocytes in the knockout mouse. And it's only present in the nonparenchymal cells. The data on the righthand side shows the wild type and the knockout mouse that have been challenged with paracetamol. The wild type is shown in black; the knockout is shown in blue. You can see there's a clear decrease in ALT activity in the knockout mouse, a clear positive impact on survival in that knockout mouse and a clear decrease in the score for necrosis in the livers of these knockout mice. What I also haven't shown today, but it's part of the publication, is that in these knockout mice we prevent neutrophil infiltration into the livers of these mice. So what we believe we've done is knocked out that key molecular signal that links hepatocyte cell death to inflammation.

4.4#5 DA: This is a concept really developed by Kevin Park at the University of Liverpool. And

he's noted it as what we call closing the loop, where we really identify an adverse drug reaction in humans, try and understand that better, and then feed that knowledge back into designing safer medicines for human use. And what I've done here is applied that to the concept of the role of HMGB1 in drug-induced liver injury. You can see at the top of the circle there we've identified the particular candidate molecule from the literature. We've defined its performance as a potential biomarker. We've shown proof of it being a key mechanistic driver in certain contexts of drug-induced liver injury and chronic liver disease. And really where we are now in 2016 is really to try and complete that virtual cycle if you like and close the loop. The pharmacologist in me is telling me that I really want to develop a therapeutic intervention. And we know that HMGB1 can't really be considered as one molecule anymore. There're multiple molecules. So it's quite likely that we're going to have to move around this virtual cycle a number of times before we get the therapy that we actually want, and understand and advance the mechanistic basis of the science.

4.4#6 DA: So, this is the first data that we've reported on this topic and the first attempt to try and design strategies to target HMGB1 itself. I've been very fortunate to be funded by the European Commission through the FP7 program. And through that program we developed a novel chimeric monoclonal antibody specifically targeted towards HMGB1. So, we took the RNA, converted it to CDNA from mouse hybridomas that produced a specific antibody directed towards HMGB1. We cloned that onto -- those mouse variable regions onto an IgG backbone with human sequences. So we were able to produce that first antibody. And then we've shown that the binding of that chimeric antibody was highly equivalent to the murine form using SpR or the Biacore-based analysis. And as a negative control we used the E2 antibody, which is raised against the tetanus toxin. And then we looked at the actual binding domain of HMGB1 and the full protein itself. And what you can see here from the data on the bottom of the screen is the response of binding of this antibody to HMGB1 itself in a dose-dependent way, You can see that there's no binding to the box A portion of HMGB1, but in the box A domain of HMGB1

we see clear binding there. So we believe that's the epitope for this particular antibody of interest. So we've produced the antibody and validated its binding at the heart of HMGB1.

4.4#7 DA: So the next step was to test its efficacy in our well characterized animal model of liver injury. You can see here from the data, if you just focus your attention on figures B and C, we see that in the control animals treated with paracetamol we see a robust ALT increase. But then with the two antibodies, the murine and the human version we see a decrease in ALT activity and a decrease in miR-122. When we look at the histological scores for necrosis shown in panel D, at the lower part of that you can see a decrease in the scores for necrosis, but importantly, you can see the prevention of neutrophils not being infiltrated into the liver. And we also see a decrease in pro-inflammatory cytokine production on administration of the antibody. And this antibody is administered in a delayed fashion after acetaminophen treatment. So, of course as I mentioned I'm a pharmacologist, so dose response relationships are almost everything to me. So we wanted to look at the dose-dependent

relationship on the effect of this particular antibody in these mice.

4.4#8 DA: And what you can see here in panel A and B is a clear dose response protection of this particular chimeric antibody in response to paracetamol treatment backed up by miR-122 and the depletion of pro-inflammatory mediators as well.

4.4#9 DA: So of course we're already blessed with a treatment for acetaminophen overdose in the form of N-acetylcysteine which is very, very effective when given early on. So, in this experiment we compared the efficacy of our novel antibody with N-acetylcysteine. So, you can see here all these mice are treated with paracetamol or a controlled antibody and the antibody of interest. At two hours after the paracetamol treatment you see the N-acetylcysteine is completely effective. It prevents the ALT increase as we all are familiar with. But our novel antibody when given two hours after treatment only resulted in an ALT depletion of about 30 to 40 percent about two hours after treatment. When we look at six hours after treatment in these mice, we can see that N-acetylcysteine is completely ineffective. That's the second group

of animals on that figure on the righthand side. We can't prevent the liver toxicity associated with paracetamol with N-acetylcysteine at six hours after treatment. But our novel antibody still provides that robust effect that we saw at two hours. And we believe that it's because we're targeting that later delayed mechanism, those immune-mediated effects.

4.4#10 DA: So what we believe and a pathway forward is that you can combine N-acetylcysteine and anti-HMGB1 therapy in these mice. So, the lesson that we learned is anti-HMGB1 therapy is more effective than N-acetylcysteine when given at late time points. Of course, we wanted to understand the mechanistic basis. There's a number of potential mechanisms how this antibody can work. One is through complement activation and the other one is through Fc gamma receptor mediated effects. So, just in summary complement activation really facilitates phagocytosis, chemotaxis and cell lysis. And there's a key amino acid actually, lysine 322, which is essential for C1q binding.

4.4#11 DA: So what we did is produce effector function deletion mutants where we replaced alanine

for that lysine 322. And we looked at its binding on normal human serum. And what you can see here, particularly if you focus your attention on panel B, you can see that the antibody of choice binds to C1q, but the mutated version, that there's no binding at all of that particular group. The second effect we wanted to look at was the role of Fc gamma effects, which control cytokine release and antibody-dependent cell site toxicity. An essential for this is actually glycosylation on asparagine 297. And what we did is we actually treated this particular antibody with EndoS which deglycosylated that particular asparagine moiety. As you can see here from the shift in the Western blot there to show the depletion, also the reduction in the molecular weight following EndoS treatment. And then we looked at the binding, so the plant lectin, LCA, and shown that that was completely prevented with EndoS treatment. We prevented binding to CD64 and also prevented binding to live THP1 cells. So we have those functional effector deletion mutants to test further forward.

4.4#12 DA: So we tested them on our standard paracetamol overdose model. What you can see from

this figure, particularly from panel A, is that the effector function deletion mutants were all responsive. They all worked in this particular model with respect to ALT depletion, TNF response and also some other pro-inflammatory mediators. So the lesson that we learned from these appraiser studies is that the mechanism of action of this particular antibody is likely through analyte neutralization. We know that HMGB1 is not one protein, it's multiple different proteins, and we know that the different redox isoforms have different biological effects, and they're all mutually exclusive being from cytokine production, chemotaxis, or no function at all.

4.4#13 DA: So what we really want to do is try to identify a therapy that can target those specific forms. Unfortunately, the antibody that we produced at the moment picks out all those different molecular forms equally as you can see from the data here. So we really need to be a little bit smarter about the strategies that we approach to try and identify functional specific redox isoforms of HMGB1.

4.4#14 DA: And one strategy that we've undertaken

to try and do this is through inhibitory peptides. So we know that the disulfide form of HMGB1, that pro-inflammatory form which is prominent in acetaminophen overdose, that works to the TLR4-MD2 axis. So what we did is operated a computational approach to identify novel inhibitory peptides that really fit into that pocket, that MD2 interaction site for HMGB1, and also negative controls as well. So again we used those to challenge our acetaminophen overdose mice. And you can see there's a clear dose-dependent protection shown by ALT increase, TNF, and also survival, and also their histological scores for necrosis in these mice as well. So we now for the first time have a potential strategy which needs further work to target specific isoforms of HMGB1.

4.4#15 DA: So, just to summarize this portion of work, we've shown that HMGB1 is a promising biomarker first of all in acetaminophen overdose and now as Rachel has presented in idiosyncratic DILI. We've shown it's a mechanistic driver of drug-induced liver injury, and we've established it as a potential therapeutic target. We produced the first chimeric antibody targeted towards that and

shown that it's functional with respect to its dose-dependent effects. We've gone some way to identify its mechanism of action through analyte identification, and shown where it could potentially fit in its place in the clinic following acetaminophen overdose in a delayed manner to N-acetylcysteine. And we've also started a program of work through funding through the FP7 European Commission program to develop specific therapies for HMGB1-specific isoforms. And there's also ongoing work now in other forms of liver disease that we've established as HMGB1-dependent, such as alcoholic liver disease and carbon tetrachloride-induced fibrosis.

4.4#16 DA: So with that I'd like to leave it there. And, of course, thank you.

(Applause)

DR. SZABO: Thank you very much, and thanks for the speakers for staying on time. So, with this I'd like to open the panel for discussions and questions from the audience.

DR. WATKINS: Mark, I don't know what question you're going to ask, but the question I'm

going to ask you is what is the FDA's -- what comments do you want to make either from the agency or your own perspective on these new biomarkers and how they could move towards regulatory acceptance.

DR. AVIGAN: Well, I have the same disclaimer as everybody else in the audience, which I'm a citizen of the country, but I don't speak on behalf of the agency. I think it's very interesting, exciting work4. I think there's still a lot of questions about kinetics. There's also issues I think -- I'll play psychiatrist, I'll ask you questions in return. But one of the things that comes up is that you did show with the SAFE-T data the idea that there was some crossover in the discrimination which raises a question of sample timing. And there are two kinds of biomarker categories. And my question is one actually is a signature for the drug -- the drug will be in some individuals a problem versus not a problem, as opposed to a prognostic marker that individuals who have a certain finding have a prognosis that they will go for worsening and a serious outcome. Those are different. So my question is with the acetylated product that Rachel was talking about and you were

talking about, the signature for a bad outcome with the acetylated version from the DILIN data, does that preclude the idea that a negative result wouldn't necessarily mean the drug wouldn't be a problem for other patients who just don't happen to have that particular signature. Because of this crossover that you showed. So that's, you know, I just wanted to hear what you thought about that. What more data you would have over time to show, to give more confidence in the result that this is a more generalizable measure for a signature for the drug.

DR. CHURCH: Well, I think the more patient data we get and the more drugs that are implicated in their DILI, if we can measure the acetylated HMGB1 and see similar results. I think definitely measuring just at one time point is not going to be sufficient. We've seen that depending possibly on timing of when you measure it can give you different results. I think it will definitely have to be measured at least maybe once, and then once again a week or so later. And I think like I said, the more drugs that are implicated that we see these results for we can either move forward with

it showing good sensitivity, or we can eliminate it.

DR. WATKINS: Just the nature of the DILIN network is these cases are found out in the community, referred to us. And of the 1,500 cases we have, only a few hundred were within the first two weeks. So the delay is a big issue for us. But Rachel did look, for instance, at all the INH cases. And within that group, acetylated HMGB1 was predicted for chronic and transplant liver failure. But the majority of them had low levels of acetylated HMGB1. So that might argue against this being a marker for a drug rather than the individual. On the other hand, I think the only way we'll find this out is prospective collection which DILIN doesn't do.

DR. AVIGAN: The reason why I'm pressing on this point is with the acetaminophen, Dan's data, clearly shows in that cohort of individuals in the emergency room who had acetylated product were actually bad actors. Yet in the human data that you showed of adaptation in the dose range that was the recommended dose range you didn't get the acetylated version. So that looks more like not a drug signature, but a signature of

prognosis based upon dose effects.

DR. WATKINS: It may be the sought after marker for adaptation. Right?

DR. AVIGAN: Right.

DR. WATKINS: At least for an individual, not for the drug. We don't know that.

DR. AVIGAN: Right. So anyway, but the answer to your larger question of is this something that the agency is interested, we're very interested in markers, both on the prognosis end for sure, but also as development occurs with regards to drug signatures for questions around.

DR. WATKINS: One other quick question. What would be the agency's position if a company came in and said we see all these transaminases, but we just think it's transaminitis because it's apoptosis, there's no acetylation of HMGB1, no release of the --

DR. AVIGAN: And I don't want to give you a definitive answer, except one of the issues I would be concerned about is the sampling kinetics, the timing of sampling. And what Rachel showed was that actually it's very critically important to know where you are from the time of dosing to when

the effect occurs to the later outcome. And that's a very important issue that will have to be thought of very carefully.

DR. WATKINS: And the kinetics are actually being put in the DILIsym model, the release and clearance, kinetics, all this. The only issue to make is that it may be awhile before regulatory acceptance, but certainly in terms of internal decision-making and in one case external funding by venture capitalists the data was key.

DR. SZABO: Okay, so let's go to the next question.

PARTICIPANT: Thank you. I think they were exciting presentations. I share a similar comment with Mark, basically. I also say the timing issue versus biosensitivity for the biomarker. And I see the DILIN result come out, the average time is eight days after the onset as a safety concern, basically much earlier. So I'm just a little concerned if we get the biomarker after the eight days, you know, some patients may be in some disaster, you know. I think it's a little late. If you can put it earlier, I'm not sure. Since from the same SAFE-T data if you put it earlier, on the first

day your specificity starts to go down, you know. Basically your data. So I think this may be a very important issue. Basically you face a very time sensitivity. That means your threshold will be very difficult to cutoff. And this is my comment.

Another question basically is from your sample, your DILIN sample, you get a 147 sample. Is this the drug all caused by acetaminophen or by diverse drugs? Because the different drug may be caused by different, you know, the DILI.

DR. CHURCH: In the DILIN data set, none of the patients had acetaminophen overdose. They were all multiple drugs.

PARTICIPANT: So, multiple drugs.

DR. CHURCH: Yes, there was many different ones. But none of them were acetaminophen. There were acetaminophen data in the SAFE-T patient data set, but not in the DILIN.

PARTICIPANT: Okay. This drug, for example, onset is pretty low and the high, there's some difference there, or just randomly selected drug? I know the DILIN had close to 1,000 samples, right? So how you select -- the criteria for how you select this 100 sample?

DR. CHURCH: So, we wanted to use the ones that had been collected within two weeks of DILI onset. So that's why the N was --

DR. WATKINS: You can enter the DILIN network up to six months and your liver chemistries can be normal. It was a minority of samples that were collected within two weeks of the onset of drug-induced liver injury. That's why there's a smaller number of 150.

PARTICIPANT: Okay, thank you.

DR. SZABO: Thank you. Let's move onto the next question.

PARTICIPANT: Yes, so two questions, one for Rachel and one for Dan. Rachel, you just kind of answered very quickly there was a bunch of different drugs. I'm going to press that. How many different drugs amongst the 147 samples?

DR. SZABO: Can you please speak closer to the microphone?

PARTICIPANT: Sorry. How many -- among the 147, and especially among the 16, and then the other cohort. I forget. There was like 20. How many different drugs were there? It wasn't like dominated by one or two drugs, was it? Were

there 16 different drugs here, 20 different drugs there?

DR. CHURCH: No, I would say both groups had multiple different drugs. Neither of them was dominated by any one. In both groups, there was also different forms -- cholestatic as well as hepatocellular represented in all three of the groups as well. So I would say there were at least 10 different drugs.

PARTICIPANT: Okay, 10 different drugs. Okay.

DR. CHURCH: It could be more. Off the top of my head, I'm not sure. But it certainly wasn't overwhelmingly one drug.

PARTICIPANT: And presumably you looked and made certain there wasn't some other bias, like age, or I don't know, some other bias that could be responsible for the segregation. There was no age bias.

DR. CHURCH: Yes, not that I could determine, no.

PARTICIPANT: Okay. So now I'm -- this is really nice data. This is what we've been looking for for a long time. This is really

exciting. I agree with you, Paul, this could be a breaking point here. But I'm trying to put it together with Dan's data with acetaminophen. And the model I'm kind of coming up with is that all drugs that induce this acute liver injury in any patient are going to look the same with acetylated HMGB1 initially. And Dan's data says if you can get in there and block that conversation real fast, you can limit damage. It'll be interesting to see if you can do that with every drug, or just acetaminophen. So that's going to be really important to do. But it sounds like that discussion is taking place that HMGB1 is participating with immune cells, and trying to get everybody to join the conversation in that initial stage. Some drugs will keep that conversation going on and on and on and on. Other ones tend to like shout it out, and then be done, and then leave the podium. They just walk away, right? And then there are some drugs that want to do that, and in some patients they're allowed to keep that conversation going for a long time, but other patients say get out of here. And so, Dan, your work is wonderful. You're trying to get at some of the fundamental underpinnings for understanding

that. But this biomarker seems to be able to tell us that. But what we really need -- this is the question I want to ask Dan, because progressing with the science in people is going to be tough. Then you get guys like Jack Uetrecht with his models of trying to modify the immune system. Maybe that'll keep the conversation going longer, and you can look and test this hypothesis, whether that HMGB1 signal can persist over time. So Dan, in animal models, any other animal models with any other drugs with repeat administration -- even with acetaminophen with repeat administration -- what happens to that HMGB1 signal? Does it go up and come down, or does it climb? What happens over longer periods of time, besides that two-hour, six-hour time point? Can you tell us a little bit about that?

DR. ANTOINE: Yes. So, I mean, in terms of the time, of course, we got obviously really detailed on the standing of the events following acetaminophen overdose --

PARTICIPANT: You've got to get closer to the mic. I did too.

DR. ANTOINE: We've got a real good understanding of the time course of events in

acetaminophen overdose. And essentially what happens is that you get a peak very early on of the necrotic form followed by a second peak of the acetylated form. And those both actually resolve within 24 hours in that particular model. That's if you like the only drug model we've looked at. But we've looked at chronic models of fibrosis, alcoholic liver disease, and where you see a persistent elevation, particularly of these inflammatory forms of HMGB1 to a much lower degree as you do quantitatively, as you see with acetaminophen overdose. But it is there bubbling around in the background and participates in those chronic inflammatory events. But I think it's also important to note out the -- just to understand what these biomarkers are actually telling us as well. So, the acetylated HMGB1 indicates the active release of this protein from alive cells participating in this conversation as you've suggested. And I think that's only half of the story as well. I think what we also need to do is go back and revisit the data, and look at the redox forms of HMGB1 in these same individuals, because that is really what's going to give us the mechanistic

driving information about whether this molecule will participate or not in those conversations.

DR. SENIOR: Dan, what's the significance of the acetylation process? What's that telling us?

DR. ANTOINE: So, that's telling us that there's been an activation of an immune response. There's been acetylation of HMGB1, so therefore it can't translocate to its normal nuclear position if you like within the cell and participate in transcription translation. It's packaged up and secreted as an active inflammatory mediator. So that's really the significance of that mechanistic understanding. And that's really dependent on histone acetyl transferases.

DR. SZABO: And following up on the question, so is there any way to know if this is coming from hepatocytes or macrophages? Because it's a little bit confusing that there's a lot of referral to acetyl but then HMGB1 being a marker of inflammation, which can you clarify that? That's a little confusing.

DR. ANTOINE: Yes. So, no, you're completely right. So this is a non-hepatocyte

specific biomarker. We believe it's a mechanism-specific biomarker. So we always pair this with a miR-122. And we've started to make some inroad into understanding where it's come from using different conditional knockout mice. So we have a macrophage-specific knockout mouse and a hepatocyte-specific mouse. So we believe that the initial signal is from the hepatocyte, but the acetylated form in acetaminophen overdose is specifically derived from the macrophage or the Kupffer cell. But in chronic liver disease actually the hepatocyte itself has the potential to acetylate HMGB1 and secrete that as an active messenger. And we've shown that and published on that in alcoholic liver disease.

DR. SENIOR: Dan, is that association between the acetylated form and the RNA, miR-122, is there some message in the RNA that's causing the acetylation?

DR. ANTOINE: We don't think so at the moment. I mean, the interesting concept actually is if you actually knock out HMGB1 from a cell, that cell itself acts more like the cell it's supposed to be. You actually free up the transcription

factors to interact with DNA. So if you knock out HMGB1 from a macrophage, those macrophage phagocyte has a lot more than they do when they actually do have HMGB1.

DR. SZABO: Thank you. Dr. Bonkovsky?

DR. BONKOVSKY: Dan, congratulations. Beautiful work. You're not really thinking that this is a marker-specific or selective even only for drug-induced or toxin-induced injury. Tell us about viral hepatitis, autoimmune hepatitis. There are lots of other liver diseases. What can you tell us about that? That's the first question.

The second one is: tell us a little bit about the assay. I mean, right now you're the only one in the world that really can do this, is that right? And how much blood do you need? What's the turnaround time? If this were ever to be sort of useful as a marker for, okay, these are people who are going to be in trouble and we need to do a treatment trial we would need a much more rapid turnaround time than sending 20mls of blood to Liverpool and hoping for a result after a month or two.

DR. ANTOINE: That's a great question,

Herb, I really appreciate it. So, the first part of your question is looking at the role of HMGB1 in different liver diseases. So, we believe that it's not specific for drug-induced liver disease at the moment. The data is telling us that these biomarkers really reflect conserved mechanisms of pathophysiology within the liver. So we've looked at acetylated HMGB1 in alcoholic liver disease, carbon tet fibrosis. We've also looked at HMGB1 in patients with hepatitis C infection. And the interesting thing about those patients -- we recently just published that in Gut -- is that patients that -- when they have their first liver biopsy we can see they all have pro-inflammatory forms of HMGB1. But on treatment, those that respond to treatment and don't progress in terms of fibrosis, we look at the immune histochemistry staining in HMGB1 for those individuals. The progressors have a higher increase of HMGB1 expression in the liver. They have a stronger translocation from the nucleus to the cytosol, and they also have acetylated HMGB1 in blood. The guys that respond to treatment, the expression of HMGB1 in the situs, all goes down. They have the oxidized

form of HMGB1 in blood. So it looks really quite interesting as a prognostic biomarker for those scenarios. I think the thing that's really pairing it apart from normal liver disease and DILI is the time series of events, something that's been commented on previously and something that John's really pushed forward is the concept of time with these biomarkers. And really with drug-induced events, what we're seeing is the necrosis form really followed by the inflammatory form. But in a lot of these inflammatory-based hepatocyte diseases, if you like, we see the inflammatory form concomitant with the necrotic form as well. So I think time is a key element for that. With respect to your question on the assay itself, unfortunately we're the only lab in the world at the moment that is running these assays. That doesn't mean that we're not the only lab that can run these assays. It's not by far and away any sophisticated mass spectrometry at all. And there is scope to improve that assay. So we're really quite actively discussing with other people to try and transfer this assay out of our hands and to work with other people. What we have is an assay that we've

developed and validated quite rigorously through the safety bioanalytical validation guidelines. But there is scope to improve that and improve the turnaround time. At the moment, we do need about 50 microliters to sample at the moment.

DR. MATTES: Bill Mattes, FDA NCTR. And I'm kind of embarrassed after such great talks and Frank's insights to come up with something far more pedestrian. What I'm hearing in all of the talks is the importance of biomarker kinetics. And it really comes out actually as John came up with the time course for any individual patient. So that makes me ask a very simple question. As we drive to biomarkers that will deal with drug-induced liver injury, are we actually thinking about ending up with something like the insulin monitors -- a patient, a person does a finger stick every day, and we sort of track things as they go? And I ask kind of anyone who would be interested.

DR. WATKINS: Well, I think the first part of the question, you know, the kinetics of these things, the release and clearance has been ignored in ALT, AST, alk phos. Not such an issue in chronic liver disease, but when you've got an

acute and then resolving disease, it becomes very critical. So that's why it was actually Frank Sistare very early in the DILIsym Initiative that said biomarkers, translational biomarkers should be a major focus, and it has been. Now, in the individual patient, whether you'd want to be able to retrospectively look at the kinetics in say blood drops or something from finger pricks, something that I haven't -- don't know what's practical or thought about. But obviously the more data points you have, the better.

DR. MATTES: Well, I guess what I'm thinking is, if you think down the road that you get a combination, or one or two biomarkers, that will give you the indication that you either are going to adapt or need help. And if you are going to apply this on a more generalized basis, then if kinetics is an issue --

DR. WATKINS: Oh, I see. Yes, okay. So once you have an injury you're talking about, right?

DR. MATTES: Yes.

DR. WATKINS: Well, you know, clinically the treatment for drug-induced liver

injury is you stop the drug. That's why there's only a handful of hepatologists who do it. You can't charge for it clinically. It's a minority of patients. The 1 in 10, 1 in 5 that go onto chronic. And so you can wait in that sense. I would say clinically, in terms of clinical management, you can wait two weeks to see if they're getting better or not, and then do an acetylated HMGB1 test if that all marks out. I don't know that you would need daily kinetics for a therapeutic intervention, for instance.

DR. MATTES: Some of this has to do with really, is it something where you just need an occasional blood draw, or is it something you need more frequent?

DR. WATKINS: Well, certainly for the science of it, the more blood draws we can have, the better. That's why phase I makes a lot of sense in that period. But out in phase III and things, I just don't know what the practicality of that would be. But again, the more data you have, the better.

DR. MATTES: But perhaps I'm thinking of the curves Rachel showed, where you're seeing --

DR. WATKINS: Oh, yes.

DR. MATTES: You don't know where you are. You don't know where you are in that.

DR. WATKINS: Sure. If you want the earliest therapeutic intervention, daily measurements. If this hypothesis turns out to be true of a short half life of acetylated HMGB1, that would make sense.

DR. SZABO: Great. So, in the interest of time let's move on. There are three more questions. Short questions, short answers, if possible.

DR. MEHTA: Sure. Ruby Mehta, FDA. Excellent presentation. My question is related to the assay. Are there any differences if you were to run the assay on fresh blood sample versus on the stored and thawed samples in the assessments? Are they similar? Are they different?

DR. ANTOINE: Short answer is -- it's the same. We've been through quite a robust bioanalytical validation protocol even before we were able to get hold of these samples through -- via SAFE-T. So we know that quite well, and it's the same.

DR. MEHTA: Thank you.

DR. AVIGAN: Dan, I had a question about the stoichiometry. So, these antibodies that block. These are putative neutralizing antibodies. So, when you count molecules that you inject and then they're circulating over a period of time, and you're trying kind of to neutralize HMGB1 molecules -- which are presumably cytokines that are secreted -- what actually is the stoichiometric consideration? I mean, how many molecules are you actually binding to? And then the time effect of these circulating antibodies over a long period of time. So that's the question.

Then the second part of the question is: what's the down side? In other words, let's say you block necrosis effectively. Is there a down side in terms of delaying some regenerative process in the liver that actually might, in the long run, be to the benefit of the patient? Let's say in a viral infection situation or something like that.

DR. ANTOINE: So, the first part of your question, we're still working that out. We don't completely know the answer. But for the second part of your question, that's an important point as well. Because we know that these antibodies in

circulation have a long half-life. And there is some evidence now in the literature that HMGB1 can participate in -- intracellular HMGB1 can participate in regeneration as well. We believe that that's isoform-specific as well. So that's why we're really going down the route of trying to produce these specific isoform-dependent therapies. Something that's actually quite promising is we've developed sort of aphamas of 6 kildon-like antibody molecules which have a lot shorter half life and you can actually target those for specific isoforms.

DR. REGEV: So, actually two short questions, one to Dan and another to John. So, Dan, first of all, congratulations. This is very impressive. And happy birthday, again.

DR. ANTOINE: Thank you.

DR. REGEV: On the potential treatment, everything goes well in the future and we have this potential treatment for acute drug-induced liver injury, where do you see the timing-wise as far as when do you use that as treatment to stop the process into acute liver failure?

DR. ANTOINE: So, again, I think my

ultimate ambition really is to develop a biomarker-based therapy package if you like, and to use HMGB1 itself to guide the actual therapeutic intervention. So intimate knowledge of that time course of the right biomarker at the right time, the right HMGB1 isoform to target with the right therapy. That's really how I envisage that, rather than as a blanket treatment in post-injury events. But to use the biomarker to guide the right therapy.

DR. SENIOR: The hard problem is picking the right one at the right time. The sequence of the biomarker information is going to vary with the situation, and it's difficult to create an overall plan.

DR. ANTOINE: No, I agree.

DR. REGEV: So a question for John. I've been asked this many times, and you're the closest person to this statement. So, when the Hy's Law states the 10 percent. So people with hepatocellular injury and jaundice, will they have 10 percent mortality if they continue on the drug? Or it is even if they discontinue the drug, they still have this 10 percent mortality? What was the exact statement?

DR. SENIOR: It's not a fixed number, that 10 percent. It's an approximation, developed on a really small sample. I don't think we can answer that question at all. It's not a fixed number. All we do know is that a moderate injury is more common than a very severe, life-threatening injury. But the exact number is difficult.

DR. REGEV: But you mentioned if it was on the drug or off the drug.

DR. SENIOR: Well, this is what we're trying to find out. This is what it's all about. And that's what we need. We need the serial data, as Paul said, in order to find out.

DR. SZABO: Okay. Last question.

PARTICIPANT: Okay, last question. So, after I left, John, you were saying something about the source of the HMGB1. And Dan, you were making it clear that it isn't hepatocyte-specific. But in the experiment you did with the conditional knockout, where you saw the dramatic effects, that was hepatocyte-derived, right? There's still HMGB1 expressed in Kupffer cells and every other cell, right? So, at least in that particular case,

that argues that the HMGB1 is coming out of the injured hepatocyte and getting things going. And when you block that, you block that progression. Correct?

DR. ANTOINE: That's completely correct. That's the conclusion of that study is that the hepatocyte HMGB1 is the molecule that kicks it all off.

PARTICIPANT: Okay, so then I got that right. So then I'm going to ask you: when you do a conditional knockout and you knock out HMGB1, have you followed those mice out for a long period of time to kind of see what happens to the immune architecture of the liver? Does it change because you're not getting like little background blips of HMGB1 over a period of time? Do Kupffer cells go away? Some of these other cells we talk about, do they abandon the liver?

DR. ANTOINE: The quick answer to that question is the longest we've followed these mice is for about 20 weeks, 16 weeks for subchronic if you like carbon tet experiments. There's no change in those animals compared to wild type at those time points, but it is an interesting concept that you've

mentioned. And we're really trying to do inducible conditional knockout mice at the moment.

DR. SZABO: Thank you very much. Congratulations on a great session. And we have 15 minutes. We can now reconvene at 2:30 sharp. Thank you.

(Applause.)

(Whereupon, the above-entitled matter went off the record at 2:15 p.m. and resumed at 2:31 p.m.)

DR. WATKINS: Okay. It looks like our numbers are dwindling a little bit here, but to stay on time, because I know everybody has to get out of here for planes, our next speaker is Kathleen Gura, who is clinical research program manager at Boston Children's Hospital -- we've not had pedes yet -- is going to tell us about pediatrics and liver disease among infants receiving parenteral nutrition. Thank you.

4.5#1 KG: DR. GURA: Thank you very much for having me. This has been a great meeting, and I've learned a lot. I hope to educate you all on a disease you probably never heard of, parenteral nutrition associated liver disease.

4.5#2 KG: These are my disclosures.

4.5#3 KG: Parenteral nutrition associated liver disease, PNALD, for short -- this little girl's a classic example of a patient we used to constantly treat. She is not typically bronzed. She's actually quite pale. She had a direct bilirubin level of over 20 when we met her. Luckily, she's still with us, but there's been a long history.

4.5#4 KG: Parenteral nutrition, for those of you not aware, it's a form of intravenous nutrition that we give to patients who have inadequate intestinal length or intestinal failure. It's a very complex solution. It's comprised of dextrose, amino acids, vitamins, trace elements. We give lipid emulsions with it as a source of essential fatty acids and to supplement non-protein calories, so we can decrease carbohydrate load. It's a life-saving therapy. Before 1968, children with intestinal atresias often died. But it does have known complications.

4.5#5 KG: What happens if you develop PN liver injury? We define it as cholestasis, as a direct bilirubin greater than two milligrams per deciliter. It's a progressive disease, and

continuous, as long as the child remains on parenteral nutrition. What we do know is if we stop the PN, it does reverse when you get into full enteral feedings. We also know that if you decrease the lipid dose or eliminate it entirely for a short period of time, children get better. However, you've got to be careful because if you develop essential fatty acid deficiency, that also can cause steatosis and de novo lipogenesis. So again, the lack of fat is just as bad as too much fat. Patients with cholestasis can go on to develop fibrosis, which can progress to cirrhosis and to liver failure. A lot of these children used to go on to liver transplant or die waiting for a new liver.

4.5#6 KG: The risk factors are multifactorial. Preemies are at greatest risk because of their immature hepatic function, low birth weight infants. Septic children, every septic event puts another hit on their liver. It's a classic case of the two-hit theory of steatohepatitis. The longer you're on PN, the greater the risk. Intestinal failure, short gut syndrome.

Because of bacterial overgrowth,

bacterial translocation increases the risk of sepsis. There might be a component of the parenteral nutrition itself, either a deficiency or a toxicity that could cause this condition. These children are prone to multiple OR visits, and every OR visit increases the likelihood of developing PNALD, and also the lack of enteral nutrition because of the villous atrophy, which leads to bacterial translocation, sepsis, etc.

4.5#7 KG: So it's a deadly condition. It's not at all uncommon, especially in the pediatric world. Forty to sixty percent of all children with short bowel syndrome go on to develop cholestasis. Dan Teitelbaum's group in Michigan, back in 2000 or so, reported that it was 78 percent fatal if your direct bilirubin remained above 3 mg/dL for three months. More recently, Paul Wales' group in Toronto, SickKids, said that PNALD was 90 percent fatal if the cholestasis continued to progress and the child remained on PN for a year. Until 2006, it was responsible for 1.4 percent of all deaths of children less than four years of age, and it was the leading cause of liver transplant in that population. But we do know that the cholestasis does

resolve if you stop the PN and you're able to give full enteral nutrition. For us, in pediatrics, it's a race against the clock to achieve full enteral nutrition, stop that PN, and the child will recover. But oftentimes, they may not have adequate bowel length to do so. The question for the group is, is PNALD a form of DILI? Being the pediatric person, we do what we always do. We take what the adults do, we try it and see if it works, and if it doesn't, we'll make it better.

4.5#8 KG: What we did was we took the 2014 adult DILI criteria and we saw if we could apply it to the pediatric population.

4.5#9 KG: We had a pre-existing database of children that we treated at Boston Children's with PNALD. The reason we selected that cohort was because we thought the PNALD is associated with the use of phytosterol-rich soybean oil lipid emulsions that is very commonly used in parenteral nutrition.

4.5#10 KG: So the method we used, we actually applied the criteria at baseline. Baseline to us was when we stopped the use of the soybean oil, phytosterol-rich lipid emulsion, and then began our treatment for PNALD, which was a fish oil-based

therapy. We applied the adult criteria, as you see listed there, but because we already had concerns about alkaline phosphatase levels, we decided to try another marker GGT, in lieu of alk phos, to see how that behaved.

4.5#11 KG: The results. We had a total of 214 children that we had treated for PNALD at Boston Children's. Of those, we had to exclude 46 because they were over a year of age, or they transferred in to us already on the fish oil emulsions. We were able to analyze 168 children.

4.5#12 KG: When we looked at each component of the DILI criteria, we found that most of the children didn't really conform. In fact, one of the worst behaving ones was alkaline phosphatase. Only 11 percent of the children actually met that criterion. In fact, the best criterion was GGT, which is not currently part of the adult DILI criteria, and that was 62 percent.

4.5#13 KG: When we looked at the proportion of patients that met the criteria for DILI, when we compared the classic components of ALT, total bili and alk phos, it was much lower. It was only 39 percent, compared to -- substituting alk phos for

GGT and combining ALT total bili and GGT. That was 69 percent. That suggests that kids obviously behave a little differently.

4.5#14 KG: However, we know the study's terribly flawed. We knew that going into this. It's a retrospective analysis of prospectively collected data, the very heterogeneous population. PNALD is not an acute liver disease. The onset's gradual, can occur over a period of weeks to months. It doesn't happen overnight. Even though we assume that it's due to the phytosterol component of the soybean oil lipid emulsions, we don't have serum phytosterol levels to compare to see response, so we use a surrogate biomarker. We use direct bilirubin to identify patients with PNALD, and those are the children we treated with our therapy. Of course, not all patients with PNALD will have an elevated direct bilirubin level, so of course, we could be accused of selection bias.

4.5#15 KG: Again, let's remind everybody about the limitations of using adult DILI criteria in children.

4.5#16 KG: There are many concerns. We don't even have pediatric DILI criteria at present, and we also

know that elevations in hepatic enzymes in children may not be due to the liver itself.

4.5#17 KG: It may arise from another tissue besides the liver. Let's look at alk phosphatase. It doesn't just come from the liver. It comes from bone, as well as the kidney, placenta, and small intestine. The problem in clinical practice is most centers, because of cost, only look at total of alkaline phosphatase levels. They don't fractionate. It's too expensive.

4.5#18 KG: When we see alkaline phosphatase, the child has a bump in it, oftentimes it's not because of the liver. It could be due to growth, or it could be due to metabolic bone disease because of handling fractures, so it's a different cause. However, if you have cholestasis, you will see a rise in your alkaline phosphatase levels also. There's also gender, as well as age variations, and as I mentioned before, most people don't look at the fractionated alk phos.

4.5#19 KG: At our institution, as part of our study in PNALD, we did look at 15 patients at baseline and fractionated their alk phos just because we weren't comfortable responding to those alkaline

phosphatase levels and assuming they all came from liver. When we fractionated them, we found that 80 percent came from bone, and only 20 percent came from liver. So again, makes the use of alk phosphatase suspect when you're trying to decide if a child has DILI.

4.5#20 KG: When you talk about bilirubin, bilirubin's just not one lab. It's actually three different markers. It could be indirect, direct, or delta bili. Some centers like to report it as total bili. But when we talk about bilirubin levels in PNALD, we talk about the conjugated bilirubin.

4.5#21 KG: The direct bili being above 2 mg/dL, that came to consensus at the 2012 FDA GREAT Workshop. However, we also know that patients who have fibrosis and cirrhosis can have normal bilirubin levels.

4.5#22 KG: So again, even this lab is not perfect. This slide just shows you we had a group of 40 children who had biopsies that showed that the children had cirrhosis. It's not typical that we do biopsies in this population, but when we have them, we like to look into them further. We noticed

that these children had cirrhosis on biopsy, at the time of baseline, they all exhibited elevated bilirubin levels. However, over time, we were able to get the bilirubin levels down to normal and remain normal. The group on the left is all converse patients on parenteral nutrition, as well as the ones who subsequently came off. The group on the right were all the children who could continue to receive parenteral nutrition with fish oil ad infinitum. This is going on now over five years. Again, we didn't stop the PN, and they still remained with normal bilirubin levels.

4.5#23 KG: What about ALT and AST? Again, not all of it comes from liver. It also can come from the heart, skeletal muscle, kidney, brain, and pancreas, and the levels don't follow normal, nice bell-shaped distributions.

4.5#24 KG: We also know that the absolute levels don't correlate properly with disease severity or the extent of hepatocellular damage. You can't use it for prognostic information. If a child has a burnt out liver, they're going to have very low levels. That's only because there's very little viable tissue left to actually excrete the enzymes.

You could have a child with a normal ALT and AST, and the only way you know they have liver dysfunction is because they have a prolonged PT, and they have decreased synthetic function, as exhibited by a low albumin.

4.5#25 KG: So it's very common to see mild elevations, especially in PNALD, as well as NAFLD. The numbers can lie, so we always look at the whole patient. We don't just respond to numbers because we know that elevated ALTs and ASTs can happen without liver disease, celiac disease, thyroid dysfunction, adrenal insufficiency, very commonly seen in the premature infants. We also know that liver disease without elevated enzymes can occur, such as hemochromatosis or chronic hep C, so again, can't just look at the numbers.

4.5#26 KG: We looked at ALT in our PN population and children who actually had normalization of the direct bilirubin level and were on full enteral nutrition. We saw that the ALTs continued to remain elevated. So we were wondering is it ongoing hepatic dysfunction, perhaps ongoing hepatic inflammation? But we also just didn't know if maybe this is continued disease progression,

despite coming off PM. We just looked at the relationship between the ALT and direct bilirubin.

4.5#27 KG: We noticed the bilirubin levels always normalized months before the ALT would, and the ALT would bounce around and remain elevated. It got better, but never got to normal. So that's something we learned is that over time, the trends got better, but they never normalized.

4.5#28 KG: When we looked at our children with cirrhosis, same pattern happened in this group. These children had normalization of the bilirubin levels, but their AST and ALT never quite hit normal. The trends improved, but they actually did not normalize.

4.5#29 KG: When we looked at GGT, the marker that we thought was better than the others, in looking at a new and improved DILI, even that number is suspect because even though some of it comes from liver, it can come from other sources, as well, including the proximal renal tubule, pancreas, heart, lung, and brain. Preemies often develop intraventricular hemorrhages, so of course they have elevated GGT for a different reason. Also, breast-fed children, they get mom's GGT.

Apparently, there's no fractionated GGT in the U.S. However, there's a team in Italy that's currently looking at this, and that might be very useful to look at, just like we like to look at fractionated alkaline phosphatase.

4.5#30 KG: Again, GGT and DILI, GGT appears to be better, not perfect, more sensitive, but still not perfect.

4.5#31 KG: As a reminder, we showed this before.

4.5#32 KG: One thing I did add just because of yesterday's discussion, we do have a PELD score. It's like the MELD score. It's just for kids, but it's different. We don't look at creatinine; we look at growth. We did look at the improvement in the PELD score of these cirrhotic children over time, after -- they started at baseline when they started our therapy. Over a period of 12 months, it normalized. So again, it was a nice way to trend our patients' response to treatment over time by following the PELD.

4.5#33 KG: In conclusion, we have to consider all these limitations when we look at the different criteria when we're evaluating the pediatric patient. Obviously, there's more work needed to be

done in children. In fact, maybe we should toss the term out the window when we talk about PNALD.

4.5#34 KG: Maybe DILI shouldn't even be used when we discuss this disease state, maybe something else, like multifactorial induced liver injury, MILI, or something else. Maybe that's more descriptive of this condition.

4.5#35 KG: With that, I'd like to thank my team, Boston Children's Hospital, Mark Puder and Paul Mitchell and Alex Potemkin, and actually the FDA, Dr. Mulberg, Drs. Yap, Kim, and Chen, who they inspired us to look at our database a little differently to see if we could come up with a pattern to understand this condition a little better.

4.5#36 KG: Hopefully, after this meeting, I'll learn more, so thank you.

DR. WATKINS: All right, our next presentation's from Yvonne Dragan. I've known Yvonne for more than three decades, when we were in the same lab together at VCUMCV. Her current position is director of global discovery toxicology at Takeda. She will talk to us about TAK-875.

4.6#1 YD: DR. DRAGAN: I'd like to start by

introducing the players on the team. John and Neila are pharmacovigilance clinicians who contributed to this program. Nizar performed the pharmacogenomics data that I actually won't speak about today. Mitch Friedman is the development toxicologist. Juliana is a statistician. Francis is the investigative toxicologist, and Ohira-san is the medic who was involved with this program.

4.6#2 YD: TAK-875, as I'll call it throughout this talk, is a GPR40 agonist. It has a unique mechanism of action compared to other agents that have been developed for type 2 diabetes. It's orally active and quite potent and has been shown, in Phase 2 clinical trials, to be effective. However, this compound was stopped during Phase 3 clinical development due to liver safety signals.

4.6#3 YD: I want to talk about both pre-clinical and clinical aspects of development of this compound because I think it tells us something about how we need to work together; and b) that on both sides of that divide, we don't actually understand the signals and perhaps could do a better job.

4.6#4 YD: In this particular case, in looking at the GLP toxicology studies -- here I'm just

discussing the 26-week study -- we actually saw no histological evidence of liver toxicity, although we did see some minor rise in transaminases. This was at a multiple of 72X, the expected efficacious dose in the clinic. We did see some centrilobular hypertrophy, but again, that would not be consistent with the small degree of rise that we did see. The NOAELs were not based on that, but rather on the submandibular gland toxicity that was observed.

4.6#5 YD: However, in the GLP tox studies in the dogs, a different pattern arose. Actually, this was both dose and duration dependent. In this particular case, I have the information for the 39-week study plus 13-week recovery. In fact, we saw a number of effects in this, both time point and dose. We saw both hyperplasia of the bile duct, some inflammatory cell infiltrate, some necrosis of the bile duct gland, and in addition, there was granulomatous formation with inflammation in certain of the cases, as well as we could see precipitate in the biliary tree. Other types of toxicity were observed, and NOAELs were set with sufficient safety margins to move forward with the

compound.

4.6#6 YD: This is two dogs that looked -- this is the response of two of the dogs that one could see in a very short-term investigative study. In these two animals, we saw marked ALT, AST, GGT and alkaline phosphatase increases. Again, this was at a high multiple relative to the human efficacious dose.

4.6#7 YD: This is the pathology picture with regard to the impact, with respect to the formation of the granulomatous response, again in the portal area.

4.6#8 YD: The other pathology that was observed was, again, this crystalline formation in the biliary tree. This is a higher magnification.

4.6#9 YD: What's important is then taking these sections and doing MALDI mass spec analysis, we could identify that within the granuloma was TAK-875 and its glucuronide. In further analysis of these samples, we found that there were no dog-specific metabolites in either the plasma, the bile, or the liver, and that there wasn't any evidence of covalent binding to liver proteins, despite the fact that this is an acyl glucuronide.

But again, the reactivity level is very low, and no protein binding was uncovered. However, there was found that there was a high level -- because we had found this high level of both the parent and the glucuronide in the biliary tree, that part of the issue seemed to be that because dogs, relative to rats, have a slower bile flow rate, that we actually had precipitation of the compound, due to exceeding its solubility in the bile in these animals. This is what resulted in the granulomatous formation with inflammation.

4.6#10 YD: We did some in vitro studies to try to rule out some other potential confounders, so very typical in vitro studies to look at the glucose galactose inhibition pattern to see whether or not you might have an agent that has a mitochondrial toxicity. While certainly, at very high dose, one could observe such toxicity, if one took into account the exposure, there actually is minimal effect at the serum plasma levels that are projected for people, and also with a 10X margin.

4.6#11 YD: The other side of that is to look at the effects on the biliary transport mechanisms. Again, if one considers just the transporters

important for movement into the canaliculus, both for the bile slots that go through BSEP, but also for the conjugated bile slots that can utilize MRP2, it's important to consider not just the effects on a single transporter, but rather on multiple transporters.

4.6#12 YD: What's shown in this slide is actually inhibition patterns for multiple transporters by TAK-875. Typically in a development program in relatively early discovery, we'd be looking primarily at the parent compound. If you compare in the red box, the micromolar inhibition level for human, dog, and rat BSEP, they're not spectacularly different. However, if one takes into account the glucuronide in addition, one can look at the human and see that the glucuronide is much less potent, but in the dog, the combined parent and glucuronide has some activity. This is very different from what would be observed in the rat. Again, we did not see the same type of histology in the rat that we saw in the dog.

4.6#13 YD: This may be one contributor. The other thing to look at would be MRP2 and the glucuronide can inhibit the MRP2 at approximately the serum

level. The clinical program was stopped in 2013. Approximately at that time, this really nice paper by Morgan, et al. came out, indicating a way to consider what that in vitro BSEP or other transporter binding should be taken into in the context of the plasma serum or serum levels. In doing so, one can see that on TAK-875, which is in the aqua color in the upper box that indicates a higher risk zone, that TAK-875 would fit into that zone.

4.6#14 YD: But that alone seems to be an insufficient argument in this case, in part because we have a much higher level of both the parent and the glucuronide in the liver of the rat, so we would have expected to have overwhelmed that system in the rat if that were the only contributing factor. Still, it's probably one of many factors. I placed in this diagram to remind me to say that we are working closely with DILIsym program in order to try to model in the context of exposure cross-species, what are the risk factors that are involved for this specific compound? We have agreed, as a company, to provide that dataset to DILIsym. While this is a way over-estimate when one doesn't take into

account the actual exposure level or inhibition level, and it's not done per species, it does suggest that we should be looking at these very carefully for each of the species to understand what they tell us about potential risk for human.

4.6#14 YD: Bile salts can cause toxicity due to their detergent properties. Particularly MRP2 can compensate for BSEP inhibition, but an inhibition of both, again, in the context where your primary metabolite and elimination method is a glucuronide is an increased risk, for certain.

4.6#15 YD: In addition, the alteration in bile acid levels may be one of the other contributors to the decreased solubility of the compounds within the dog bile and have contributed to its crystallizing out of solution.

4.6#16 YD: Again, this is just a slide that indicates there is a decrease in the measured bile acid concentrations in dog. I want to switch gears and talk about two of the clinical programs that provide some evidence for DILI.

4.6#17 YD: First, I'd like to state that the Phase 1 clinical trials did not indicate elevations of transaminases or any indication of concern. Phase

2 studies were performed, one in Japan and one in the U.S. and South America. Again, there was not overwhelming evidence that DILI would be a concern.

4.6#18 YD: A cardiovascular outcome trial study is required for any new type 2 diabetes compound, and the design of the study is shown here, in which the placebo versus the higher dose of 50 milligrams TAK-875 was provided. The exclusion criteria are provided at the bottom and are fairly common. Here is the dataset from that clinical trial.

4.6#19 YD: Again, for the TAK-875, relative to placebo, you can see that for the indication of 3X, upper limit of normal for the transaminases, that there is an increase in the TAK-875 group, again at the 5X, again, albeit it low, and at the 10X. I'd like to then show you the index case for that particular example. In this trial, there was one individual who presented at, I believe it was 29 days after start of compound administration, with a high increase. You can see the ALT in red. You can see, in blue, the AST elevation, and the orange color is GGT. Alk phos is a gray color, and the T bili is in black. Again, this is our index case in this study that suggested that a Hy's Law-like case

has certainly occurred. Again, this went to adjudication and was suggested to be possible.

4.6#20 YD: A Phase 3 study that was performed in Japan, designed as follows, in which there were two dose groups, 25 and 50 of TAK-875, with the exclusion criteria as shown, was performed.

4.6#21 YD: In this study, again, the increases in ALT, three times greater than upper limit of normal, are shown here, with three cases for 25 milligrams and for 50 milligrams.

4.6#22 YD: If you look at the cases that are either five times upper limit of normal or that are three times upper limit of normal with a total bili of 2X total bili, there is one case that I'll present. In this particular withdrawn case, what can be shown here is the ALT is in blue, the T bili is in purple. This individual had gallstones and was on a number of concomitant meds, which are shown here. Again, the adjudication suggested that this is unlikely to be associated with TAK-875. However, these two cases, in part, led us to look at an aggregated assessment of all of the ongoing clinical trials, and it's based on that analysis, in order to be protective of patient safety, that we stopped the

development of this compound.

4.6#23 YD: The key messages that I'd like to provide to you are that the liver was identified as a target organ in non-clinical GLP tox studies, that the crystalline formation was thought to cause liver toxicity at high doses in dog, but sufficient margin existed to take the compound forward, relative to the human efficacious dose, and that we did not see a similar crystallization in the rat. In post Phase 3 termination, additional investigative studies were performed. These included demonstrating the effects on the hepatobiliary transporters, including inhibition of BSEP and MRP2. We also demonstrated that bile acid homeostasis was altered in vivo in these studies. In the in vivo dog studies, the serum bile acids were elevated earlier than the overt liver injury was observed. Bile acid quantification in these treated animals also indicated that the solubility of the compound was exceeded in the dog, in the dog bile, but calculations suggested that even under a worst-case scenario, at least a 15X margin to human at the 50-milligram dose should have been sufficient to protect patient safety. Again,

TAK-875 development was terminated based on the liver safety signal.

Thank you.

DR. WATKINS: Great, thanks. I want to comment that obviously it was a big blow, I'm sure, or disappointing to Takeda, late in Phase 3, with a promising first-in-class effective drug for type 2 diabetes, to call it quits. But to their credit, they have really made an effort to engage academics, our group in particular, with the genetic mouse studies, as well as DILIsym, with the desire to put all this in the public domain, so that new knowledge will come from all the patients that were exposed, as it turned out unnecessarily, to the drug.

Our next speaker is Scott Siler. We heard a little bit about DILIsym initiative. He's co-director, along with Brett Howell, who's in the audience, who's going to talk to us about modeling drug-induced lipotoxicity.

4.7#1 SS: DR. SILER: Thank you for the invitation to present today at this fantastic meeting. I really appreciate and am thankful to have the opportunity to do so. I'm hopeful that I'll be able

to, with this presentation, build on some previous presentations, both from this year and prior years.

Dr. Watkins has alluded to and provided a little bit of description of the DILIsym platform, which is a mathematical, mechanistic model of drug-induced liver injury. In prior years, my colleague, Brett Howell, has given similar presentations describing the platform, and last year Dr. Regev gave a nice presentation on some of the clinical aspects of drug-induced lipotoxicity within the liver. Taking all those bits and pieces together, I'd like to share with you at least our attempt at modeling drug-induced lipotoxicity. Before we enter in the realm of mathematics and predictions, let's just take a step back and remind ourselves of the underlying physiology, how it interacts, and how drugs might participate in dysregulating the system. Within the liver, there are multiple pathways that participate in regulating the lipid levels.

4.7#2 SS: When I say lipids, I'm referring to fatty acids and triglycerides, not so much considering cholesterol. There are four primary pathways for partitioning fatty acids within the liver: 1)

delivery of fatty acids to the liver via the adipose tissue and the circulation; 2) changes in fatty acid oxidation that can lead to the accumulation of lipids; 3) disruptions in release of triglycerides from the liver by inhibiting VLDL triglyceride release; finally 4) steatosis, from increases in de novo lipogenesis, the production of fatty acids from carbohydrates. Steatosis isn't necessarily a result of any one of these factors, but could be any of these factors combining in a variety of different ways leading to the increase in both fatty acids and triglycerides. Drugs have been shown to participate in stimulating each or many of these pathways. I think disruption in mitochondrial function, fatty acid oxidation causing steatosis is a biomarker thereof. Now in the world of treatments for metabolic diseases, we may see unintended consequences of disturbances in these lipid pathways. With respect to lipotoxicity, there's a wealth of data singling out saturated fatty acids, probably two dozen in vitro studies in hepatocytes and other cell types, where palmitic acid, the primary saturated fatty acid in the circulation, is implicated in driving up the production of reactive

oxygen species, so inducing oxidative stress within the cells. Saturated fatty acids have potential to cause oxidative stress. But unsaturated fatty acids do not have this effect. The main fatty acid in this group is oleic acid, found in olive oil. Downstream are disruptions in mitochondrial ATP production, and/or induction of apoptotic pathways. This is the qualitative scheme. To build a mathematical model, you need to capture all the quantitative elements.

4.7#3 SS: We've gone in the literature and pulled out as much as is known about these pathways, assembled that in those quantitative pieces within our platform that we call DILIsym. Again drug-induced liver injury is our focus here. We're really trying to develop a platform that allows us, collectively, to anticipate where we might find some challenges as we develop our drugs. It's a mechanistic mathematical model of DILI and includes what we determine, at this point, to be the primary contributors, so the underlying chemistry, biochemistry, physiology. It is then assembled in mathematical equations, and we have developed a nice graphical user interface so that we can pull

together these numbers, punch a button, and we'll have predictions of DILI generating simulation results that are compatible with what's measured in the clinic, what's measured in pre-clinical studies. In order to confirm that we have these qualitative and quantitative components assembled properly, we use what we call exemplar drugs to help optimize and validate the model.

4.7#4 SS: There's a nice panel here, and this list is ever growing. With our next release, there will be another half dozen or so compounds added to this list. We have them categorized, but there is a multitude of compounds that have potential signals or mechanistic components in multiple categories. For today's talk, we're clearly focusing on what's highlighted in red and highlighted in purple. In purple, in the mitochondrial toxicity, I'll talk about etomoxir in the latter half of the talk, which has the potential to be disruptive of mitochondrial ATP production directly, due to its effects to inhibit fatty acid oxidation. It also has the potential to induce lipotoxicity, and we'll be exploring that. It's an exemplar in both groups.

4.7#5 SS: Then there are two other compounds,

Juxtapid and Kynamro, that we've used as exemples for lipotoxicity aspects. Juxtapid is available on the market. You may know it as BMS-201038. It is now available to treat patients with homozygous familial hypercholesterolemia. It's an inhibitor of the microsomal transfer protein, so it perturbs the assembly of the VLDL particles within the liver; hence, presumably less particles in the circulation. There was a fantastic clinical study, a dose escalation study, where every four weeks the dose was escalated, enhancing a greater pharmacologic effect, less VLDL triglyceride released from the liver. However, it was found to increase liver fat. Patients who were not steatotic to begin with became steatotic either during or after this clinical study. Coincidentally, many of those same patients also developed increases in ALT. Correlation or causation, hard to say. With the Daisy platform lipotoxicity with saturated fatty acids was driving the changes in oxidative stress. We captured this data by simulating the different levels of triglyceride accumulation, with increases in ALT, by design. We used this data to help train our

model, to make sure that we were confirming those quantitative interactions.

4.7#6 SS: We brought that forward into simulating Kynamro, also known as mipomersen. It's an apoB100 antisense oligonucleotide that wipes out the primary protein backbone of the particle, and it's also available now to treat familial hypercholesterolemia. However, these patients also showed increases in steatosis, increases in ALT, and as this graph shows, increases in cleaved cytokeratin 18, which, of course, is indicative of apoptosis. The clinical data are given in the gray diamonds, the simulation results in red, again, confirming that we have captured the quantitative elements of lipotoxicity appropriately within this clinical paradigm.

4.7#7 SS: Now we've established by targeting one of the four pathways, perturbed steatosis or VLDL triglyceride release, we were able to predict lipotoxicity. Let's explore a different pathway.

4.7#8 SS: To do that, we used etomoxir. This simulation-based study was done to determine if that clinical hepatotoxicity of etomoxir was due to mitochondrial toxicity or lipotoxicity. At this

point, I should probably point out, etomoxir was originally developed to treat type 2 diabetes, thinking that inhibiting fatty acid oxidation would enhance carbohydrate oxidation. Efficacy was so-so. Basically, it was terminated for that program. However, it was applied later for congestive heart failure, a pretty large Phase 2 trial in Eastern Europe, the ERGO trial, was taken on. That was terminated maybe three or five months into the program because a number of early patients receiving treatment presented with increased ALT and AST. The investigators stopped the trial.

4.7#9 SS: The question has always been looming, then: what was the underlying mechanism for it? Was it because of direct disturbances on mitochondrial ATP production, or was it due to kind of a slow burn, a slow accumulation of lipids, hence lipotoxicity? We believe our platform is well suited to explore this question.

Just a reminder here, I think I more or less described it, etomoxir is a very potent inhibitor of fatty acid oxidation, which can lead to changes in mitochondrial ATP production, in purple, and/or the accumulation of lipids, hence oxidative stress

and apoptosis, in red.

4.7#10 SS: In order to do this properly within DILIsym, we needed to know a bit more about the compound, so we needed to know, on the exposure side, the pharmacokinetics, the properties, so that we can do some physiologically based pharmacokinetic modeling within DILIsym, and some mechanistic in vitro data. What is the exposure response relationship for fatty acid oxidation? We did a good job of capturing the exposure. It was also one of those compounds for which liver concentrations are greater than plasma concentrations.

4.7#11 SS: We also did a good job of capturing the fatty acid oxidation exposure response relationship, put those two together. Then we had to consider what are some of the characteristics of the patients that might have been in this trial, accounting for interindividual variability.

4.7#12 SS: To do that within DILIsym, we use what we call simulated population, or SimPops. In this case, we included mechanistic variability in the oxidative stress component in the apoptosis induction component, the mitochondrial dysfunction

pathways, assembled them in various different ways combinatorically, and collectively used this simulated population, these SimPops, for our simulation, our predictions of DILI. In the ERGO trial, they observed ALT increases after six weeks, and those are the gray points approximating the ALT increase within that time frame, sometime between six and ten weeks.

4.7#13 SS: As we did our simulations, only including the direct mitochondrial toxicity hypothesis, what I described in purple, you can see that we predicted zero cases of DILI, zero cases of predicted ALT increases.

4.7#14 SS: However, if we instead included the lipotoxicity hypothesis, we predicted a similar incidence, with a similar timing, of the ALT increases. This really seems to -- it's simulation. It's not hard data. It doesn't say, "Ah-ha, we have it," but it really helps us gain a little more confidence in the hypothesis that lipotoxicity was participating. Following up on that, taking these same results, these 200 to 250, I think it was, simulated patients within this SimPop, plotting them all together and looking at,

then, liver fat versus ALT, you can see, again -- these are basically the same data in that same way, illustrating how some patients were really particularly responsive to the accumulation of steatosis, hence lipotoxicity. Also, looking at our output of cleaved cytokeratin 18, you can see that clearly, the injury was -- there was apoptosis occurring in these simulated patients, which is consistent with that oxidative stress hypothesis.

4.7#15 SS: To summarize, the hepatotoxicity that was observed in those clinical trials, the simulation results really seem to suggest that lipotoxicity was the underlying mechanism, and had we been able to use these simulations in advance of the clinical trial, we would have been able to inform their decisions, potentially minimize the liver safety concerns, and maybe the periodic measurement of liver fat during the trial might have, again, helped separate patients, monitor patients, such that we could have avoided that injury.

4.7#16 SS: With this, now, we believe DILIsym is well poised to explore some of these other mechanisms that disrupt fatty acid lipid

partitioning within the liver, hence lipotoxicity.

4.7#17 SS: Here are the members of the DILIsym modeling team.

4.7#18 SS: Thank you.

DR. WATKINS: I might just mention, though the public/private partnership is now within the company, the goals remain the same, which is public disclosure, make availability of the model, et cetera. Our next presenter is Lans Taylor. Lans is the director of the University of Pittsburgh Drug Discovery Institute and Allegheny Foundation professor of computational systems biology. There are a number of liver models out there from the DARPA-supported Wyss Institute and MIT. This is the NCATS version. This is the one I, personally, am most excited about, so Lans.

4.8#1 LT: DR. TAYLOR: First of all, I'd like to thank the organizers for allowing me to take part in this very exciting meeting, and particularly those of you who have stayed past a logical time to avoid the traffic in this area.

4.8#2 LT: Why develop human organs on chips? We do know that there are species differences in physiology, toxicology, and response to diseases.

We know that humans aren't rats, although that begs a joke about either lawyers and/or politicians. We also have the potential to build a chip, with IPSCs to address patient heterogeneity in both efficacy and tox testing, in parallel to doing traditional animal testing. If this is successful, we have, potentially, the ability to minimize the amount of animal testing that would need to be done, which is driven both by financial pressures and societal pressures.

4.8#3 LT: This program is a combination of work between DARPA and NCATS of the NIH, with cooperation and collaboration with the FDA and the EPA. The goal, in a very short period of time, was to build microfluidic 3D human organ constructs on chips. The concept, from the beginning, was ultimately to get to the point where you could incorporate iPS-derived cells, so you could make these models very specific to individuals. Another kind of DARPA-esque activity here is to take the advanced individual organs and physically couple them to start looking at, at least, partial human organ-to-organ interactions.

4.8#4 LT: We've built a whole platform of a human

liver on a chip. It involves four cells; iIt's 3D; it's a microfluidic platform that we use for both drug discovery and development. In A, in the upper left, you can see the microfluidic component. We use four cell types. In this first generation I'll describe first, we're using primary human hepatocytes. We select a lot of cryopreserved cells that we've been following in testing a variety of things over time. Then we use three human cell lines for endothelial cells, for Kupffer cells, and stellate cells. We also have the option to add in other kinds of cells for disease. One thingsI'll show you is having the liver used as a metastatic niche for breast cancer. At the bottom of A, on the left, you can see the active area of the device. The green are hepatocytes that have been labeled with a fluorescence-based biosensor for apoptosis, under continuous flow, so there's an influx side and an efflux side. In C, right middle, from that efflux media, we can make a variety of biochemical and metabolic readouts. Because the devices are thin and designed to be optically transparent, we can also do real-time measurements of these fluorescence-based biosensors to look at

mechanism, like apoptosis or ROS production, calcium transients, etc. The other component of the complete platform which is critical is in the upper right, D, an MPS database we've constructed to allow us to acquire, process, manage, analyze, and ultimately model the data, but we can also draw in from external databases information we would need for modeling and making predictions.

4.8#5 LT: This is a simpler diagram, focused on this first-generation device. In scale, it's about a 0.3 microhuman. You can see, in the upper left, the plastic plumbing. There's a dime sitting next to it to kind of show the size scale. You can see the green active area, containing hepatocytes that have been labeled, and down in the lower right, you can see a diagram of the organization of the four cell types. We actually labeled the different cell types and, through confocal imaging, could define where the cells were. This isn't made up.

4.8#6 LT: You can see in this first model, since we call it the SQL-SAL, it's a sequentially layered self-assembly model, we lay down the cells layer by layer, and then allow them to interact with one another, and based on natural interactions between

the cell types, they form the structure. We're already using this first-generation model in some human toxicology testing, and we're also working on three liver diseases, liver cancer, hepatocarcinoma, as a niche for metastatic breast cancer, and non-alcoholic fatty liver disease. I'm not going to show you a lot of the data that we've generated over the last year and a half, but in summary, we've been looking at these by contract with NCATS over the a period of a month of activity. In the first day or so, as the cells have been layered and are interacting with one another, some LDH leakage, and then that flattens out. Since it's under constant flow, we get pretty good output for urea and albumin. You can see on the right, in B, under flow, you've got an increased output. That's been demonstrated in a lot of organ systems that when they're used to seeing a flow, they have a mechanical stimulus that changes their physiology. Some additional functions we've demonstrated, we've taken a panel of hepatotoxic drugs that have demonstrated a variety of mechanisms of hepatotoxicity. We've demonstrated, during this first month period of time, CYPs activity, so both

Phase 1 and Phase 2 metabolism. We also were able to induce fibrosis in the model with methotrexate, where we got an increase in collagen production and a stimulation of smooth muscle actin. We also have been able to demonstrate immune-mediated hepatotoxicity using the standard LPS trovafloxacin combination that induces apoptosis in the hepatocytes. Because we've built a panel -- and I'm not going to take the time to show you the panel of biosensors we've built -- we have drugs where we've positively tested the activity of a variety of physiological biosensors. This just shows one.

4.8#7 LT: This shows, on the left again, the active area in the flow of the device. In the center, we have, in this case, the hepatocytes that have been labeled, or a subset of them, with an ROS biosensor. On the right, we've used a mitochondrial toxin, where we've kind of double labeled them. We've used mitochondrial membrane potential sensitive dye that's in red, and the ROS biosensors in green. So you can see on the bottom, on the right, as the membrane potential is lost in the mitochondria, upon challenge, then you get a rise in the ROS

production. The value of these fluorescence-based biosensors, these are real time. You can look spatially and temporally within the device over the month of the activity, at least so far, of these devices.

4.8#8 LT: I mentioned the database. This is crucial to our ability to analyze the value and continually improve the model. On the left, you can see the organ model. We have a whole array of different readouts that we make over time. Those are captured in the database, in the center there. We can also draw in data on compounds from Open FDA, from Stitch and DrugBank and any database that's available -- draw in information that we can use in beginning to do the modeling. We can also build classifiers, as we build up the number of drugs that we study and their activities. So we can make predictive models of potential toxic liability because obviously, the goal is to make a projection on what would occur in the human.

4.8#9 LT: I'm going to make you read this. This just is an example of one of the readouts of our compound report, where we have the drug, the clogP, then we have information on pre-clinical results,

which we've pulled down from external databases, clinical information, and then graphs summarizing some of the key data.

4.8#10 LT: This is an evolutionary process, I might say right up front. I have drunk the Kool-Aid of organs on chips. On the other hand, I believe strongly that there needs to be validation, and that's where we are now in this whole process.

4.8#11 LT: I think these are lofty goals, and the progress made by all of my colleagues that are in this program on different organs is really quite spectacular, but we have a ways to go to make it a truly powerful tool.

4.8#12 LT: We've already started on Version 2.0 of the SQL-SAL. We've started focusing on the maturation of iPS-derived hepatocytes. We've optimized, to another extent, the nonparenchymal cells and the media that we're using. We also, because zonation is crucial in the liver, we've established an oxygen zonation model, and we've started implementing some of these disease models. I won't go through all the details of this. You've seen iPS cell processes for maturation. You start on the left with human skin cells, go through a whole

variety of magical steps, with drugs, as well as other treatments. But today, for iPS-derived hepatocytes, we still don't have fully mature hepatocytes that have been developed.

4.8#13 LT: If you look on the lower right, in the red box, that's a whole array of different functional biomarkers that characterize adult human hepatocytes. In the blue box is where the field is, essentially, now, where they're partially mature. They're still more fetal than they are mature hepatocytes. One of the things we've been doing, which I think is simple minded, but important, is -- of course, our hepatocytes don't mature from the fetal stage to the adult stage in the dish, in the presence of drugs; they do it within the developing liver. What we've done is to put these partially matured hepatocytes into our devices and let them cook for a month, and then -- we're in the middle now, so I can't tell you the answer -- the goal is to see if we can't have the whole environment that we have those cells in drive them the last stage to fully mature hepatocytes. I won't go through these details, but comparing the SQL-SAL 1.0 to 2.0, we've changed a

variety of things. Again, I emphasize this is an evolutionary process. I suspect we'll go through Version 2, 3, 4, and 5 over the next couple of years. A key thing that we've added is our cell layering. we've added an equivalent of a space of Disse, with a matrix between hepatocytes and endothelial cells. By controlling the flow rate across a physiological range, we can change the oxygen tension within these devices to create either Zone 1 or Zone 2.

4.8#14 LT: This just shows, in the upper left, the Nortis device, the plumbing. In cross-section, we can see that we've improved some of the structure, but a key here is the presence of this matrix that we've placed between the hepatocytes and the endothelial cells. Then this is looking down on top of that matrix, the endothelial cells make a nice sheet. Because oxygenation is so important in the liver, we wanted to create a model where we could control the oxygen tension and create at least Zone 1 and Zone 3. We wanted to model that, as well as measure it. RTP, it's a ruthenium dye, which is quenched by oxygen. It's been used for a number of years for a variety of applications. We harnessed that for making measurements in real time within the

device. We've also modeled, based on biophysical properties of diffusion of oxygen and the consumption of the cells that are in the device. In fact, just by controlling the flow rate from 15 microliters per hour down to 5 microliters per hour, we can create, essentially, conditions of oxygen for Zone 1 and Zone 3. Our goal now, when we're in the middle of those experiments, since there's a lot of activities that are distinct in Zone 3 and in Zone 1, to make those measurements and demonstrate that, in fact, we have created zonation. The next step, in Version 3.0, is a redesign of the device, so we could get zonation within a single device, so we could have Zone 1 at one end and Zone 3 at the other.

4.8#15 LT: I'll just show one slide one of our disease models. This is the case of using the liver as a metastatic niche for breast cancer. About a week into having the liver model function, we add the red cells, which are fluorescently labeled with a fluorescent protein. These are aggressive cancer cells. We put those into the device.

On the lower right, one of the things we found out pretty quickly is that we have two subpopulations of these cancer cells, one in red, which become

dormant -- they don't migrate; they don't divide -- and the line in blue are rapidly dividing cells. If you treat these devices, now, with standard drugs like Doxorubicin, we can actually knock down that rapidly growing population, but as you can see in the lower left, you can see some red cells that remain. They'll remain dormant for a while. In fact, we're working on ways of how can we go after those cells, either re-awaken them and then hit them, or find a separate treatment?

4.8#16 LT: To add insult to injury, from the DARPA NCATS program, in addition to having to build an individual organ with increasing capabilities, we have to start combining organ systems. We've put together a team coupling the gut, liver, and kidney, which makes a lot of sense for tox. The gut is a collaboration with people at Hopkins, Mark Donowitz, and Baylor, Mary Estes. We're doing the liver. The kidney is led by Jonathan Himmelfarb from the University of Washington. John Wikswo at Vanderbilt is building the toys for coupling. On the left, you can see the various interconnections. One of the things that we have done is functional coupling with a combination of some drugs and some

other kinds of metabolites, where we take it through the gut first and take the output from that and give it to the liver and let it act on it, and take the medium from the liver and put it to the kidney. In three of the cases, out of three, where we've done that, these organ systems are doing what they're supposed to do to the molecules. Now we're dealing with the nightmare of scaling and the common medium across these organs, but we're starting to do that. I might add we wouldn't have done it if it wasn't a requirement in the program because we still have a lot to do with the liver. I'm not going to walk through this, but we are implementing, at the University of Pittsburgh, quantitative systems pharmacology for everything we do, all of our drug discovery programs, including safety testing, using an iterative computational and experimental approach.

4.8#17 LT: On the in vitro experimental approach, more and more we're using the organs on chips from humans as a better, although more complicated and lower throughput model, to look at efficacy. We also were using them in the early safety. Of course, because we can't rely on these devices fully

yet, we also, of course, use animal testing. I might add, if you continue the arrow around, you get to computational models, and DILIsym is in that space. Because if you really begin to understand what's happening with the experimental data, you can mathematically model it. If you can mathematically model it, you can then make predictions and go back experimentally and test whether those predictions are true.

4.8#18 LT: So in summary, our initial liver MPS systems show very promising results. We believe that developing the liver and any of these organ systems is an evolutionary process. They're going to get better every year. We think a complete platform is required to acquire, analyze, manage, and model data, as well as to compare it to pre-clinical and clinical data.

4.8#19 LT: With that, I just acknowledge colleagues that we've worked with.

DR. WATKINS: Great, thanks. So we saved best for last, Gyongyi Szabo, who is the professor and vice-chair of medicine associate provost at University of Massachusetts, also

recently stepped down from being president of the ASLD. She is going to talk about exosomes; I think next year there'll be a lot more on at this meeting.

4.9#1 GS: DR. SZABO: Thank you so much for the invitation. When John and Paul asked me to give a talk, I was elated. They didn't tell me that I'm going to be the last talk in the entire two-day meeting, so bear with me. I think exosomes are somewhat of a new kid on the block. In fact, in biomarker discovery, I think there is quite a bit of attention outside of the hepatology field. I just came from the Bioselect biomarker conference from Boston, where a lot of presentations focused on exosomes on liver disease.

4.9#2 GS: Exosomes are a subset of extracellular vesicles that are produced by various cell types under steady-state condition, but most of all under stress or disease conditions. On the left-hand side, it shows that there are microvesicles and exosomes that actually have different biogenesis mechanisms, and I'm not going to have time to get into that. Furthermore, the understanding of the intricate details of the biogenesis of these vesicles is not fully clear. But we end up with the

smaller vesicles, these are called exosomes, in the size of 4,250 nanometer, and larger vesicles that are called microvesicles, and those are 152,000 nanometer size. These all are coated by membranes, and the membranes have certain features and characteristic markers. For example, for exosomes CD81 and CD9, always present on these exosomes, a phosphatidylserine is one of the markers of the larger vesicles, the microvesicles. I'm going to mostly talk about exosomes today. I'm going to talk about the exosomes as looking at them as potential biomarkers. I'm going to show you a little data about the biodistribution of exosomes, cellular sources, and potential functional effects in intracellular and intercellular communication.

4.9#3 GS: These exosomes can actually have various materials in their cargo. These include various nucleic acids, DNA, messenger RNA and micro RNA, and also certain proteins. In fact, these could be very specific, but also for the disease condition that induces the release of these exosomes. Indeed, the literature has some publications that exploited exosomes as potential biomarkers of liver disease.

4.9#4 GS: I don't have time to go into details, but it has been shown that exosomes isolated for either urine or serum of patients with various liver conditions can have cargos that include either certain surface markers, such as the CD10 or 26 that are listed here, but they also can have certain micro RNAs in their cargo that have been found to be changed, either decreased or increased, compared to parental cells or normal conditions, suggesting that there is some noise here that deserves further evaluation. What I'm going to talk about is actually related to the micro RNAs that are found in exosomes.

4.9#5 GS: I'm not going to have time to go into details about micro RNAs, but you heard about micro RNAs, particularly micro RNA 122, a lot in this meeting so far.

4.9#6 GS: In a previous study, we have shown that in APAP-induced liver injury, there is a rapid release of not only plasma ALT increases, but there also goes along, in a linear correlation, with micro RNA 122 in the plasma. We took it a step further and asked the question what is the portion and the fragment of the plasma where this micro RNA 122 is

found? In this particular case, in the APAP-induced liver injury, we found that the majority of the micro RNA actually was in the protein rich fraction that was outside of the exosomes, although there was some increase in the exosomes, as well, in the APAP-induced liver injury. This is somewhat different from many other types of liver injuries that we tested, including alcoholic liver disease, fibrosis, or nonalcoholic fatty liver disease, where most of these increases in micro RNA is actually happening in the exosome-rich fraction. If these exosomes are produced under liver injury, then one of the questions is what happens to these exosomes? What is their biodistribution?

4.9#7 GS: What we know is that composition of the exosomes can be different from the composition of parent cells, so certain compounds can actually either get concentrated or not be present in exosomes in comparison to the parent cells. It has been suggested that exosomes can also function in mediating cell-to-cell communication, both in normal physiology, but also in pathologic conditions. It has also been suggested that exosomes can actually be taken out by various cell

types. We have shown that hepatocytes are actually exosome-releasing cell types, and they can also be targets for exosomes.

4.9#8 GS: Another question is what happens if we introduce exosomes using a mouse model? Here, we used the mouse that is deficient in microRNA-155. We used exosomes that were actually enriched and loaded with microRNA-155 mimic. After intravenous injection of these exosomes, loaded with miR-155, we found that the presence of the microRNA =-155 was detectable in the liver and in the adipose tissue as early as ten minutes after the IV injection. Even after 40 minutes after the injection, it was detectable both in the liver and the adipose tissue. There was some noise in the lung, muscle and kidney, but that was very, very minimal.

4.9#9 GS: So what happens in the liver? We perfused livers to make sure that we are not measuring something that is still in the plasma, and isolated hepatocytes and liver mononuclear cells. We found that these exosomes actually were readily taken out by hepatocytes and liver mononuclear cells within ten minutes, and a little still hung around after 40 minutes. This told us that

exosomes actually are distributed very rapidly. They can go to the liver and are taken out by hepatocytes and mononuclear cells, but the turnover of these exosomes appears to be relatively short, suggesting that this is a pretty rapid and dynamic process.

4.9#10 GS: In this particular study, there are some additional data. We show that exosomes introduced into the intravenous circulation show an organ distribution that predisposes to enrichment in the liver and the adipose tissue, although it could be found in other tissues. Indeed, they enter hepatocytes mononuclear cells.

4.9#11 GS: One of our other interests is alcoholic liver disease. In alcoholic liver disease, we have shown that micro RNA 122 increase actually occurs similar to what you see in drug-induced liver injury. The increase in micro RNA 122 in alcohol-induced liver disease in mice, at least, here shows a linear correlation, but if we look at the distribution of this micro RNA, we find that the micro RNA 122 actually is enriched in the exosomal fraction in the circulation. That's also true for some of the inflammation rate in micro RNAs that are

increased in alcoholic liver disease, particularly micro RNA 155.

4.9#12 GS: Again, we were interested in the question of how would alcohol-induced exosome release? We took a very similar experiment that we asked normal individuals, essentially healthy controls, to consume alcohol. After a binge drinking episode, we took blood samples, half an hour, one hour, to four hours, and 24 hours later, and found that, in the top left panel, we find a significant and gradual increase in the number of circulating exosomes in these individuals after the binge drinking.

4.9#13 GS: This goes along with a rapid increase in micro RNA 155, and a somewhat slower but significant increase in micro RNA 122 in these individuals. The lower panels show that the increase in these extracellular vesicles is also reflected in patients with alcoholic hepatitis. In the lower left I show you an alcoholic hepatitis patient's plasma where there is a significantly higher level of circulating exosomes, compared to normal controls. These exosomes contain micro RNA 122 (on the left side of the right panel). In order to do

mechanistic studies, we returned to the animal model, and we found that if, indeed, we feed mice alcohol for four weeks, the extracellular vesicle numbers are increased compared to the pair-fed nonalcohol controls. That goes along with an increase in ALT. The electromicroscopy image of these exosomes is shown on the right lower part, making these measurements by nanosight analysis.

4.9#14 GS: In order to do biomarker discovery, obviously we're interested in the signature and cargo of these microvesicles and exosomes, so we isolated the RNA and micro RNAs from exosomes isolated from either alcohol-treated or control mice, and analyzed the extracellular vesicles from these mice.

4.9#15 GS: I'm just going to show you the results that we found and confirmed that micro RNA 122 was enriched in exosomes from the alcohol-fed mice, but we also found that certain other micro RNAs, particular micro RNA 192 and micro RNA 30a, showed a significant correlation, in terms of potentially serving as a marker for alcohol-induced liver damage in the exosomes, compared to the pair-fed, nonalcohol-fed mice.

4.9#16 GS: Then from these mice, we went back to the human situation, and now wanted to validate all of this in the patients with alcoholic hepatitis and isolate the exosomes from patients with alcoholic hepatitis. We could essentially reproduce the data that we found in mice, that exosomes isolated from patients with alcoholic hepatitis were specifically enriched in micro RNA 122, micro RNA 30a, and 192, suggesting that this approach in looking for enrichment and changes in certain micro RNAs in exosomes could be a kind of good way to identify disease-specific markers. I just wanted to show you this from the literature that actually looked at exosomes in a liver-toxicity model.

4.9#17 GS: This was the galactosamine-induced liver injury, where these investigators now looked at protein content of exosomes, actually extracellular vesicles, and showed the comparison what these various proteins 90, 70, and CLUSTERIN -- you see the entire list of these proteins -- and they compared liver extracts. On the right panel, it shows the serum extracellular vesicles for the expression of these proteins between controls on the left, and the diseased liver

toxicity model on the right. You can appreciate that many of these proteins that they feature here show an enrichment after the liver toxicity induction. This happens in the extracellular vesicles, suggesting that not only the micro RNAs from our studies, but potentially protein markers, could also be exploited for biomarkers in these extracellular vesicles. Going back to the hepatocytes and the liver damage related exosome release, we asked the question what is really the source of these exosomes.

4.9#18 GS: By studying human hepatocytes, we found that alcohol exposure induces release of exosomes over time. Those electron microscopy images give you an idea how these little vesicles form, and I suppose that some of those are exosomes. We find that in these primary human hepatocytes derived exosomes, there is enrichment for micro RNA 122.

4.9#19 GS: The question comes what does these exosomes and these little vesicles do? Do they have any function? We essentially took these exosomes that were derived from alcohol-exposed hepatocytes and we put them on normal monocytes. What we found was that the monocytes quickly took

up, essentially, and got these exosomes.

4.9#20 GS: But it seemed like these exosomes had a little more than just taken up by the macrophages and monocytes because we found that there was a functional difference in those monocytes that took up ethanol exposed hepatocyte exosomes versus just nonalcohol treated normal exosomes when we combined this with LPS stimulation. Essentially, the boxes with the red boxes indicate that the micro RNA 122 content actually increased in the monocytes when they were exposed to these exosomes.

4.9#21 GS: I must mention that micro RNA 122 actually is almost undetectable otherwise in monocytes, in immune cells. These exosomes transfer the micro RNA 122. But they not only transfer it, but it appears that these micro RNA 122 may have a functional effect. Because what we find, that after the transfer of these hepatocyte derived exosomes, and particularly in the hepatocytes that were exposed to alcohol, we find that the micro RNA 122 target, hemeoxygenase-1, is reduced, and hemeoxygenase-1 also has a role in induction of pro-inflammatory cytokines and kind of an adverse regulatory effect on it. So in those

cells that were exposed to the hepatocytes that were exposed to alcohol, now these exosomes are given to the monocytes, we find that there is an, actually, augmentation of pro-inflammatory cytokine production at the level of TNF and IL1beta, suggesting that these exosomes from hepatocytes actually can modify the function of immune cells, potentially, through micro RNA 122 transfer.

4.9#22 GS: In summary, what I showed you is that exosomes could be unique signatures, in terms of the exosomes cargo, in drug-induced liver injury, and potentially in alcoholic liver disease. We certainly need to learn way more, and potentially in other liver diseases, as well. It appears that the biodistribution of the exosomes is in a way that they're rapidly taken up in the liver into hepatocytes and immune cells, and the hepatocytes certainly are a very profound cellular source, but also a target of exosomes. Functionally, it appears that exosomes taken up by targets can actually alter the function of the recipient cells. This particularly is true in the context of hepatocyte derived exosomes, how they can modulate immune cell functions in the liver.

4.9#21 GS: I'd like to thank NIAAA and NIDDK for funding my colleagues in the lab. Thank you.

DR. WATKINS: Okay. The hour is getting late, but we still have 20 minutes for questions. One thing about exosomes that's very intriguing is they're small enough to get out of the hepatocyte through the fenestrations. If they're containing certain key messengers and things that would predict a DILI or severe DILI, you should be able to sample them, even in a totally healthy liver. There is one publication, Natalie Holman's publication just came out in Tox Sci, showing that with acetaminophen, prior to toxicity, in the absence of stress, the exosomes that are released have changes in their contents, which may be the first step towards exploring what these exosomes are doing. Questions?

PARTICIPANT: I've got a question for Lans. That was really elegant. You've got these chips; you have all these different cells. The only one that I was really troubled by is you dropped in a monocyte -- it wasn't really a monocyte. It was a tumor cell line, THP1. You're taking a risk,

I think, by putting in a tumor cell, letting it go for a month, and asking whether -- whereas, you can get the same monocytes from the same patient and add those.

DR. TAYLOR: It's an important question. Let me -- this isn't an excuse, but just a statement. In this program, we had strict milestones to meet, in terms of performance of the models, so we took a lot of shortcuts. This is, remember, a DARPA-like program.

PARTICIPANT: Oh, you mean it meant money here or something.

DR. TAYLOR: We wouldn't continue to be funded unless we made these steps. Now that we've kind of proven that we can make the models work and it does a lot of things, now we're focused more on using patient cells. In the early stages, we just had -- the goal was to get the system functioning for a month and have a proof of concept, so we took some shortcuts. I agree with you.

PARTICIPANT: Okay. Then that same cell, actually, in the last talk, it was referred to as a normal monocyte, but it really was a THP1. You did call that a normal monocyte in your talk,

at least I thought I heard that. Same question to you. Why can't you use monocytes --

DR. SZABO: I'm sorry.

PARTICIPANT: Yes, your talk.

DR. SZABO: Those were for normal individuals. They were not from alcoholic patients.

PARTICIPANT: All right, but your slide said THP1 on the slide.

DR. SZABO: We did both of them, actually may have showed that, but we validated it in human monocytes, as well.

DR. WATKINS: By the way, in the DILI network, those 1,500 people, we also have frozen peripheral blood monocytes from them and the ability to contact them. We've already made iPS cells from one woman, and Cellular Dynamics made hepatocytes and macrophage-like cells. So as the technology progresses, there is a group of very well phenotyped DILI patients that you could go after iPS drive cells or even primary cells. Yes?

DR. ROSENBERG: I'll ask the same question that brought riots of laughter last night to you, that is you're beautifully modeling an organ

on a chip. What would it take to scale up, to have an artificial liver?

DR. TAYLOR: Great question, and we have the McGowan Regenerative Institute at the University of Pittsburgh. There are people there building bioreactors, which are 1,000 or more fold larger. I really think we have a ways to go in the micro devices, where we can understand the physiology, get the right cells, finish the iPS cell derived system, so we can have the right genetic backgrounds from different people, as well as disease backgrounds. If we get to that point in the micro devices, where we could do lots of experimentation rapidly -- that's one of the values of it -- then I think that door is open for the future.

DR. WATKINS: Frank Sistare.

DR. SISTARE: Question about the exosomes. What do you have to do to process the sample? Can you just freeze it and come back to it, or do you have to process it immediately, do ultraspin? What do you have to do to get the exosomes? How heroic or how difficult is it?

DR. SZABO: The isolation itself is

quite labor intensive, but they are stable. So the good news is that you can freeze the samples.

DR. SISTARE: Right away, you don't have to process it right away?

DR. SZABO: Yes, and the recovery -- and we find that the function, even, of the exosomes is fairly retained after thawing. Repeated freezing/thawing probably doesn't help.

DR. SISTARE: But at least the first thaw, the exosomes is stable?

DR. SZABO: Right.

DR. SISTARE: But there's a kind of an intensive process that has to --

DR. SZABO: Right.

DR. SISTARE: To me, I wonder whether they'll catch mainstream use, other than research use, if they have to go through that kind of process?

DR. SZABO: This is a developing area, as I stated. This is kind of a new kid on the block. When it comes to the diagnostic arm of this, there are new methods coming out, so I don't think that in the future, particularly for diagnostic purposes, you necessarily need any of the very complicated methods.

DR. SISTARE: I also had a question for to Yvonne. I'm struggling exactly how to put it into words. You have a process in the dog which took some time. When did you begin to see that signal where it was the drug coming out of solution in the bile? When did that start to happen?

DR. DRAGAN: We could certainly see this process -- certainly, in our four-week pivotal tox study, we could see this with one gram per kilogram. We knew then that we had an issue, so that when we went further on and realized what was occurring, we could reproduce this in a two-week period, in the dog, at that very high dose that I showed of 600 milligram per kilogram.

DR. SISTARE: Okay, but at a lower dose, you saw in a 39-week study --

DR. DRAGAN: Correct, so --

DR. SISTARE: -- you saw it late into that study. How late was it? The signal, like an alk phos, started to come up pretty late.

DR. DRAGAN: That was very late, yes.

DR. SISTARE: The picture that emerges is you're giving the same drug for all that time, but it's taking a while for it to come out of

solution.

DR. DRAGAN: I think there are two separate processes occurring here, and it's both dose and time dependent in the dog, so that you have your overall solubility issues related with the compounds themselves, so that's one piece, so a higher dose, you see it sooner.

DR. SISTARE: Understood, but I'm going to focus on the lower dose.

DR. DRAGAN: Okay, so then on the lower doses, then, you have the concurrent process of inhibition transporters and alterations of amount of compound in the bile at any given time. I'd say that combination of those two, and possibly other effects, are what are contributory there.

DR. SISTARE: But you're thinking that the amount of drug being transported into the bile is increasing over time, I guess. The changes that are occurring are taking a while to occur.

DR. DRAGAN: As you have a lower dose of drug, yes.

DR. SISTARE: Yes, but it's taking many days.

DR. DRAGAN: What's interesting is that

cholestatic process that you see very clearly in the dog is not the picture we saw in the clinic. It's more hepatocellular.

DR. SISTARE: Yes, so let's get to that. The thing about the gallstone disease, now you're starting to think maybe are they so different. Something in solution is coming out of solution. It's not the drug; it's an endogenous substance. You talk a little bit about perhaps -- I think you kind of suggested that maybe, over time, you are altering the solubility, perhaps indirectly through some other transporter, something that's perhaps responsible for phospholipid content or something like fluid content or something like that. You wonder if they're not unrelated?

DR. DRAGAN: I can't say that they're not. We didn't look into that. We did not do any experiments to address what are the endogenous substrates of these transporters, and what is the impact of the concurrent administration of this amount of drug.

DR. SISTARE: We've got a Scott Siler to model that for us. He's going to model that. Then the bigger question, I guess, is here we are in drug

development, finding one or two cases like this, and then trying to make this question about do we continue to invest millions of dollars to try to bring this across the finish line? You've got these two Hy's Law cases. One said maybe, one said probably not, but yet -- and one of them is like, "Maybe the drug is actually altering the constituency of the bile in such a way that people who are prone to bile acid diseases or gallstones may actually get these attacks. Is that another form of DILI that we have to worry about at the end of the day? Those are questions that we face when we develop these drugs.

DR. DRAGAN: But at the end of the day, the aggregation of all of the available Phase 3 data and the liver signals therein, the fact that the duration, until we saw them, these were contributing factors to our decision.

DR. SISTARE: I know. No, I know they're difficult decisions. The other thing I wonder about -- Greg is not here still, but I wonder if something like -- I don't know anything about this HepQuant, whether in the dog, prior to demonstrating any increase in alk phos, if there's

some sort of measurement of overall ability to take an endogenous bile salt constituent and transport it? I don't exactly know how that works, but I wonder if something like that is something -- again, Scott, you can model for us. We can give you data in such a situation.

DR. DRAGAN: In these really high dose exposures in mouse, rat and dog, we can see an increase in serum total bile acids. Again, whether that's contributory or not, I can't say.

DR. SISTARE: Yes. Okay, thanks.

DR. WATKINS: Yes, next?

DR. LUFFER-ATLAS: Debra Luffer-Atlas from Eli Lilly. I actually wanted to ask a different question to Yvonne about the Takeda story. That is first of all, you guys were amazing in the amount of data you shared in the public domain, so all this is published -- not the tox, not the animal studies, but all the clinical data was published, and there was a poster at the ADA from Steve Nissen's group on your cardiovascular safety study. We did a deep dive on what's published. Having looked at the whole picture -- the last line of questioning was all about risk, risk, risk. We

honed in on the fact that you had really limited benefit at the end of this longer period of dosing, especially given that the GPR40 mechanism was supposed to be a safer mechanism insulin, glucose-dependent insulin secretion without the risk of hypoglycemia, so that you're going to be able to differentiate from sulfonylurea. At the end of the day, your hemoglobin A1C is eh. Want to bring up the perspective here, especially with the FDA, that there is still a benefit/risk argument to be made. If you were gangbusters on benefit, the way that you had hoped you would be -- and yes, you still have your two cases, although I would argue can you really say the gallstone case was caused by, or it was perhaps a predisposition or a pre-existing condition? I saw 1.6X on the T bili. That doesn't actually technically meet Hy's Law. So you have these two cases, you also have some other background incidences, but again, how did this play into your thinking, in terms of a benefit versus risk ratio, rather than just looking at them independently?

DR. DRAGAN: Absolutely. That's always a consideration here. It's about the patient and what benefit they would derive from

this, in the context of the risks that you observe. Again, on initial treatment out to 24 weeks, there's pretty good efficacy signals. With longer term, it becomes less clear.

DR. WATKINS: Last question before we close.

DR. TREEM: Will Treem from Janssen. I want to congratulate Kathleen and her colleagues at Boston Children's, Mark Puder, Chris, Doug, and others for this work because it certainly has changed our ability to care for these infants with short bowel syndrome and maintain their livers, so that they don't need either a liver transplant or a combined small bowel/liver transplant. The only question I have is that I was fascinated by your data that persistence of ALT elevations -- as you, I'm sure, know, the people in Nebraska have reported on liver biopsies going out on children who were treated with fish oil instead of omega 6 -- omega 3, instead of omega 6 -- show that their bilirubins came down to normal, and then had some biopsies which showed either persistent fibrosis, or even worsening fibrosis during the time they were followed on PN with omega 3 fatty acids. There's

been some animal data that suggested that omega 3s ameliorate steatosis in animal models of fat overload to the liver, but not steatohepatitis. I'm just curious how are you following these kids that now have normal bilirubins, maybe still on parenteral nutrition, omega 3s. What are you looking at, and how are you following them, and what's your thoughts about what else we need to do to prevent progression of fibrosis?

DR. GURA: Thank you for your comments. What we're doing is just trying to prevent -- it's PN-associated liver disease. At least we know better than the conventional soy-based lipid emulsions. We don't think it's perfect. We're actually thinking maybe we need a better ratio of oils. It could be, also, the absence of phytosterols. It may simply just be better than what we used to have. We're looking at other factors, also, like prevention of sepsis, avoiding other hepatotoxic meds. People forget that the Zantac that's put in the PN could also be contributing to these elevations in transaminases. We also make sure they don't go to the OR while they have any kind of hyperbilirubinemia. It's a big

package. We're just continuing to learn, and that's the beauty of this. We buy ourselves time to learn more. We don't biopsy unless we're in the OR. We won't take a child just to have them -- because the babies will bleed out if they're still sick, so they only get a biopsy if they happen to be in the neighborhood. A lot of it, we're still learning, trying to perfect the lipid emulsions, trying to get the kids off, trying to get their bowels to adapt. That's our biggest challenge is that we want to get these kids of parenteral nutrition. It's still a work in progress.

DR. WATKINS: Thanks, everybody, for staying. John already is working on the next one next year, and I can tell you it's going to be the best ever, so round of applause. Thanks, everybody.

(Whereupon, the above-entitled meeting was concluded at 4:10 p.m.)

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