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

I am going to tell you today about the Tolvaptan Initiative, which is an effort to identify a personalized DILI risk management strategy.
Tolvaptan is a vasopressin antagonist developed by Otsuka, already approved for the treatment of hyponatremia. It is a candidate as well for the treatment of Autosomal Dominant Polycystic Kidney Disease (ADPKD). Liver injury was observed during clinical trials, with approximately 4% of patients experiencing ALT elevations greater than three times the upper limit of normal, and there were three Hy's Law cases. FDA approval for this indication has not yet been received. I show this figure here, which is LFT plots that I know a lot of you are familiar with. I just want to draw your attention to the ALT values in black. The gray shading indicates where this particular patient was on drug. This is the time course of a liver response for an actual tolvaptan-treated patient. I want to point out that this patient was on drug for several months before there were any elevations in ALT. Then after the drug was stopped and the ALT values returned to normal, when the patient was re-challenged with the drug, ALT elevations occurred much faster during the second exposure.

This kind of profile is suggestive of an involvement of an adaptive immune attack as sort of the critical event promoting the liver injury. There is quite a bit of evidence to support the role of the adaptive immune system in these liver injury profiles, including, as we had just heard from Dr. Urban, really strong genetic associations between susceptibility to these kinds of liver injuries and the HLA region of the genome. Demonstrated HLA risk allele associations have not been clinically useful in risk management. We believe this is because there are
actually unaccounted for susceptibility factors and a risk that occurs at the level of the liver.
Illustrating the steps here, where drug elicits some hepatocyte stress. This results in an innate immune response and release of danger signals that, in combination with the adaptive immune attack, are actually responsible for the liver injury. But non-HLA risk alleles have not been clinically useful in DILI risk management.

We believe there is need for both genetic and non-genetic biomarkers in order to develop a personalized medicine strategy. While it was unfortunate that liver injury was observed in the clinical trials for tolvaptan, one really positive thing to come out of this was that Otsuka was really diligent in collecting samples from patients in the trials, including genomic DNA. Plasma and urine were collected at baseline, at three weeks, and then annually for up to three years on drug treatment, from both controls and cases in people that experienced the liver injury.
Samples with Consent Available from Tolvaptan Clinical Trials

- Genomic DNA
- Plasma and urine
  - Baseline
  - 3 weeks
  - Annually for up to 3 years on drug treatment
- DILI causality assessment by 5 hepatologists
- Opportunity for IDSS to collaborate with Otsuka and other partners to identify a personalized medicine strategy
Examples of sample collection from the cases are illustrated in the figure on this slide. And you can see there was plasma and urine collected at baseline, on three weeks on drug but before there was any sort of liver injury, and then also at the time of event. And then for all the cases, there is a DILI causality assessment by five hepatologists. Given this really rich sample set and the kind of tools and approaches, we realized this would be a great opportunity for us at the IDSS to collaborate with Otsuka, as well as their other partners, to identify a personalized medicine strategy for tolvaptan.
Objectives of the Tolvaptan Initiative are to manage the risk of DILI in tolvaptan-treated patients through the identification of both genetic and non-genetic risk factors for tolvaptan-induced liver injury and to provide a mechanistic understanding of the tolvaptan toxicity, in order to further direct discovery efforts and to provide biological plausibility for any empirically-derived biomarkers.
The integrative approaches that we are using to develop this strategy really begin with the clinical data and samples collected from the patients in the clinical trials, where more unbiased approaches have been taken, such as metabolomics and genetic analyses to identify risk factors associated with susceptibility to the liver response. We are also coupling these unbiased approaches with more targeted approaches. For instance, using in vitro models to identify the activation of stress response pathways in primary human hepatocytes exposed to tolvaptan. We are also using some cutting edge genetically diverse mouse population models. And then we are taking data from all of these different approaches, including some others, and using it to guide the development of a computational model for tolvaptan-induced liver injury, using the DILIsym software. But what is really cool about this approach is that we are taking data from all of these different studies and actually then using it to guide a targeted hypothesis base approach to biomarker discovery in the clinical data and samples collected from the patients in these trials.

I don't have time to tell you about all the different studies today. In fact, I feel like I barely have time to tell you about the mouse population-based approach we are using but that is what I am going to talk about mostly. Some of you may know that at the Hamner we have been working for a while with genetically diverse populations, which have allowed us better to model adverse responses
observed in humans, even when there is no toxicity observed in traditional non-clinical models, as was the case for tolvaptan.
But recently, we have transitioned to working with the next generation of these genetically diverse mouse populations, a genetic reference population called the Collaborative Cross. The Collaborative Cross is a superior resource for this kind of work because of the rationally designed breeding scheme that has been used to develop this population. It has resulted into just a really extremely diverse population of mice and this allows us to not only model these kinds of toxicities that are observed in humans but also do high resolution genetic mapping to identify risk factors and to study mechanisms that are associated with the toxicity susceptibility. We have been fortunate to work with this population that is currently only available through UNC. And we have hypothesized for this work that evaluating the liver response to tolvaptan in a genetically diverse population like the Collaborative Cross could allow us to identify sensitive strains, which could be used to both study mechanisms and identify risk factors for tolvaptan DILI.
One other point I want to make here before showing data from this study, is just going back to this figure I showed earlier. As you heard this morning from Dr. Uetrecht, it is difficult to model the adaptive immune response in non-clinical models. So, we are actually focusing on evaluating these very early events, the hepatocyte stress and potentially innate immune response. But we believe these initial events may not actually involve cell death or hepatocyte death. So, we may not see a response by measuring traditional non-clinical markers alone, markers like ALT. What we have learned that liver gene expression profiling, after an acute high-dose exposure of a drug can actually be able to be used to identify these very early events, even in the absence of overt toxicity. For this study here, we are actually combining a mouse population-based approach with toxicogenomics to identify mechanisms and risk factors associated with the toxicity.
This is the study design here. We treated 45 Collaborative Cross strains, eight male mice per strain; four getting vehicle and four getting tolvaptan, with just a single dose. And then 24 hours later, we necropsy the animals. I want to make the point that the dose of tolvaptan that we are using is 100 mgs per kg. The human equivalent dose in AUC for this dose in a mouse is actually not that different from the dose used in the clinical studies. At necropsy, just 24 hours after this single dose, these are the endpoints that we are measuring. So, after the single dose of tolvaptan, we weren’t expecting to see liver injury by measuring traditional biomarkers like ALT alone. But I think as you can appreciate here, we did see elevations in ALT in three of these 45 strains. We also did histology. Not surprisingly, we didn’t see any changes after just 24 hours.
We did find that these ALT elevations were well-correlated with AST and miR-122. We did a global gene expression profiling in the liver of all of these animals. First we looked at were gene expression changes that were associated with treatment across all of the strains, independent of a liver response. And you can see here in those genes we found enrichment of pathways that were suggestive of mitochondrial dysfunction. We also looked for gene expression changes that were associated or correlated actually with the ALT fold change. And here we found enrichment of pathways suggesting some alterations in bile acid homeostasis.
And then we looked for gene expression changes that were not only associated with treatment but that would differentiate our resistant and sensitive genes. And the most significant gene to come out of this analysis was actually a gene that is involved in the loss of immune tolerance. The really cool thing about this gene here is that the protein product produced from this gene gets secreted in the liver. It goes into circulation and it may be a serum biomarker.

**QTL Mapping with ALT Fold Change Identifies Significant Genetic Associations**

- Focus on chromosome 14
- Narrowed down genes in QTL interval to 6 high priority candidates
  - Includes genes with biological relevance: apoptosis and innate immune response
We also did QTL mapping, using ALT fold change. And I know you have seen a bunch of these Manhattan plots in the last talk, so I won't describe what this is here. I just want to point out that the strongest genetic association we saw was on chromosome 14. We looked at the genes within the interval on chromosome 14 and narrowed it down to about six high priority candidates, some of which have a biological relevance in showing some association with apoptosis and innate immune response.

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### Conclusions

- Tolvaptan-induced liver response was observed in 3 CC strains
  - Animal models for additional mechanistic experiments
- Toxicogenomics work identified treatment-induced adverse outcome pathways across all strains and specific to sensitive strains
- QTL mapping identified genetic associations with susceptibility
- All discovered with single dose comparable to human exposure

- Candidate genetic and non genetic biomarkers have been identified
- Will guide hypothesis-based approach to biomarker discovery in samples collected from clinical studies
I know I went through this quickly. I will just summarize the major findings from this work. A tolvaptan-induced liver response was observed in three of the Collaborate Cross strains. So, now we have animal models for additional mechanistic experiments. Our toxicogenomics work identified some treatment-induced stress response pathways that occurred across all strains in response to the treatment and some that were specific just to the sensitive strains. We did QTL mapping and were able to identify some genetic associations with the susceptibility. And all of this was discovered with just this single dose of tolvaptan that is comparable to that used in the clinical trials.

Going back to this figure one last time here, I just wanted to point out that we saw some evidence for mitochondrial toxicity and bile acid toxicity, apoptosis, and loss of immune tolerance. We have identified both genetic and non-genetic biomarkers and these will now go on to guide a hypothesis-based approach to biomarker discovery in the samples collected from the clinical studies.
This illustrates that point here. I told you about the cutting edge preclinical models. But we are generating this kind of data from all of the approaches that we are including in this initiative. And all of this data is coming together and is being used to guide a really hypothesis-based approach to biomarker discovery in the clinical data and samples from the tolvaptan studies. I think I have shown you that we have really transitioned from using these approaches to explain problems to now, hopefully, solving them. And we have learned a lot about how to do this work now and we believe that we can do this kind of study, a Collaborative Cross study, as well as some of the other approaches that I wasn’t able to tell you about today, in as little as six months.
There are a lot of people to thank that are part of this effort. And before Paul cuts me off here, I will just thank a few people that are in the audience today: Paul, who directs our Institute; some other folks like Dr. Urban, who is heading up the genetics work; Brett Howard, head of the DILIsym team; and then our partners from Otsuka, mostly Dr. Bill Brock and Sharin Roth, who have been extremely helpful in doing this work.