Thank you, Paul. Thank you for the nice introduction. I would like to
congratulate Dr. Senior and thank him for the invitation to give me the opportunity to talk about this today.
A few years ago, I became interested in microRNAs mostly because, as you all well know and talked about during this conference, we have very poor markers of liver injury in our armamentarium. Currently and for many, many years, use of transaminases certainly gave some information for us in clinical practice but have very severe limitations. They are not specific. They really don’t correlate well with the progression of liver disease, cannot distinguish between hepatic injury and inflammation, and, certainly cannot distinguish between drug-induced liver injury and other type of liver injuries. So there is clearly a need for more specific and stable biomarkers. And I do like to hear that that work is being undertaken in new biomarker discoveries for liver disease.

Currently used markers of liver injury

- Alanine aminotransferase (ALT) is not specific for the type of injury
- Aspartate aminotransferase (AST) is not specific for the type of injury

- ALT/AST are not specific:
  - Poor correlation with progression of liver disease.
  - Cannot distinguish between hepatic injury and inflammation.
  - Drug induced liver injury (DILI) from other causes of liver injury.

There is a need for sensitive, specific and stable biomarkers.

Circulating miRNAs, exosomes?
So, one of the potential targets and candidates for biomarkers could be potentially microRNAs for several reasons. The microRNAs regulate various genes and they also are found in a very stable form in cell-free body fluids, including the serum and some of the microRNAs actually are packaged into small vesicles, either exosomes or microvesicles, or apoptotic bodies and can be found in the circulation. Therefore, all of these characteristics make them attractive new non-invasive biomarkers.
For hepatologists, microRNA-122 is particularly exciting because very uniquely this particular microRNA represents about 80 percent of the entire microRNA pool in hepatocytes. Now, if you consider that there are more than a thousand different type of microRNAs, it is pretty remarkable to have one in that high kind of propensity in liver cells. But it turns out that microRNA-122 regulates various mechanisms including cholesterol biosynthesis and it has been identified as major host factor in hep C viral replication. And I am not going to talk about that part today.

But interestingly, there has been a lot of attention to microRNA-122 changes in liver diseases, particularly in the circulation, in the plasma and serum compartment. And various studies demonstrated that in drug-induced liver injury there is increase in the serum levels of microRNA-122. It has been shown to increase in chronic hepatitis C infection and also in non-alcoholic fatty-liver disease and in hepatocellular carcinoma. So, it certainly marks at the same time that this is possibly and very likely not going to be a specific marker but certainly deserves additional attention.
If one looks at, for example, acetaminophen-induced drug liver injury, in a mouse model, what we find is that on the left various time points and changes in ALT levels in mice. And, as one would expect, a few hours after a sublethal dose of acetaminophen, ALT levels increased. But at the same time, if you look at microRNA-122 levels in the same plasma specimens, then it appears that at one hour, microRNA-122 shows a significant increase at the point when ALT hasn't changed yet, suggesting that potentially the timing and the sensitivity of this marker could be a little more sensitive than ALT.
Also in a different study in a rat model, in a fulminant hepatitis model of Wilson's disease, investigators found that kind of the similar phenomenon that on the top panels you see on the left, the microRNA-122 increase that is at week ten is already significantly increased when AST is still normal. And at a later time, again, the ALT and bilirubin changes show differences but really, the microRNA-122 shows up and increases earlier on, suggesting that this could be an early marker.
Definitely changes in the serum microRNA-122 levels in various model of liver injury appear to correlate with ALT. So, on the left of upper part is an alcoholic liver disease model in mice; in the middle, acetaminophine-induced liver injury; and on the right is an infectious and inflammatory model in mice that is an autoimmune disease induced by the CpG, DNA and LPS administration. The extent of the
increases and even the magnitude of microRNA-122 changes are different between the different models. And the highest kind of level both in ALT and miR-122 were found in the APAP model, where there is the largest extent of hepatocyte damage.

In chronic hepCV infection in humans, we also found that there is a linear correlation between ALT changes and microRNA-122 in the circle they think of plasma in patients.
So, moving on to a different kind of model, actually we were interested in the role of microRNA-122 in the non-alcoholic fatty liver disease. And here, again, if you use a mouse model of methionine-deficient diet or a control diet, what we find is that over time between one to eight weeks of administration of this diet that induces massive steatohepatitis and actually fibrosis by week eight, we find that increasing the serum microRNA-122 but, interestingly, the correlating levels of liver microRNA-122 actually were decreased. So, that really was intriguing to us and made us question the potential role of microRNA-122 in the liver. So, microRNAs are included by DNA and, essentially, in the biosynthesis there is a pre-microRNA-122 form. And that essentially indicates the formation of new microRNA-122. And interestingly what we found was that this pre-microRNA-122 was severely reduced, compared to normal animals in the mice with steatohepatitis. And one of the factors that actually drive the promote the region of microRNA-122 have an HNF6 side, which essentially is one of the promoters and inducers for microRNA-122. Interestingly, we found that that was reduced also, suggesting that there is a transcription regulation of microRNA-122 in this model of non-alcoholic fatty-liver disease, leading to the lower levels in the liver. In addition to the regulation of cholesterol synthesis, relatively little is known about the role of microRNA-122 in hepatocytes liver diseases. So, various studies show that there is new 122 expression human NASH in the liver.

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

Decreased liver miR-122 is associated with transcriptional repression and concomitant increase in the serum. Liver and serum was collected from mice fed either MCD or MCS diet for 1, 2, 3, 6 or 8 weeks. Primary miR-122 (pri-miR-122) expression was measured in the livers and hepatocytes of MCS or MCD diet-fed mice (n = 6–8) using TaqMan primer assay, normalized to GAPDH (A). HNF6 mRNA was quantified (B). Total RNA was isolated from 50 μl serum samples and equal volume of RNA was used to measure miR-122 levels using TaqMan miRNA assay. Spiked synthetic Cel-miR-39 was used for normalization (C). Serum from 6-week MCS- or MCD-fed mice was fractionated into exosome and protein-rich fractions using ExoQuick method and miR-122 expression was determined using TaqMan miRNA assay (D). * indicates \( P < 0.05 \) vs. MCS.
And another kind of known background is that the MAP3K actually regulates NFKB in cell survival and tissue remodeling processes. So, these potential correlations led us to the hypotheses that potentially the decreased level of microRNA-122 in the liver in NASH could have some specific pathogenic roles.

So, to explore this, we started at evaluating the MAP3K kinase and we found that at the messenger level it was increased in the non-alcoholic fatty-liver disease model. And it was increased at the protein level not only in the total liver but also in isolated hepatocytes. Now, I showed that potentially these MAP3K kinase is a target of microRNA-122 regulation. And so that question be used an inhibitor of microRNA-122 in isolated hepatocytes. And then we found that if, indeed, we inhibit microRNA-122, then the levels of the MAP3K actually increased. I probably should clarify that there is actually most of the microRNAs act in a way that they inhibit the target messenger RNA. So, in this case, if microRNA-122 is reduced, that means that the inhibition of the MAP3K is really meaning that then it is expected that by limiting microRNA-122 we actually find the metric K3 kinase RNA being increased. That suggests that microRNA-122 targets the MAP3K kinase.
Now, bouncing from this MAP3K3 is NF kappa B, which is another major regulator of inflammation. And in it, we find that in the MCD diet-induced model, in the liver there is a massive induction of NF kappa B and this also is seen in the nuclear binding level in the total level but also in hepatocytes and that is on the top right side. And if we inhibit the MAP3K kinase, then we can actually attenuate and NF kappa B activation, suggesting that, indeed, there is a causal kind of relationship between these various kinases and regulatory factors.

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MAP3K3 regulates NFκB in hepatocytes. C57BL/6 mice (n = 6–8) were fed either MCD or MCS diet for 3, 6, or 8 weeks. Primary hepatocytes were isolated from MCS or MCD diet-fed mice (6 weeks). Nuclear proteins of 5 μg extracted from total liver (A) and 20 μg of whole cell lysates from hepatocytes (B) were used to detect NF-κB nuclear binding using EMSA (n = 3). MAP3K3 was inhibited using MAP3K3 siRNA (20 nM) in hepatocytes isolated from C57BL/6J mice. Some samples were exposed to LPS. NFκB nuclear binding was determined 48 h after transfection using nuclear protein extracts (C). * indicates P < 0.05 vs. MCS.

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The other potential target for microRNA-122, as I told you earlier, is HIF-1, the hypoxia inducible factor 1. And this is interesting and potentially clinically relevant because those of you who treat and see patients with non-alcoholic fatty-liver disease, many of them actually have sleep apnea. So, hypoxia is happening at the macroscopic or physiological level. But there is also a lot of speculation that even at the liver tissue level, hypoxia could, potentially play a role.

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

And as you can see, both the RNA levels of vimentin and also the immunohistology staining suggests that the protein levels are increased in mice with steatohepatitis compared to controls. To come back and show the causal relationship here, we used an anti-microRNA-122 SINRA transected to hepatocytes in the left upper corner you can see that the HIF-1a levels actually are increased when we inhibit microRNA-122. Therefore, essentially leaving the repression effect of microRNA-122 on the HIF-1. And on the right-hand side, you can see that the same things happens at the biological activity in the nuclear binding.
HIF-1α upregulation correlates with fibrosis. C57BL/6 mice (n = 6–8) were fed either MCD or MCS diet for 3, 6 or 8 weeks. Formalin-fixed liver sections (8 weeks of diet, n = 3–6) were stained with Sirius Red to assess the collagen accumulation (A, left panel) or subjected to αSMA (A, middle panel) or vimentin (A, right panel) immunohistochemistry. Total liver RNA was used to measure HIF-1α expression by qPCR (B, left panel). HIF-1α nuclear binding was detected using EMSA (B, right panel). Liver mRNA expression of the HIF-1α target genes lysyl oxidase (C) and the mesenchymal marker vimentin (D) were measured by qPCR. * indicates P < 0.05 vs. MCS. # indicates P < 0.05 vs. 3 weeks MCD.

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And the same thing happens in hepatocytes on vimentin, if we inhibit microRNA-122, then the SRNI against microRNA-122 and not the control increased the vimentin levels at hepatocytes. That kind of left us with the conclusion that microRNA-122 in non-alcoholic steatohepatitis has multiple roles. First of all, it appears that there is a reduction at the transcriptional level by reducing the pri-microRNA-122 levels, most likely through HNF6 and potentially other mechanisms. And this leads a reduction in the mature microRNA-122 in the liver. But at the same time, there are some mechanisms that are not very well known but certainly result in increased levels of serum microRNA-122 so that kind of contributes to this consistent dichotomy. It appears that in the liver the microRNA-122 actually has, in addition to cholesterol metabolism appears to regulate HIF-1 alpha and the MAP3K3 kinase and those processes can contribute to inflammation, fibrosis remodeling and certainly the circulating microRNA-122 potentially could be at least one of the biomarkers indicating liver damage.

HIF-1α and vimentin are miR-122 targets in hepatocytes. MiR-122 was overexpressed using pre-miR-122 (10 nM) or inhibited using anti-miR-122 (20 nM) in hepatocytes isolated from regular diet-fed C57BL/6 mice. Forty-eight hours after transfection, HIF-1α mRNA levels were measured by qPCR (A). HIF-1α nuclear binding was assessed using EMSA in primary hepatocytes after transfection with anti-miR-122 (50 nM). Some samples were exposed to TNF-α for the last 2 h of the transfection (B). Vimentin mRNA expression was measured after miR-122 overexpression (10 nM) or
miR-122 inhibition (20 nM) in isolated murine hepatocytes (C).

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What I wanted to come back to is that the microRNAs in the serum are often actually packaged in exosomes. And as exosomes are small extracellular membranes vesicles on the size of 50 to 100 nanometer in diameter that are produced by most cell types.
They are found in the extracellular space and various biological fluids, not only serum, saliva, and in all kinds of other biological fluids. They contain various nucleic acids and proteins and among those are microRNAs. There is increasing evidence that these exosomes actually can function as kind of messengers between cells and potentially may get to various organs and could be having a beneficial and harmful pathological effect. Certainly hepatocytes are one of the sources of exosomes that can also be targets.
And indeed, there are various recent publications that indicate that exosomes could be considered as like biomarkers of liver disease. So, for example, in various types of liver injury, the presence of an increase in exosomes have been noted in various biological fluids, as described here. Many of those microRNAs actually did contain microRNAs as well. That is certainly of interest.
So, we ask the question if exosomes serve as therapeutic vehicles and could potentially these actually have some function and effect. And the way we approached this was that we took a B cell line. So, there are B cells that produce large amount of exosomes after stimulation at IL-4 and CD40. And then we took those exosomes and isolate them. Now one of the characteristics of exosomes is expression of CD63 that allows the purification of these exosome compartments.
And then we used those exosomes and either loaded them with various microRNAs. Or particularly for microRNA-155. That was the kind of system that we used or we used an inhibitor of micrRNA-155 and these kind of modified exosomes were then tested for functional activity.
We tested them by delivering this microRNA-155 inhibitors to macrophages and that was because normally microRNA-155 actually can regulate inflammation or they tried to deliver a precursor of the microRNA-155 into hepatocytes and this was two hepatocytes were chosen as targets because typically hepatocytes microRNA-155 expression is very low. So, what we found was that if took macrophages and stimulated them with LPS and that is the first two bars on the left compared to the one very much to the left, no treatment. Then LPS stimulation induces a lot of microRNA-155 in this side. And on the right-hand side in that kind of graph, you can see that this goes along with an increase in TNF production.

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

And that suggests that, indeed, these exosomes could be actually vehicles to bring on to us a type of modulation. In this particular case if this was an inhibitor, again with the microRNA-155 and this inhibitor actually was biologically active. I don't have enough time to go into details but it was shown as in our publication that actually what they find is that the exosome-mediated delivery of these inhibitors is more
efficient than just doing a regular transfection inside with an inhibitor, which I think is very intriguing and certainly brings a little more attention to the exosomes in this system. The opposite side of this is that we actually made exosomes and then and loaded them with microRNA-155 precursor, essentially to see what was the effect of these exosomes on hepatocytes that normally don't express much microRNA-155. We injected these loaded microRNA-155-loaded exosomes into mice and then we evaluated the liver and also isolated hepatocytes for the expression of microRNA-155. And these were mice that were microRNA-155 deficient. So, normally they didn't have natural microRNA-155. By giving these exosomes loaded with miR-155, we found that we couldn't detect the miR-155 in the liver of these knockout mice. And if you isolated hepatocytes that the miR-155 actually was found in hepatocytes, suggesting that, indeed, again, these exosomes are capable in vivo to deliver these either inhibitor or a precursor for macroRNA into the liver and into hepatocytes.
To summarize, I want to leave you with the idea that there is evidence that exosomes actually could be therapeutic vehicles. It could be that depending on, so on the left side with the black kind of RNA and microRNA, that is an inhibitor. And if we put that into an exosome, then actually that has an effect on macrophages to potentially inhibit the microRNA-155 activity and the contrary of this, if we take the exosomes and put the precursor on it with the blue kind of microRNA marking, then that potentially can deliver a functional microRNA to tissues in mice and particularly to hepatocytes. That suggests that certainly exosomes are a new and exciting area from the standpoint of cell-to-cell communication or potentially, organ-to-organ communication. They also potentially deserve to be evaluated as therapeutic vehicles.
And I want to thank our funding agency and my colleagues who contributed to this work. Thank you. (Applause)