WEBINAR
Detecting Disease in Blood: What miRNA Biomarkers Can Tell Us
30 May, 2012

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Sean Sanders: Hello and welcome to this *Science*/AAAS audio webinar. My name is Sean Sanders and I'm the editor for custom publishing at *Science*.

Our webinar today will look at the very interesting and topical subject of microRNA biomarker analysis in biofluids. The information potential held by microRNAs combined with the fact that they are stable in the bloodstream has led to a rapidly growing interest in using microRNAs as diagnostic and prognostic biomarkers. This webinar will present the latest advances in technology and strategy that are allowing researchers to move microRNA biomarker research from discovery in the lab to application in the clinic.

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Our expert panel on the line with me today will guide us through this fascinating topic. They are Dr. Colin Pritchard from the University of Washington in Seattle, Dr. Monty Montano from Boston University School of Medicine in Boston, Massachusetts, and Dr. Adam Baker from Exiqon in Vedbaek, Denmark. I'm very pleased that you could all be with us today. Thanks for joining us.

Before we get started, I have some information that our audience might find useful. Note that you can resize or hide any of the windows in your viewing console. The widgets at the bottom of the console control what you see. Click on these to see the speaker bios or additional information about technologies related to today's discussion or to download a PDF of the slides.

Each of our speakers will talk briefly about their work. After which, we will have a Q&A session during which our guests will address the questions submitted by our live online viewers. So if you're joining us live, start thinking about some questions now and submit them at any time by typing them into the box on the bottom left of your viewing console and clicking the submit button. If you can't see this box, click the red Q&A widget at the bottom of the screen. Please

remember to keep your questions short and concise, that will give them the best chance of being put to our panel.

You can also log in to Facebook, Twitter, or LinkedIn during the webinar to post updates or send tweets about the event, just click the relevant widgets at the bottom of the screen. For tweets, you can add the hash tag, #sciencewebinar.

Finally, thank you to Exiqon for their sponsorship of today's webinar.

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Now, I'd like to introduce our first speaker for this webinar, Dr. Colin Pritchard. Dr. Pritchard completed his undergraduate and graduate training at the University of Washington in Seattle in the United States and his medical training at the University of Washington School of Medicine. Dr. Pritchard is an assistant professor of Laboratory Medicine as well as the associate director of the Clinical Molecular Genetics Laboratory at the University of Washington Medical Center. His research focuses predominantly on oncology molecular diagnostics, particularly the source and utility of microRNA biomarkers in blood and the development of innovative molecular diagnostics for the identification of mutations that can guide therapeutic decision making. Warm welcome to you, Dr. Pritchard.

Dr. Colin Pritchard: Thank you, Sean, for that kind introduction and thank you for the invitation. So I'm going to be talking about circulating microRNA from the perspective of the clinical laboratory and what we think about in the clinical lab to get these exciting analytes ready for primetime as clinical diagnostics.

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So the outline of my talk, in the first part of the talk, I'm just going to briefly describe microRNA as circulating biomarkers and then I'm going to focus the bulk of my talk on what we think about in laboratory medicine. So those are sources of variation, pre-analytic variables, biological variation, the state of microRNA in plasma and I'm going to present a little bit of data that suggest that microRNA is present in at least two different states. Then finally, I'm going to talk about the origin of microRNA and what implications that has for disease specificity. So by origin, I mean what cells are releasing microRNA into the serum and plasma.

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So circulating microRNAs are very exciting as a lab analyte and are attractive biomarkers so it's like we've discovered protein for the first time and we have this new class of analytes in the serum and plasma that can be used for lab testing. The reason that they're so attractive is they're abundant in blood. So the most abundant microRNAs are present in copy numbers up to 100 million/ml. They're easy to measure by well-established PCR techniques and Adam's going to talk a lot about some of the methods for that in the last talk. They're highly stable, which was surprising because there are high levels of endogenous RNAs in the plasma, but microRNAs seem to be resistant to this and there are many disease these associations, which is the most important.

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So it wasn't until 2008 that microRNAs were first discovered as stable analytes in the serum and plasma, but since then in the last four years, there have been over 200 publications. There's a lot of excitement in this area and for good reason. A lot of the publications have focused on microRNAs, circulating microRNAs as noninvasive cancer diagnostics for prostate, breast, colon, many others and Adam is going to talk a little bit about some exciting data of microRNAs as potential noninvasive colon cancer diagnostics and many other diseases, pregnancy, diabetes, heart disease and others. But the sources of variation are not well studied and that's the focus of my lab.

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So the next part of the talk, I'm going to talk about sources of variation.

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So sources of variation can be broadly categorized as either analytical or biological.

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Analytical variation can be further broken down into pre-analytic, analytic, and post-analytic and even within these categories you can further break it down. So pre-analytic variables are things like blood draw technique and specimen processing. So you might imagine that if you're looking at an epithelial expressed microRNA in the plasma and there's a skin plug in the tube that maybe that will contaminate your signal so that's one area where blood draw technique might matter. Specimen processing, I'm going to talk a little bit about some data why that does matter later on. Analytic variables are things in the actual measurement process itself and the extraction process. So

clearly, the methods of RNA extraction are going to matter as well as the methods for measuring microRNA. Then, post analytic variables are things like normalization techniques. All three of these classes of analytic variation are not well studied at this point, but we're making some headway.

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So the other thing to think about in terms of analytic variables that we think about all the time in the clinical lab is interferences. Some common interferences that interfere with many different kinds of assays are hemolysis so that's lysis of red blood cells, lipemia which is basically lipids in the plasma, drugs that a patient maybe taking that might interfere with the assay, or antibodies could interfere.

Shown on the right-hand side of this slide is an example of increasing levels of hemolysis in the serum and we do see this in the clinical lab all the time. It's very common to see hemolyzed specimens. Because of specimen handling, a sample can be hemolyzed. Then, on the far right is lipemic samples and anybody's plasma can get lipemic if you have a big fatty meal before you have your blood drawn. We don't have evidence that this interferes with microRNA measurements yet, but there's reason to believe that it might.

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So the other major class of variation is biological variation and biological variation can be broken down further into with individual variation and between individual. So factors of biological variation within individual are diurnal so morning, evening, fasting/fed such as lipemia, and sick/well. So, you know, in the clinical lab, we see patients that are generically sick and we're trying to distinguish sick patients with other diseases from sick patients with the disease of interest. So it is important to study this sort of variation within individual.

Between individual are variation is also equally important. So age and gender and Monty is going to talk a lot about the effect of age and has some interesting data to show that that is an important between individual variable or within individual for that matter, the genetic variation, ethnicity, and then also chronic disease.

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So in the next part of the talk, I'm going to talk about the state of microRNA in plasma.

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So how is microRNA stable in plasma? We know that there are very high levels of endogenous RNAs and that message RNA for that reason is not stable in plasma, it's degraded very quickly. So there are three ideas, one is that microRNA be intrinsically stable. So these are very small RNAs generally about 22 nucleotides and then we thought that well maybe they're just intrinsically resistant to RNA. So that turns out to not be the case because if you purify microRNA and then spike it back into plasma, it's degraded rapidly within seconds. Another sort of leading hypothesis is that it's stable because it's protected by some mechanism and probably vesicles so that's one model and it may be protected by other mechanisms as well.

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So this is adapted from a paper by Arroyo in 2011 that showed that there are probably at least two populations of circulating microRNA. So what you're looking at in these graphs are size-exclusion chromatography of plasma specimens and then different microRNAs. So on the left, there's microRNA-142-3p, which by size-exclusion chromatography, the signal comes out almost entirely within a vesicle size population, and then on the right is miR-122, which has the opposite pattern, which comes out almost entirely in the protein population. What they showed in this paper was that that microRNAs could basically be divvied up into ones that were primarily vesicle population microRNAs, ones that are primarily protein. Complex population microRNAs are somewhere in between and this protein complex has argonaute 2 protein.

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So what are the implications of two plasma states for circulating microRNA biomarkers?

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So the biggest implication in the clinical lab, I believe, is specimen processing. So what you're seeing in this slide is successive specimen processing. So standard plasma is shown on the top graph and what we're seeing is the particles within the plasma. The middle panel a shows platelet-poor plasma. So this is plasma that has been respun and then the supernatant taken off of that so many of the platelets have been spun out as well as some of the vesicles. Then the bottom graph shows platelet-poor plasma that's been filtered through a 0.22-micron filter. So what you can see with this culture counting is that with the successive processing, you remove platelets and

vesicles from the plasma and this probably has important implications for microRNA signatures especially for those microRNAs that are predominantly in the vesicle fraction.

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So in the last part of the talk, I'm going to talk about the origin of microRNAs in the blood plasma and serum and what implications this might have for disease specificity.

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So where do plasma microRNA come from? So, three leading cell types are probably blood cells, endothelium which lines the blood, and other tissues of course, but the relative contribution of these three origins is not really well characterized.

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So this is some data from a paper that our group recently published and what you're seeing is a heat map of 79 circulating microRNAs that have been proposed as disease biomarkers in the literature. We're looking on the left is a heat map of the plasma from healthy donors and the expression of these biomarkers and then on the right is the blood cell expression of flow sorted blood cells matched to this plasma. What you can see is that there's a very tight correlation between the blood cell expression of these plasma biomarkers and the plasma expression in healthy donors suggesting, although not proving by any means, that at least in healthy individuals, microRNA biomarkers are largely derived from blood cells.

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Also from this paper, we showed that if you take consecutive clinical plasma samples and you look at blood cell counts, so you do a complete blood cell count on the whole blood prior to making plasma and then you look at plasma levels of microRNAs, you see that myeloid expressed microRNAs such as miR-223 correlate with myeloid blood counts such as neutrophil count. Whereas lymphoid expressed microRNAs such as miR-150 correlate with lymphocyte counts and the reverse wasn't true so this was interesting.

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Finally, we and others have seen that red blood cell derived microRNAs are fairly dramatically affected by hemolysis. So microRNAs such as miR-16 and miR-451that are highly expressed in red blood cells maybe increased up to 50-fold in the blood plasma in hemolyzed specimens compared to non-hemolyzed specimens. Non-

red blood cell microRNAs such as miR-122, miR-223, miR-150 were not impacted by hemolysis at all. So again, this suggests that hemolysis is going to be important but specifically for that subset of microRNAs that are highly expressed in red blood cells.

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So in summary, I believe that circulating microRNAs are incredibly promising as disease biomarkers. As I said, it's like we've discovered a whole new class of analyte in the blood and we have a new toolbox to develop exciting diagnostics from. But little is known about analytic properties of microRNAs in cell-free plasma and serum or biological variation. MicroRNAs are present in at least two states in plasma, one that seems to be in a small protein complex such as mir-122 and another that's in a vesicle-associated state such as mir-142-3p and there may be more states that we don't know about yet.

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Plasma microRNAs are likely released by blood cells. We have a correlative evidence to suggest this and we're doing more work to prove this. Most importantly, plasma microRNAs that are abundant in normal blood cells may have poor specificity as biomarkers because they're influenced by cell counts and hemolysis. I really want to emphasize this point because I believe that in our search for non-invasive biomarkers it will be best to look at those microRNAs that are not highly expressed in blood cells.

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So I'd like to acknowledge Muneesh Tewari at the Fred Hutchinson Cancer Research Center and tech Evan Kroh who did a lot of this work as well as my mentor John Tait, Jason Arroyo, Brent Wood, Liz Setran, and Hye Son Yi and at the University of Hawaii Marc Goodman and Yeonju Kim. Thank you very much.

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Sean Sanders: Great. Thank you so much, Dr. Pritchard. That's a fantastic introduction for us and we're going to move right on to our second speaker and that's Dr. Monty Montano.

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Dr. Montano received his B.A. in biochemistry from the University of California, Berkeley, his Ph.D. in genetics from Stanford University, and conducted his postdoctoral work at the Harvard School of Public Health in Cambridge, Massachusetts. He is currently a principal investigator at the Boston Medical Center in the Department of Medicine, in the sections of Infectious Diseases and Molecular

Medicine. Dr. Montano's laboratory is broadly interested in the identification of biomarkers for immune and muscle interaction in infection, muscle wasting, fibrosis, and aging. Research initiatives in his laboratory include analysis of the interaction between host immune factors such as macrophages and muscle stem cell remodeling during HIV-associated muscle wasting, and the molecular phenotyping of aging and identification of biomarkers for anabolic response. Dr. Montano is also a principal investigator on a Pepper Center project to identify biomarkers for anabolic response. Welcome and thanks for being with us, Dr. Montano.

Dr. Monty Montano: Thank you, Sean, for that introduction and thank you Colin for that very interesting initial presentation. As Colin mentioned, one of the biggest challenges in translational medicine is developing surveillance methods for monitoring not only disease but also therapeutic outcomes and to do so in a minimally invasive way. This is important because not everyone is easier to have a biopsy and for example in cases like the human brain, it's not really feasible. So this is part of the appeal of biomarkers and today, I'd like to discuss our work using microRNAs as biomarkers in peripheral blood. Granted, a weakness in this approach is that biomarkers may not be directly linked to underlying mechanisms but nevertheless, they can be useful in disease surveillance. So the purpose of this talk I'll focus on two lines of investigation in our lab, aging particularly with centenarians and chronic infection with HIV.

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So what are the questions posed in this presentation? Well firstly, is there prior evidence that microRNAs can be associated with aging in tissue irrespective of serum blood levels, sort of a proof of concept.

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Secondly, can they be used to help us to track quality of life, something that's dear to the hearts of our aging country in a concept known as health span.

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Perhaps one of the best examples of successful aging come from studies in centenarians, men and women who live well beyond a hundred years and I'll be talking about these types of individuals.

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Another key translational question is the capacity for monitoring chronic infection and treatment. This is partly motivated to track and identify changes in health or changes in therapeutic response.

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For us, this is relevant in HIV infection, which despite the profound success of HAART in extending lifespan, infected individuals remain at higher risk for multiple disorders that I'll discuss also in this presentation.

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Finally, I'll wrap up with some speculation on what this might mean for translational research in aging and infection.

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Okay. Is there evidence for microRNA changes associated with cellular or tissue aging? Perhaps a good place to begin is the brain because as my yoga instructor says, you can walk around with 0% body fat, but if you can't control that mind of yours, little else matters. So does the brain change with aging and is this detectable with microRNAs?

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Well it does and rather dramatically. In this figure, we have a graphical representation of gene expression in what's termed a heat map. The map shows expression of several hundred microRNAs in whole brain of mice aged from two months to two years. Red represents increase in expression and blue is decrease. Right off, you see collectors indicated by a tree of related expressions to the right of the heat map so aging happens. It happens in the brain and microRNAs reflect or possibly even contribute to that conflict outcome. So proof of concept, microRNAs are associated with aging.

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Now with data like that showing tissue aging, can we make a leap of reason and speculate on aging profiles in blood, which is cell free. Rather than using two months to two-year-old mice, can we use aging in the extreme of humans, i.e., centenarians who are a hundred years and more and compare them to the rest of us, which I'm guessing as everyone in this webcast.

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Well to do this we've recently acknowledged that we are using serum profiling as a biomarker opportunity and that primary microRNAs transcribed and processed, resulting in translational inhibition indicated by the red line and to a lesser extent inhibition of transcription and RNA stability.

Slide 36

With some unknown relationship between this biogenesis pathway and emergence of microRNAs in the serum, possibly occurring in exosomes or in argonaute 2 protein complexes as Colin mentioned in the first talk. Okay. So now that we know what is it we don't know, let's look at centenarians in serum profiling and let's ask these specific questions.

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One, are the levels of microRNAs in centenarians related? So in this figure, we have two centenarians with their microRNA profiles plotted against each other. You can see that centenarian 1 on the Y axis is highly correlated to centenarian 2 on the X axis using their blood microRNA expression profiles. This correlation may suggest a stable profile linked with exceptional longevity, but obviously, this can require far more centenarian datasets to make that statement a robust one. But yes, they are related, which was the initial question.

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A second question is do these centenarian blood profiles differ from the rest of us? To answer this, we teamed up with Exiqon and Adam Baker from Exiqon will be talking in the third presentation. What we chose to do was to compare our centenarian microRNA samples from peripheral blood with a reference panel of 1400 or so samples of younger individuals, which is what you see in this figure. So, shown in red are about 150 randomly selected microRNAs in plasma of centenarians compared to the average of the reference panel in blue. The Y axis is relative expression level and the X axis is just an arbitrary designation of the microRNAs we looked at.

Firstly, you can see that there's a remarkable similarity despite this extreme longevity suggesting that there's a stable, potentially a stable network of circulating microRNAs.

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However, if you look a bit more closely, you can detect stable differences or potentially stable differences and in this case indicated by the arrow a microRNA that is upregulated in both of the

individuals. In fact, if you take a closer look at this entire panel, you note that on average, there's an increase of about 2.5% to 3% in microRNA expression across the board. What does this mean? Well it's unclear, but there is some evidence based on knockout experiments of microRNA biogenesis suggesting that when you knock out microRNAs, you increase the tumor risk in those suggesting possibly that this maybe an adaptive mechanism for preventing, you know, tumor formation. Obviously, we'll have to stay tuned to these studies to figure out which microRNA families are being influenced in these individuals.

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An additional question that we're interested in is whether there are gender differences in microRNAs and this slide simply points out that if you look at the male versus the female, you can note specific differences.

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So again, with close inspection, you can find gender related microRNAs again all in cell-free plasma or serum.

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Okay. I've talked a bit about extreme longevity and serum microRNA biomarkers, now I'd like to discuss a second example of interest to our lab namely chronic infection with HIV. In principle, this maybe related and relevant to other infection scenarios, but for us we're focused on HIV.

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So it turns out there are many complications associated with chronic infection even despite therapy and some of these are outlined in this slide. You'll also notice that these particular conditions resemble aging. For example, there's a frailty related phenotype that's been described, there are declines in muscle and bone mass, there is a chronic inflammatory burden that isn't resolved with therapy, there is cellular immunosenescence that's elevated in chronic infection and there are cognitive and brain declines, functional declines.

Now, this is not really aging, it's more a phenocopy of aging than actual aging. For example, we have a paper in press by Banerjee, et al, showing that premature muscle aging appears to activate some but not all fibrotic pathways regulated by TGF beta. So it's a phenocopy of aging.

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So given these conditions, biomarkers could be useful in monitoring how this individual's, you know, quality of life continues. So, in this slide, what I'm showing is to explore blood biomarkers in infection, we've been measuring plasma and serum samples from humans with HIV infection and also non-human primates with SIV infection. Shown in this figure on the left are real-time PCR data for a nineplex of miRs, of microRNAs and on the right is the same data shown as the heat map. Both represent 7 HIV positive non-human primates and 6 HIV negative.

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If we focus on the SIV positive, you can see that in general these particular microRNAs are more highly expressed than in the negative counterparts. Whether this reflects disease pathogenesis associated with infection or infection per se is something that is unclear at the moment and it would be interesting to test in further studies. But this does give us a sense of the possibilities of pathogenic tracking made available by serum profiling.

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Now, it's worth noting for a moment analytic approaches can be critical to interpreting the data, therefore, if you do embark on these types of questions, be sure one of your best friends is a biostatistician. Now, to demonstrate this criticality of interpreting the data, above are two heat maps of essentially the same data. The left heat map globally scales the data by columns and rows whereas the heat map on the right scales the data only by columns indicating which microRNAs are most highly expressed within but not between samples. So this is the same data, but there's a radically different sort of view and each has its advantage and it all depends on the question you're trying to ask. So it's important to be mindful of that.

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Now a key question is whether serum profiling of infection compares with tissue profiling of the same microRNAs and I think Colin demonstrated quite nicely that this can be true for microRNAs in cell subsets in the peripheral blood. This slide indicates a similar outcome using serum samples in brain, tissue samples associated with infection.

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So if we box the infected, we can see that both heat maps are providing a pretty good association with infection suggesting that microRNAs in serum maybe robust biomarkers for surrogate tissues. But again a limitation is that we have very little feel for the mechanisms, but it is a proxy that we can use to flesh out potential mechanisms.

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Okay. To wrap up, well one undercurrent concept in what I've presented is that blood microRNA profiles may reflect either directly or indirectly tissue physiology or pathology.

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A second implication from our work on aging is that microRNA profiles in blood may allow us to distinguish chronologic age, how old you really are, from biologic age, how old you feel. This of course has implications for therapeutics especially those focused on rejuvenation and I view this as the molecular Ponce de Leon search for the biomarker fountain of youth.

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Another implication of this work is that blood microRNAs may help to track host's response to infection either acute or chronic and maybe monitored for responses or outcomes such as decline in muscle or bone and also brain function.

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Finally, we've noted and also Colin noted that in individuals you can see differences and I showed you differences between centenarians and also differences could be identified in the different HIV infected subjects. Given the large number of microRNAs that can be sampled in blood and the minimal invasiveness and the attractiveness of that approach, this may represent an opportunity for personalized diagnostics in health monitoring.

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Okay. So I'll stop here and I'd like to acknowledge several of my colleagues in this research. In the Aging Studies, Paola Sebastiani and Tom Perls of the New England Centenarian Study, Kim Long and Clint Baldwin, Greg Gibson of Georgia Tech, and Adam Baker at Exiqon. In the HIV Studies, I'd like to acknowledge Dan Michaels and Susan Westmoreland and all of this work had been supported by the NIH. Thank you.

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Sean Sanders: Wonderful. Thanks so much, Dr. Montano, a fascinating presentation and we've actually had a lot of questions come in for you so I hope you will be able to get to those in the Q&A coming up shortly.

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But before that, we're going to have our final speaker and that's Dr. Adam Baker. Dr. Baker holds his Ph.D. in Molecular biology and Genetics from The Research Institute of Molecular Pathology in Vienna, Austria. Following his graduate work, he undertook his postdoctoral training at Boehringer Ingelheim, subsequently obtaining a senior scientist position at Chromos Molecular Systems in Vancouver, Canada. Before joining Exiqon, Dr. Baker headed the Division for New Technologies at deCODE genetics in Iceland for seven years. He currently holds the position of director of Communications, Biomarkers, and Clinical Sciences at Exiqon. He works closely with clinical research groups and Exiqon's pharmaceutical partners to develop microRNA based diagnostic solutions based on LNA technology. Warm welcome, Dr. Baker, thanks for being with us.

Dr. Adam Baker: Thank you very much, Sean, for the introduction. I'd really like to thank Colin and Monty for those fantastic presentations.

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I'm going to finish it off by discussing some of the general technical considerations I think we need to consider when we're studying microRNAs themselves, particularly when we're considering biofluids, and briefly at the end, I'm going to introduce some of the Exiqon research programs themselves. We've had fantastic introduction or fantastic presentations from Colin and Monty talking a lot about pre-analytical variables and other factors we need to consider. I'm going to ask you to consider briefly the microRNAs themselves.

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So analyzing microRNAs is not easy at all. If we think about them, they're very, very short sequences of just 19 to 22 nucleotides in length and they've very highly homologous family members often with single based differences. Quite often as well, there's very large variation in the base-pair composition, the GC content that can vary from up to 5% to 95% GC content. So how do we deal with this from

a technology point of view? Well, the way that we've been addressing it at Exiqon is using LNA.

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So I can briefly introduce what is LNA. LNA is a technology developed and used by Exiqon to facilitate the research of microRNAs. In detail, LNA is a class of high affinity RNA analogues, where the ribose ring is locked in the ideal confirmation for Watson and Crick binding. What this actually means is that when oligos have LNA incorporated into them, they actually pair with the complementary strands more rapidly and can increase the stability of the resulting duplex and this is very critical in many hybridization-based technologies such as PCR or array technologies or in situ hybridization.

The take-home qualities you get are an increased Tm where you can increase the Tm of the oligos by 2 to 8 degrees every time you include an LNA. You can increase the delta Tm, which allows a larger difference so you can select mismatches and perfect matches with better specificity and you can use Tm normalization, which is critical when you're designing multiple oligonucleotides for multiplex assays such as in PCR or arrays. Finally, I'd like to note that you can actually develop shorter oligos than traditional DNA and allow you to keep the same high Tm and you'll see this in action when we discuss PCR later.

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But we need to reflect again searching for microRNA is challenging. Searching for these microRNA biomarkers is like looking for a needle in a haystack. I just like us to consider for a minute what we're looking at. If a cell is containing approximately 10 to 30 picograms of total RNA, only 0.3% of this is the microRNAs. Again, if you think about biofluid, it can become even more challenging. Total RNA levels in plasma range from about 6 to 300 nanograms/ml and only 5% of this is microRNA.

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So really, we have to have technologies that are very specific and very, very good at finding needles in a haystack as we say.

Well, we have developed a PCR system, which we feel is very optimal for finding these needles in haystacks particularly in biofluids in clinical samples. The real challenge is to develop a PCR system that's able to detect these very, very small microRNA and also have very, very short nucleotide sequences. So what we have done at Exiqon if I

just briefly go through the mechanism, we developed a combined first step where we had a polyA tail to the mature microRNA template. We then developed cDNA through adding a poly G primer that has a 3' degenerate anchor and 5' universal tag. In the second stage of the reaction, the cDNA template is amplified using microRNA-specific primers.

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The key points of this is because we can develop the primer so small, you can actually have two primers that land on to the mature microRNA itself, which allows a great advantage of specificity for these microRNA key pieces. We do our visualization and detection through SYBR Green.

So what are the advantages? The advantages here that we have a universal RT reaction, which is perfect when we're using small clinical, critical samples. We contain no bias when we do these first stages of the amplification. Then we're using LNA in both primers, which allows for sensitivity and specificity to really capture those needles as we call them and pull them out from the hay.

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In the next slide, I'm just trying to reflect a little bit more as well about how we need this extreme sensitivity. Why do we need such extreme sensitivity when we do microRNA profiling from clinical samples? Well in the figure I'm showing you, I'm showing you PCR dilution curves as you see many times, but also showing you the dilution curves and then in reality the PCR reactions, with total RNA input. What you can see is we're looking down to 1 picogram of total RNA input into the PCR reaction and you see that using the Exiqon PCR system, we have linearity.

But if you look at the blue circles, where you can actually see what is critical, is a sort of idea of where we're dealing when we start dealing with plasma or serum, we're actually working with picograms of input RNA. So you really, really have to have a technology that can actually pull out from such small amounts. It's very different if you're using buffy coat or PAXgene or cell lines where you have hundreds of nanograms of input material. So this is a very, very important consideration when you go forward about designing your experiments about which technology you're going to use.

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In the next slide, also just reflecting that we have a very, very simple workflow. This is actually critical and key if you don't want to

introduce biases from programs into actually analyzing this microRNA within the clinical samples. So this slide just reflects that we can take 20 microliters of serum and plasma, which equates to about 150 picograms of RNA input into a single one RT reaction and then you run this on a 96 or 384-well PCR plate and then do the normalization and data analysis. A very, very simple workflow, which avoids technical biases and allows you to get to very, very robust results very quickly, hopefully.

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Finally, this slide is just an overview to show that Exiqon has developed a whole panel of different PCR products to actually address all of those diagnostic product development needs all the way from complete miRNome PCR profiling all the way down to individual assays, which is key for us to be able to develop entire programs around this technology.

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So a few more considerations when we're considering clinical samples.

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I brought it up briefly, but I just want to show again that we have to be very, very rigorous when we do multiplex PCR or do PCR with multiple microRNAs, we have to ensure that our sensitivity in performance of all the microRNA PCR reactions is the same. This slide is just demonstrating that we've tested 647 different microRNAs. We've introduced here a 15-point dilution curve with synthetic microRNAs that we're actually testing the performance. So what you can see is we have an absolute, great equal assay efficiency between all these different PCR assays.

What I'm just showing you at the top of the graph is also where plasma and serum is along this performance. Then we've got this dilution curve all the way down to 15 copies of input template into the PCR reaction. So what you're actually seeing here is that in the serum and plasma levels, the performance of the Exiqon PCR system is very linear, very robust, and not near the edge of the technological window.

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Moving on to the next slide, I think this is a key area, it's been touched on both by Colin and by Monty and it is critical, it's the post analytical variable. It's how do we work with normalization of this

type of data and normalization is key. You've seen examples of it in Monty's clear presentation and also Colin has brought it up that it can be critical and this is one of the things that you have to consider in great detail as you carry out your experiments. There are no housekeeping genes available in this situation. U6 and 5S are not applicable in serum and plasma samples. So there are many methods available and actually I hope that maybe some of the questions will address this at the end, but we'd like to discuss in detail different approaches that you can use, but you do need to address different approaches in normalization.

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Another aspect that we've been building up in great detail is there are other sort of variables and this is we have normal reference ranges. So at Exiqon, we actually have luckily in our research programs been able to now screen thousands of different samples and this is just one example where we're looking at a normal reference range for human serum, which is based on over 1400 human samples. We have this for other biological materials and it's actually key in setting up experiments and actually also helping you get your results quicker and you're actually able to see some of the interaction where Monty referenced the fact that he was able to compare his small dataset against our reference ranges in some of his experiments. Just to see very quickly if he was actually getting differences in microRNA expression.

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Finally, I just like to introduce some of the knowledge we're developing and gaining through our own diagnostic programs.

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So one of the key diagnostic programs we're carrying out at Exiqon is the early detection of colorectal cancer using blood plasma. This definitely addresses a very, very large unmet need. There's an estimated death in 2009 the US alone of over 49,000. So what we're doing is we've been developing methods that we can actually look at microRNAs within plasma from these patients or early stage patients or individuals and actually see if these microRNA profiles can tell us whether these people have colon cancer.

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Now, we've developed our whole system to be compatible with clinical procedures, but Colin gave a very beautiful demonstration of

how you really need to consider so many different analytical variables. But as long as you take these analytical variables into account and you do very carefully control for these, I'm just showing you how we can sort of develop a system where we have a very straightforward and structured approach to actually developing very robust microRNA data using this. So you can take standard serum or plasma from a hospital, screening by isolating the microRNA, and then running the PCR experiments, but again, one has to be very, very cautious as you've heard in the previous talks.

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This is a very complex slide where I'm actually just showing you the entire development that we've carried out in the early detection of colorectal cancer on blood serum and you can also see how we developed the different technology platforms. So as this project developed from a screening, early discovery phase into a validation phase, what we did was we used initially genome-wide screening so we took no bias into the microRNAs we were looking, and did very good power control experiments with 50 controls and 50 colorectal cancer patients and then went through the processing of doing QC checking and screening, developed focus panels, and then now move into the validation using a very, very specific Pick & Mix microRNA profile that we're taking forward and running on many, many different samples.

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So this is a method that we've taken forward very, very successfully and we feel it's also a method that we recommend to our collaborators and other researchers how you can develop a whole program of microRNA analysis, which is very robust basically working your way through from genome-wide screening down into a very focused validation set for using the PCR technology without having to change technology platform and performance parameters. They're very, very key and very, very important.

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So finally, I just wanted to spend one minute on something that we haven't touched on in the first two talks just to say as well that we are also looking at how we can continue to move into new fields of looking at circulating microRNAs as biomarkers. But one of the very exciting fields that we're also moving and developing experience in is toxicology and Colin briefly brought this up where we look at microRNAs and where they're actually coming from and so coming from cells or endothelial that are also being read in the circulation.

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So on this slide, you can actually see that it's actually taken off the Exiqon home page. What you can see is the whole profile of different types of panels that we're actually developing. We've developed a cancer focus panel, serum/plasma focus panels as I've briefly talked about, and now we actually at the bottom here have the toxicology focus panel.

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What does it actually looks like in terms of this toxicology focus panel? Well it's actually made up of microRNAs that we've selected through our experience and talking to our collaborators, some of the work around that are actually present in biofluids, urine or serum and plasma that could be involved or affected in toxicology studies. The panel is also designed so that you can actually go through your preclinical or animal models and human at the same time. So what you can see here is the panel is made up of 88 microRNAs that have been selected and you can see about these if you click on the product. Then how they can transfer across from the rat, from the monkey, or from the dog and there's many calibrators and controls also present on these panels.

[0:45:09]

It's a very, very exciting time as Colin and Monty have both presented for screening microRNA in these biofluids and we're very, very excited about all the different applications.

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I would just like to finish off by thanking all of research and people that have worked at Exiqon, been involved in these research projects, and also the other speakers of the day. Thank you very much for your time.

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Sean Sanders: Wonderful. Thanks so much, Dr. Baker, and many thanks to all of our speakers for this fantastic presentations. We're going to move right on to our questions submitted by our live online viewers. A quick reminder to those watching us live that you can still submit questions by typing them into the text box and clicking the submit button. If you don't see the box on your screen, just click the red Q&A icon and it should appear.

So we have received a lot of great questions, but please do keep sending them in. The first I'm going to put out to the panel and I'm

going to start with you, Dr. Baker, and we'll see if the other panelists have something to add as well. But you mentioned in your talk on normalization of data, could you perhaps talk a little bit more about that? We have had a few questions asking how one would normalize.

Dr. Adam Baker: Yeah. Thank you very much and that's great. I think Monty actually had a data slide in his dataset, which actually represented it really well that it is key that normalization approaches are critical in understanding how you actually look at the data and actually how Colin has brought it up is pre-analytical variables—like hemolysis—can actually bring a lot of microRNA expression into your datasets especially if you're doing genome-wide screening or you're doing random screening and then if you use a global mean approach to actually normalize your data, you can affect the data maybe of your differentially expressed microRNA that's not representative of a biological endpoint, but actually represents a different endpoint like what Colin was talking about: hemolysis.

So one has to really think through this carefully: is this sort of geo mean approach, a sensible approach for your dataset or as an invariant neo approach to sensible set. I think one thing that we recommend at Exiqon or when we're working in our projects is to try many different normalization approaches and actually look at how your data is moving around, how it is performing and that will help you understand the data and if there's any potential biases there. But it's a critical, critical point and it's not trivial. I think Monty said it quite right, you need to make friends with biostatisticians.

Sean Sanders: Uh-hum. Good. Dr. Pritchard, anything to add or, Dr. Montano?

Dr. Colin Pritchard: Yeah, this is Colin here. That was a great summary that Adam had. You know, I think that Adam said it really nicely in that there's no housekeeping gene for microRNA so that's a challenge and an invariant miR approach I think can work, but it has to be validated on a sort of experiment by experiment basis. So just sort of picking one, you know, GAPDH-like miR isn't probably going to work especially isn't going to work for plasma and serum. So the approach that we and others take is we spike in non-human microRNAs and at the time of our RNA prep and that of course only controls for variables on the RNA prep and technical aspects, but it's all we feel we can really do as far as normalizing to another miR at this point.

Sean Sanders: Excellent. So I'm going to ask a quick question about lifespan, we've had some questions come in asking what is the average lifespan of

mature microRNA biomarkers in blood. Is this in terms of days or weeks and, Dr. Montano, maybe you could take a stab at that one.

Dr. Monty Montano: Well that's a very good question. As you know, I study lifespan in humans and from humans to nematodes. I haven't really studied the lifespan of microRNAs, but I think that the data suggests that they are very stable over the course of several months. In fact, one profound set of observations have been that myocardial infarction, you know, a heart attack will cause an increase of spike in microRNAs that is detectible as a residual months later and this is also true for certain cancers and so I think that they are very stable.

Now, I think also as Colin mentioned, part of that stability is certainly due to the protein complexes that specific microRNAs are associated with. Also I think in addition to just sort of that biochemical half-life, one has to consider something, whether or not there's an actual function to microRNAs in serum rather than a proxy for tissue homeostasis. Meaning that if microRNAs are traveling from one compartment to another, that may actually affect their half-life and their presence in the peripheral blood.

[0:50:25]

Sean Sanders: Excellent. Dr. Pritchard, do you want to add anything?

Dr. Colin Pritchard: Yeah. That is a great summary. I mean it's a really exciting area and an exciting question, you know, sort of what is the half-life of microRNAs in serum and plasma and what does that mean for biology, you know, what are the clearance mechanisms. So the way I think about it, you know, how are they cleared by the kidneys, all these kinds of questions, are they cleared by the kidneys, these questions are totally unanswered and very interesting. I think Monty sort of hit the nail on the head with it's a very exciting field of biology that none of us have really touched on that much about sort of microRNAs as hormones, which the jury is still out on what that's going to mean, but that's a very exciting area and a lot of active research. So if microRNAs are being actively taken up by cells or tissues, that might be another clearance mechanism.

Sean Sanders: It actually leads perfectly into my next question, which is one that asks whether microRNAs might have some biological function or whether they're simply byproducts of metabolism or cell lysis. So, Dr. Pritchard, I don't know if you want to continue on that topic or, Dr. Montano, if you'd like to jump in as well?

Dr. Colin Pritchard: I'll defer to Dr. Montano on this one.

Sean Sanders: Okay.

Dr. Monty Montano: Well I think that the, you know, fact that you can see microRNAs in for example rhabdomyosarcoma in the peripheral blood suggest that the serum profiles are proxies for activity in specific tissues. Therefore, you might be able to glean a biological mechanism sort of like a mirror on the tissue by interrogating the network of expression of microRNAs in the serum. But this is clearly an active area of research.

Dr. Adam Baker: This is Adam if I could just continue from that. I think you're absolutely right, interesting and some of the recent work we've been able to get involved with actually touching on toxicity where we've had specific drugs and things that have specific organ toxicity and then reading the profiles that are coming in serum and plasma are showing some very distinct, very interesting profiles and mechanisms, which do give us more information into this whole field. So it's a very interesting sort of added area to this whole.

Sean Sanders: Perfect. I'm going to come back to you, Dr. Montano, with a question that asks about the form in which microRNA is circulating in blood. Do we have any evidence as to whether they're precursors or mature microRNAs as they circulate? The viewer notes that this seems important for measurement.

Dr. Monty Montano: Well that's a great question and I can't honestly say that we've tried to distinguish between the different process steps and to further distinguish between canonical and non-canonical biogenesis. I think, you know, that I'll defer to the other two speakers on this. We normally tease the region of the microRNAs for quantitation that would be present in multiple forms so I think that with the advent of RNA seq technology, the answer to this question will become apparent and quite exciting. I think it's also possibly worth thinking about not only the stage of biogenesis of the microRNA in the plasma or serum but also the actual sequence. So for example, we know that there are RNA editing mechanisms that also operate on microRNAs and so therefore the sequence of microRNAs can change epigenetically and therefore their targets can change. So not only is it going to be interesting to flesh out what types of microRNAs are present but also the sequence composition and that will imply potential function.

Sean Sanders: I'm going to come back to you, Dr. Baker, for the next question. I think you might be best able to answer this. What is the best control for plasma microRNAs when detected by qPCR and also what is the total RNA necessary for qPCR?

[0:55:03]

Dr. Adam Baker: Sorry, could you repeat the last piece, Sean, the total? Sorry what was the total?

Sean Sanders: The total RNA necessary for qPCR.

Dr. Adam Baker: Total RNA necessary. In terms of controls, I think basically what we're looking at is we've discussed normalization and the controls sort of drift into that. I think we have some reference ranges that we've developed that we used to sort of give us an idea when we run new panels is we understand the sort of levels of different microRNAs, so to speak a barcode, a profile of different microRNAs that we're looking for when we run a sample.

The actual amounts of RNA present in serum plasma, I'm going to be interested to hear what Colin says about this, you know, we know is very, very low. So we can use the systems, we're not using pre-amplification systems, which are often used. We're using 20 microliters of plasma and that's containing just, you know, 150 picograms maybe of microRNAs. It's very, very difficult to quantitate so it's very challenging. Colin, what do you –

Dr. Colin Pritchard: Yeah. You see that's an excellent point to follow up on Adam's comment. So, yeah, absolutely on a sort of picogram or nanogram, microgram basis, the amount of microRNA in serum and plasma is incredibly low, which makes it very challenging to quantitate and almost impossible to quantitate for the very, very low volumes. However, because of the short of length of microRNAs and the relatively low molecular weight, the copy number is actually fairly high, which is great for PCR. So it's relatively easy to detect even the sort of moderately to low abundant microRNAs and small amounts of body fluids, but it's hard to quantitate the amount of RNA input.

Sean Sanders: Perfect. Now is there any difference between serum and plasma microRNAs in terms of content? Dr. Montano, would you like to try that one?

Dr. Monty Montano: Well, that's a very good question. In our analysis of serum and plasma in HIV infected individuals on HAART, we were interested in

whether or not which biofluid do you use. What we found was that there were consistent differences in the recovery of specific microRNAs, but there was not a difference in the composition of microRNAs. So I think you need to choose your method and sort of stick with it.

Dr. Colin Pritchard: So I can follow up on that. We've actually been studying that fairly systematically that question and can say there are definitely differences between serum and plasma, although as Monty was saying, it's sort of on a per microRNA basis. So some microRNAs are very different between serum and plasma and some are absolutely the same and I think that's going to end up having to do with some again getting back to the origin of where these are coming from, are they in vesicles or in protein complexes. These kinds of questions are going to be relevant for figuring out why microRNAs that are different between serum and plasma are different and why the ones that are the same are the same.

Dr. Monty Montano: Yeah, I agree with Colin. I think I was speaking to the specific microRNAs that we were measuring, but no doubt that it's going to differ based on the microRNA of choice.

Sean Sanders: Perfect. So I just want to let our audience know we're going to run a little bit over the hour just to try squeeze in a few more questions, we've had a lot of questions come in. So I'm going to put this out. So you were just talking, Dr. Pritchard, about the vesicle associated versus protein bound and this question asks how important is it to distinguish between the vesicle-associated microRNAs and protein-bound microRNAs for pathology?

Dr. Colin Pritchard: Yeah. Well I mean I think that's a great question and we don't know yet. I mean my sort of take is I take a sort of pragmatic approach and I think in terms of a clinical laboratorian and so simplicity is the best. So a lot of times people ask me, oh, should we be doing, you know, immunoprecipitations against Argonaute 2 to pull out associated proteins from protein complexes from plasma or should we be doing an exosome/vesicle purification to pull that out. You know, I think that for certain applications, that's going to be very useful, but from the standpoint of a clinical lab test, it's probably going to be too complicated. So my thinking on that question broadly is well if we can figure out which microRNAs are sort of most robust to a simple processing technique or at the very last figure out what the variables are then we can just do a simple technique and bulk purification of RNA and go from there as far as a clinical diagnostic.

[1:00:04]

Sean Sanders: Great. A very good question, maybe Dr. Pritchard, you can answer this one. The feasibility of using microRNA obtained from urine or saliva, are you aware of this being done?

Dr. Colin Pritchard: Yes, I'm aware of that research. I'm not an expert in that area. I know there's a lot of really interesting especially in urine and also saliva as you mentioned and I think urine is going to be very promising especially for diseases that interface with the kidneys and the bladder such as prostate cancer for example or renal diseases, but also potentially for others. But I'm not really an expert in the area. In my lab, we haven't personally done any work with urine or saliva so I'll defer to the other speakers.

Sean Sanders: Any other comments?

Dr. Adam Baker: This is Adam. Yeah, we are actually doing work with urine and serum and plasma and sort of nephrotoxicity and other sort of areas so there's some very, very interesting research and very, very interesting results. So it's definitely an active area of research, the sort of toxicity with urine and also in some cancers like prostate cancer. We also tried other biofluids, so saliva we've heard of and CSF and other fluids like this, there's definitely microRNA profiles there and this sort of research is developing. But urine is playing catch-up at the moment to serum and plasma.

Sean Sanders: Uh-hum. So I'm going to squeeze in one more question before we finish and I'll start with Dr. Pritchard and then have Dr. Montano and Dr. Baker answer this. The viewer asks whether you think microRNA is a biomarker of effect of exposure or of susceptibility. Dr. Pritchard, you want to try that?

Dr. Colin Pritchard: Affect exposure or susceptibility, that's a broad question.

Sean Sanders: Sure.

Dr. Colin Pritchard: I guess it would depend on the context, probably all three. I'm not sure exactly what, I mean again I think it probably depends on the context whether it was and it will probably be all three of those.

Sean Sanders: Uh-hum. Dr. Montano?

Dr. Monty Montano: Well I would agree with Colin, it's hard to make, you know, vast generalizations. But I think that there is good reason to believe that with any sort of pathogenic perturbation on, you know, complex physiologies that we are that there's going to be a host response that at least in the cellular level very clearly changes microRNA expression patterns. To the extent to which that is reflected in the plasma and serum I think is the question of the day. But I think also this idea that Colin touched upon which is that, you know, are microRNAs potentially hormones would speak to the possibility of them having effects. So, you know, I think that yes to all of the above and I think in terms of susceptibility that's a fascinating question.

I do know that in some cases there are mutations in certain genes like for example the myostatin gene that create a susceptibility to inhibition by miR-1, one of the microRNAs that is expressed in muscle. So this genetic predisposition or susceptibility can create an increased risk for disease phenotyping. So I think that, you know, in effect the bridging of epigenetics of microRNAs with genetic SNP analysis is going to be an opportunity for a lot of innovations.

Sean Sanders: And, Dr. Baker, you get the last word.

Dr. Adam Baker: Yeah, well that's great, isn't it? No, I just like to say yes, again and I think Colin and Monty's answers are absolutely right is yes to all the above and I think that's the fascinating point about this whole area that we're just beginning to understand and you've heard some of the things today from the pre-analytical variables, from understanding the susceptibilities, for understanding the treatment responses. We're starting to see this and it is quite complicated but we're starting to work our way through this and it's just a supremely fascinating area to be in, in so many different areas. But, you know, it is complex and you need to work carefully through, ask the questions carefully, use the right technologies, but yeah, fascinating.

Sean Sanders: Fantastic. Well that is a great place for us to end because we are unfortunately out of time. So many thanks to our speakers for providing such interesting talks and discussion, Dr. Colin Pritchard from the University of Washington, Dr. Monty Montano from Boston University School of Medicine, and Dr. Adam Baker from Exiqon.

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Many thanks to our online audience for the excellent questions you submitted to our panel. I'm sorry we didn't have time to get to all of them.

Please go to the URL now at the bottom of your slide viewer to learn more about resources related to today's discussion, and look out for more webinars from *Science* available at webinar.sciencemag.org. This webinar will be made available to view again as an on-demand presentation within approximately 48 hours from now.

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Again, thank you to our panel and to Exiqon for their kind sponsorship of today's educational seminar. Goodbye.

[1:05:38] **End of Audio**