Thank you very much and thanks to the organizing committee for the opportunity to come and
As you know, I am based at the MRC Centre for Drug Safety Science at the University of Liverpool. I work with Kevin Park. We have a great interest in the development of biomarkers that we can utilize to understand the mechanistic basis of drug-induced liver injury, and to provide tools that we can use to assist our understanding of drug-induced liver injury, alongside the currently used standards.
When I think about the development of biomarkers from my point of view, I am looking at some of the challenges and unmet needs that we have. We need to develop biomarkers with improved hepatic specificity, about which we have already seen some excellent work presented by Dr. Szabo, looking at miR-122. We need to develop biomarkers for an enhanced mechanistic understanding, particularly in that translational space, so that we can work between animals and humans to try to understand DILI better; and earlier identification of drug-induced liver injury. I discussed that last year, so I am not going to touch on that today.

The focus of my talk today is really going to be biomarkers that are linked to mechanisms that we can really utilize to understand patient responses a lot better. And by that, I mean looking at patient outcomes and prognosis but also differentiating between benign changes and ALT activity and serious drug-induced liver injury. From my mind, to try and really understand that a lot better, to develop biomarkers associated with that, you have to to really understand the mechanistic basis a lot better of drug-induced liver injury.
I want to introduce you to one of my personal favorite biomarkers. I know you are not supposed to have favorites but I do. And this is High Mobility Group Box-1. I have an interest in HMGB1 because it acts as a dominant associated molecular patent protein. It links necrotic cell death to the activation of the immune response. And it does that by acting as a chemokine or as a cytokine for toll-like receptors, in particular TLR4, and CXCR4, and also the receptor for advanced glycation end products.
With respect to understanding its utility as a biomarker, we know that it can come out from the cell in a number of different ways. There is a passive release during a necrotic response. It also can be actively secreted from cells, particularly immune cells. And that requests a set of key lysine residues within its nuclear localization sequence. And I have just highlighted some of those on that schematic across the bottom of the screen of the various structural domains of HMGB1.
Very interestingly, HMGB1 has three sustained residues, only three sustained residues and each is very important for its function. They are modulated by post-translational redox dependent modifications and it has a profound impact on its function as an inflammatory mediator and I am going to discuss that a bit later during the course of the presentation. We looked at HMGB1 as a biomarker in the paracetamol and overdose model in a mouse. And what we did is we initially tracked its progression from the loss and the release from the centrilobular region following necrosis, following paracetamol treatment, to its appearance in blood.

All this sounds quite a straightforward and an easy concept but it has not been actually presented in the literature, tracking the biomarker from the tissue to the periphery. We also looked at identifying the two different molecular forms in our animal model of paracetamol overdose. If you remember, I told you that two distinct molecule forms, which correlate with the mechanism of release is the hypo-acetylated form, which is shown in green, which is indicative of a necrotic response and the hyper-acetylated version of HMGB1, which gives us an indication of an active immune response. We were able to develop and validate a mouse-based approach to identify and quantify these different isoforms of HMGB1 in blood. And what you can see from the data on the bottom right-hand side of the screen shown in green is the necrotic version of HMGB1, followed by a release of the inflammatory version of HMGB1. And essentially, what we see in mice is we see by indication of these two biomarkers, a biphasic response. We see necrosis, followed by inflammation.
Of course, we are very interested to see if these observations hold true in man. And of course what you can see there on the left-hand side it he data from the mice. We further developed this assay to quantify HMGB1 in the blood of humans from acetaminophen overdose. And what you can see there is essentially we see the same pattern and response. We see the release of the necrotic version of HMGB1, followed the inflammatory version. So, the mechanisms hold true from both mouse to man.
Of course we want to know if this is important. We know that inflammation plays an important deleterious role in animal models, following paracetamol overdose but can we use this biomarker to try and predict patient responses better? And that was the hypothesis. The acetylated version of HMGB1 would be upregulated in the blood of patients that had a worse outcome.

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

And what you can see from the data, this is old data now but what you can see that the patients that spontaneously survived, their levels of acetylated HMGB1 circulated in blood was not significantly different than healthy volunteers. Both the guys that required a liver transplant or in fact died, their level of acetylated HMGB1 was significantly increased in blood.
So, we show the HMGB1 can act as a biomarker but, of course, we are very keen to know that it is not just a -- it doesn’t just act as a biomarker. We want to know if it plays a key role in the mechanism of the pathology and the mechanism of the drug-induced liver injury.
One strategy we adopted was to see if by neutralizing circulating HMGB1 in blood we could reduce the adverse effects associated with the drug in a mouse model of drug-induced liver injury. So, what we did is we treated mice with acetaminophen and you can see the profile and the time course of the lethality over time. And what you can see there on that data on the top left-hand side of the screen is that coadministration of HMGB1 neutralized an antibody in fact has a positive outcome on outcome in these mice. And what we have done now is we have gone on to develop that a lot further and developed a humanized version of that antibody. We could also see a positive outcome on ALT activity and then when we looked in detail at the livers, the histological sections of the livers from these mice, in the mice treated with paracetamol in a control antibody, we saw both necrosis and inflammation, characterized by an infiltration of neutrophils within the liver. But if we co-treated those animals with a neutralized antibody for HMGB1, we saw necrosis and knocked out, essential the infiltration of inflammatory cells into the liver. So, we essentially broke that cycle between necrosis and inflammation by knocking out HMGB1.
But of course, these are antibodies and to really confirm the important role that HMGB1 might play in the pathogenesis of drug-induced liver injury in these mouse models, we had to create an HMGB1 knockout mouse. But, unfortunately, if you knockout HMGB1 from the whole body, it is embryonic lethal. So, we had to design a strategy to produce a conditional knockout approach.

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

We challenged these mice with acetaminophen and on the top left-hand side, you can see the ALT/AST data. And as you can expect from our antibody study, the mice that had HMGB1 knocked out from hepatocytes had a significantly reduced rise in ALT activity compared to the wild type. They also performed better, with respect to survival.
We looked at the livers of those mice histologically. We could also see that the HMGB1 knockout mouse had a significantly lower score for necrosis in the liver, compared to the wild type mouse. Of course, if you utilize acetaminophen as you model hepatotoxicity, you have to look at metabolism. So, we looked at 2E1 expression, gultathione depletion, and the formation of paracetamol protein. And what you can see from the data here that 2E1 expression was comparable between both strains. The ability for the acetaminophen reactive metabolite to reduce glutathione was the same between both strains and also reacting metabolite to hepatic protein was the same across both strains.

We looked at the mechanism in a bit more detail and I will just briefly give an overview of these sections. I know they are quite detailed. But essentially what we saw by knockout HMGB1 from the hepatocyte, we prevented neutrophil infiltration into the liver but not macrophage infiltration. And that was what also supported our previous studies, using the neutralizing antibody to HMGB1. But of course we wanted to really push this model and test this hypothesis further and really confirm whether or not HMGB1 played a significant role in the development of drug-induced liver injury following an initial hepatic necrotic response.
To test that hypothesis, we expressed HMGB1 in hepatocytes that were normally not expressed in HMGB1, so a conditional mouse model, using an adenoviral gene delivery system. So, by restoring hepatocyte HMGB1 expression, we could restore the toxic effects that we saw with paracetamol shown by ALT activity on the top right-hand side of the screen. We have restored the neutrophil infiltration response into the livers and also the increased necrotic response we saw in the livers by re-expressing HMGB1 back into the hepatocyte. So, that is all from paracetamol overdoes and it is all from a mouse model.
But recently, we have begun to show the utility and the importance of HMGB1 in other forms of liver disease. We published on HMGB1 in obstructive cholestasis with Helmut Jaschke. We published on the role that HMGB1 plays in alcoholic liver disease both in humans and also in mouse models. I was very fortunate to present that as a Webex at the AASLD and a hepatotoxicity special interest group in January earlier this year. And also we have got HMGB1 and its role in ischemia reperfusion.
But of course, we want to know if we can utilize HMGB1 to explore the concept of the development of serious drug-induced liver injury. And these are the concepts that have been widely discussed over the course of this meeting. The role of Hy's Law and its potential to identify and predict serious drug-induced liver injury. So, I won't talk about that in too much detail but we know that is really what we have at the moment and it is our best assessment, according to the current standards.

So, of course for the development of new drugs, the increase in ALT activity is an important problem and one that we don't really fully understand, whether ALT is just a benign change or indicates a serious drug-induced liver injury.
I am sure most people in the audience would recognize this paper published by Paul in 2006 in JAMA. He showed that about a third of those patients in that study developed a transient change in ALT activity. We have applied the mechanistic biomarker panel to those individuals in that study and we have shown a predominant increase in the M30 fragment of keratin 18, the apoptotic component. So, we concluded that the major form of cell death in this particular patient cohort in this particular setting was apoptosis.
But if we look at quantifying HMGB1 levels in the blood of these individuals, we also see an increase in total levels of HMGB1 in blood. So, these patients or these volunteers have quite significant value of HMGB1 circulated in blood had quite a potent dominant associated molecular pattern but they don’t develop a serious drug-induced liver injury. They recover and they are okay. So, why don’t they develop that serious reaction, despite having a high level of that potent inflammatory mediator in blood?

So, to understand that in a bit more detail, we need to understand HMGB1 biology itself. So, if you remember, I mentioned that HMGB2 has three cysteine messages and I have a biochemistry background. So, when I think about that, I start to get a little bit excited. Maybe some of you guys won’t. But what I thought is put this schematic on the screen here, just to show you the importance really of cysteine residues and how they play in biological systems.
If you think back to your biochemistry days, you know that cysteine can form disulphide bonds and that is quite important for structural integrity of proteins and thiol residues are particularly important for protein-protein communication. But also, if you oxidize cysteine residues on proteins, that actually makes proteins targets for degradation and can actually inactivate proteins.
This slide summarizes quite a significant amount of work led by my laboratory with some collaborators across the globe, where we pooled resources and we have all of an interest in HMGB1. And what we did is we utilized mouse-based technologies, coupled with cell biology and molecular biology to determine what post-translational modifications with respect to redox status impact on HMGB1 function.

What we showed is that the functions of HMGB1 are mutually exclusive with respect to cytokine induction and chemotaxis. For HMGB1 to act as a chemoattractant agent, all those cysteine residues must be reduced in a thiol state. If there is a disulfide bond present between cysteines 23 and 45 and cysteine 106 is reduced, then HMGB1 can act as a cytokine inducing agent as a lead-in for thiol receptor 4, in fact MD2 associated with thiol receptor 4. But if you continually oxidize all those cysteine residues to sulphonates, then HMGB1 has not function at all with respect to a cytokine and also a chemoattractant. We also know that these oxidation modifications of HMGB1 appear to be cell death mode-dependent and specific as well.

Previous to this work, another group showed that mitochondrial cleavage -- a caspase-mediated cleavage in mitochondrial complex one can induce ROS production and join apoptosis and can inactivate HMGB1 through terminal oxidation. Sort of an innate response to prevent the control and spread and damage associated with molecular patterns in and around secondary necrotic response. We tested the hypothesis that during apoptosis HMGB1 is oxidized and that could potentially one reason why you don't see a necrotic response.
So, we simply tested that head to head in our murine model of acetaminophen overdose, where we see a mix of apoptotic response with necrosis and also necrosis only.

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

We know that those different isoforms of HMGB1 are cell death mode dependent. So, the next obvious question we asked ourselves is could, through looking at HMGB1 isoforms, can we explain why we see one cohort of patients develop serious drug-induced liver injury and those develop a benign change in ALT activity by really understanding the mechanistic basis.
If we divide our cohorts of patients into those that have a serious injury or the large overdose group could host the transient injury from Paul’s study. And when we look at the mechanistic biomarkers, we know that the serious overdose guys have a really small portion of apoptosis, whereas the guys with the transient changes in ALT activity have a significant proportion of apoptosis. We looked at the HMGB1 isoforms in blood. If we first focus our attention on the serious injury, we see when we have isolated H and G, we want to characterize that by electrospray ionization mass spectrometry. We see many different isoforms of HMGB1 in blood.

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

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

But if we characterize the cysteine residues in more detail for the benign changes in ALT group, we only see the terminally oxidized form of HMGB1 or the form that has no inflammatory function, according to current theory. This led us to believe that HMGB1 isoforms could potentially not only act as a biomarker for serious overdose of serious liver injury versus benign changes in ALT but also could be a key mediator in these processes.
We took that a little bit further with pharmacologists at the University of Liverpool. So, we like to put a number on everything and quantitate things as much as we can. We quantified those different isoforms of HMGB1 across those different cohorts. And what you can see by looking at that graph there, you can see that the patients with the therapeutic indication of paracetamol only had the terminally oxidized form of HMGB1. The guys that spontaneously survived, they had a mixed bag of HMGB1 isoforms but the guys that died or required a liver transplant, their redox balance was shifted towards the reduced form or the proinflammatory active forms of HMGB1.
Lessons that we learned from these cohorts, these retrospective cohort analysis is that functionally distinct HMGB1 isoforms can determine if paracetamol liver injury is serious or benign. And of course, we can add an extra mechanistic understanding to that and link that back to the form of cell death.

And in this figure we have taken those three different groups of patients, the spontaneous survivors, the guys that died or required a liver transplant, or the guys with benign changes in ALT and we have correlated the redox ratio so that the values associated with the inactive form of HMGB1 over the proinflammatory from of HMGB1 and we correlated that against the so-called apoptotic index using the M30, M65 ratio.

You can see from these data that those patients quite nicely separate. And what we see is that those HMGB1 isoforms are linked to cell death mode dynamics as well.
I summarize there that we have shown that HMGB1 can be a key mechanistic biomarker in experimental and also clinical drug-induced liver injury. We have shown that in paracetamol overdose, and in other forms of liver injury. We have developed conditional knockout mouse models to explore the mechanism of pathology. We have looked at different HMGB1 isoforms to inform patient outcome and prognosis and also try and differentiate between benign changes in ALT to serious liver injury.

And now we believe that HMGB1 is not one protein, but it is a number of different proteins and isoforms.
I would like to thank some of these people that here in the audience, particularly Kevin Park from the University of Liverpool and, of course, the external mentorship from Paul Watkins and his lot at the Hamner. Thank you.