

Gideon: Hi, if everyone could take their seats thanks for joining us in a steamy Washington DC on a summer day, we're glad you picked us over any political conventions. My name is Gideon Blumenthal I'm from the center of drugs at the US FDA, and on behalf of the co-organizers I want to thank everyone for attending what we hope will be an engaging and productive workshop. It's clear people have voted with their feet to come down here in big numbers, we also have a lot of interest on the webcast over nine hundred people enrolled, so this is clearly an exciting technology, a lot of interest in the field and we are excited for the day. I want to give a special thanks to the hardworking staff at the AACR particularly Nicole Boschi and Anna Sadusky, they've been working tirelessly in the planning and execution of the meeting. I also want to thank Pam Bradley from the FDA who has been critical on our end in making sure everything goes smoothly.

We have a very data rich and ambitious agenda today, ultimately we want to bring all the stakeholders together, thought experts from academia, the device industry, pharma, regulatory both US and internationally, other government agencies, payers and patient voice as well. Ultimately we want to explore how we can develop this technology and bring it to patients to improve cancer patient care and I think at the end of the day we'll ask what can we do now, what types of trials do we have to design now so we can embed these exciting technologies into clinical trials going further.

I'll just walk you through a little bit on the agendas, this session one will be chaired by Julia Beaver from the FDA and will have a state of the various technologies. Session two will be chaired by Dr. Janne from Dana-Farber and he'll bring together other clinicians in lung cancer to talk about issues in drug development as well as in the clinic from a drug development and clinical standpoint. After lunch we're excited to have a case study, we had a recent FDA approval of a ctDNA assay the cobas test to detect EGFR mutations as a reflex test for erlotinib. We're very excited that we'll have a tangible case to discuss to potentially template going forward for other developers and other drug development programs.

That will be followed session three will be chaired by Dr. Philip from CDRH and she will talk about CDRH considerations for development of these assays, and then we'll hear some perspectives from various developers within the device industry. Then finally session four will bring together a lot of different stakeholders, we have Dr. Sumimasa from the PMDA in Japan, we have Andrea Ferris from LUNGeivity, we have Gary Kelloff from the NCI as well as Dr. McDonald from the, to get the payer's perspective.

Again we'll try to keep it on target, we have a lot of slides, a lot of data to present we will also have opportunities for panel discussion question answer and if you are listening in on the webcast there is an opportunity to ask questions via the web and we'll try to, if we do get questions from the web we'll try to embed it into the program. With that I'll turn it over to Dr. Janne for some introductory remarks as well.

Dr. Janne: Thank you Gideon and good morning everybody and welcome to our first Liquid Biopsy in Oncology and Device Development Workshop. As Gideon mentioned there is an enormous amount of interest in liquid biopsies and in the technologies not only for non invasive diagnostics, but for; disease monitoring, interrogation of tumor biology, early detection, evaluation of a drug resistance etcetera. The field is rapidly evolving over a

very short period of time, I think it's hard to not go into ASCO or AACR any clinical conference, liquid biopsies and non invasive technologies are at the forefront as they are in many publications in journals worldwide.

I think that there is a large degree of interest and yet I think the field is still evolving, and I'm hopeful that the workshop today will be instructive and informative to all of you here from various backgrounds, and will inspire us to think about what the opportunities and challenges are and how we move this technology further and further into the clinic, and ultimately for our patients where this may be very useful in all the aspects that I have mentioned. I wanted to just introduce our first session and our session chair Julia Beaver and welcome and we'll try and keep on time for our discussions and presentations today, so Julia welcome.

Julia Beaver: Good morning, so we have a great first session today with three leaders in their fields, they will be taking us through the current state of the science of liquid biopsies each focusing on a different liquid biopsy biomarker and describing various technologies. Each presenter will give approximately a twenty minute talk followed by a few minutes of question and answer to that presenter from the audience and the webcast, I'll do my best to triage the questions from nine hundred listeners. There will not be a panel discussion for this first session, so without further ado we begin with Dr. Howard Scher who is the chief of the genitourinary oncology service at Memorial Sloan Kettering Cancer Center, professor of medicine at the Weill Cornell Medical College and the D. Wayne Calloway Chair in Urologic Oncology. He will be speaking on circulating tumor cells and giving examples from prostate cancer, thank you.

Howard Scher: Thank you very much for the invitation, it's actually very confusing because it's very rare for the prostate person to be the first speaker at really any conference. Just by way of disclosures these are some of my collaborators in the industry without whom really this work would not be possible, and I think that's one topic for discussion and how far can an academic lab go over seeking collaboration in groups that can do things at scale.

I'd like to start with a bit of a context of prostate cancer as many of you may not be familiar with it, but it typically starts most commonly with localized disease then there is patients who are treated and may have a period where there is just a biochemical re-occurrence as the only manifestation. Metastatic disease follows and typically patients are treated with hormones, but these are not curative and as in green the patients ultimately become castration-resistant.

For years treatments were focused on around chemotherapy that was the only approved drug through 2004. As you can see since then there have been a number of agents and now we have six different drugs of diverse mechanism in our therapeutic armamentarium, and one drug [inaudible 00:13:51] that recently was given a breakthrough designation by the FDA, again the focus was around chemotherapy as the center. With the understanding of biology of series of oncogenic changes were developed and identified in the androgen receptor one of which was AR over expression, probably the most common alteration in castration-resistant disease. It was also learned that the prostate cancer cells can upregulate the androgen biosynthetic machinery and essentially become androgen factories. Two drugs; Abiraterone Acetate

and Enzalutamide which target these pathways have recently been approved and really show that if you understand biology, you develop drugs that target the biology you can improve patient outcomes.

We assume somewhat naively that we'd be able to give these oral agents which are relatively non toxic sequentially and that chemotherapy would get pushed further and further back. Obviously patients who are given the choice would rather have the pill than intravenous injections. There have been a number of groups that have looked at this question predominately metro respectively in terms of how best to sequence these agents, but as you can see this is a PSA water flow pie each line representing the present decline in prostate specific androgen level following treatment; first with Abiraterone in orange, second the same patient then treatment with Enzalutamide in blue. You can see there is really no relationship and you can't predict whether a patient would respond to one and not the other, not respond and then respond to the second but you do notice there are some patients who are primarily resistant. Their PSA is going up indicating essentially no effect of the drug.

Right now in the choice of treatment of which drug to use first is largely empiric and we do have six life prolonging treatments. We do know that if we give hormonal agents in sequence, the response in the second line setting is significantly lower and of shorter duration than if given in the first line setting. The real question is when do you use chemotherapy and there are a number of trials that are addressing this question, but obviously we want to select the right treatment for the right patient at the right time. In typically patients who are progressing on first line hormones will receive one of the two hormonal agents and this is used pretty much fifty-fifty depending on physician preference. The real question is when do you use the cytotoxic drug and this has become a major focus of investigation, and really a key point when trying to develop a predictive biomarker to inform a medical decision.

We do know however that if we only rely on the primary tumor which is the typical material available, we can be significantly misled as to what the actual biology of a patient's disease at that particular point in time is. The question is what material should we be profiling, tissue we'll hear a lot about cell-free DNA today our focus has been on circulating tumor cells. If you look at this OncoPrint of patients with localized tumors that have not been treated, those that are presenting with metastatic disease, those that are castration-resistant disease, you can see that there is a significantly higher degree of alterations. Simply looking at the androgen receptor, the frequency of over expression and the primary tumor very rare a little bit more common in the non-castrate tumors have an upwards of 40% of patients will have over expression of the androgen receptor in the castration-resistant state. We would argue that to be most informed as to the biology of a patient we need to focus on the castration-resistant disease if that's the point in the illness we are treating the patient.

We have a particular interest in CTCs because we know that these are more likely to represent multiple metastatic lesions, and as we do more and more PET scans we're learning that the multiple sites in an individual patient can be quite different. Our disease primarily goes to bone, it's very difficult to obtain tumor and even with directed biopsies we are only about fifty-fifty in being able to profile.

There has been considerable interest of late in the AR-V7 and the first assay that was developed was at Johns Hopkins by Jun Luo and Dr. Antonarakis, and they were the first to show that the presence of AR-V7 associated with the response. As we start to understand the mechanisms of resistance to the AR drugs they are really falling to three categories; the first is persistent androgen receptors signaling despite multiple agents that target. Activating mutations can occur, AR over expressions recovered as well as increased energy and bio synthesis, but the AR splice variants are quite unique and I'll talk about those shortly.

There is a bypass pathway with the glucocorticoid receptor essentially coops androgen receptor signaling and there are a cohort of patients who are completely androgen independent with RB deletions P53 mutations and these are quite resistant to treatment. Looking at the structure of the androgen receptor there is an end terminal domain you can think of that as the accelerator, a DNA binding domain, a hinge region and a like and binding domain where we are frequently when we'll see specific mutations that directly relate to the specific treatment that the patient has received. The splice variants have truncated C-terminus and if you notice in the AR-V7 there are specific amino acid coding sequence which has now become the target to which a specific antibody has been developed. You can see without a C-terminal a drug that needs the C-terminal to function will not be able to bind.

The AR-V7 assay that was developed by Dr. Jun Luo at Johns Hopkins essentially was a capture method based on the Adeno Test which uses cytokeratins and [inaudible 00:20:03] conjugated to magnetic beads. The messenger RNA for the AR-V7 splice variant was detected by PCR, this required minimal on hands time, to measure restriction is that the sample had to be processed within four hours. If we look at the relationship between the presence of the splice variant and response, again on the top left you can see the N-terminal domain, a full C-terminal domain where drugs typically bind. The splice variant without the C-terminal domain and if you look at the response to Enzalutamide if the splice variant was present then there was no response, PSA levels going straight up and the same held for Abiraterone. The presence of the splice variant again predicting for a non-response.

When the same test was applied and patients were treated with taxanes, you can see there was really no relationship between the presence of the AR-V7 and PSA declines and the patients seemed to do better. Again this was not a randomized prospective study; these were patients who came to the clinic who were sampled at various points in their illness. Well subsequently the Johns Hopkins test was licensed to Qiagen who developed a clinical grade test that passed FDA and is now being incorporated in the first precision medicine registration trial in prostate cancer using the AR-V7 test to identify patients who would potentially respond to a drug galeterone which had shown activity in this particular subset of patients. This is the essentially design of the trial, the patients are relatively early in their hormonal course, they have to have detectable V7 in their circulating tumor cells using the Qiagen assay in order to be eligible for the trial.

Which means that over ten patients are screened in order to get one who is potentially eligible, so a huge data set is being created of the patients who are screened for the trial

but are not enrolled because they do not have AR-V7. What is also unique about this trials at the primary end point is radiographic progression so that came out of the work of the prostate cancer working group two, where an imaging progression biomarker was validated over a period of several years and shown in phase three trials to strongly associate this survival. More recently we have been working or I have been collaborating with investigators at Epic Sciences looking at an antibody based approach to detect AR-V7 in single cells, in single circulating tumor cells. I'd like to start first with some of the process of how the assay was developed.

Essentially this is a non-selection based assay that enables one to identify virtually all cell types that may be present, depending on how the type of staining or specific properties that a cell may have. Again there was no selection and one of the key focuses of our efforts was to focus on obtaining samples at a point that a treatment decision was required, so we will typically see a patient who is progressing on one drug who is in need of a new treatment because his disease is progressing, and we would obtain duplicate baseline samples at that decision point. We would then be in a position to associate what was found in the circulating tumor cells in relation to specific clinical outcomes.

Again with the Epic Sciences no selection platform essentially the sample is collected in a Streck tube, there is a red cell lysis procedure, the buffy coat is then deposited on slides and distributed so there is approximately three million events various stains can be performed. There is scanning software which is easiest to think of in terms of facial recognition software and then various features of each of these cells can then be defined and identified and cells classified accordingly. The course based on morphology of the cells cytokeratin expression and in this case androgen receptor expression.

The important thing is that a range of cell types can be identified and not simply the [inaudible 00:24:39] occurring dependent. Again with trained pathologists the software can be taught essentially to recognize various cell types. Here is what we call a traditional CTC following the definition that was developed with the cell search assay, and here you can see again surrounding red amount of nuclear cells, these cells standing positive and what we would call a classical CTC based on cytokeratin positivity an intact cell CD45 negative and again they are not shown.

Some of the additional cell types that are identified in addition many of these might be missed, these would include looking first at the traditional CTC, some of the nontraditionals are simply called small cells which is strictly related to the size and comparison to the amount of nuclear cells present. There are a number of cytokeratin negative cells, these are believed to be the ones that are undergoing an EMT. There are various patterns that can be discerned, for example speckled nuclei in this particular case you can see what appear to be large nucleoli within the cells and each of these cells is now undergoing a genotypic characterization and some of the features that are coming out are in fact quite interesting. Again we also see clusters but these can be of different cell types as well; epithelia, cytokeratin and negative or simply containing micro nuclei.

The test that was developed is used in antibody specifically to the amino acid that was,

amino acids that came out of the cryptic axon that defines the AR splice variant, and the scoring was based on the level of expression relative to controls as well as nuclear localization. The nucleus localization again was critical because this is where the AR is actually functioning, you can see in some cases cytoplasmic staining but as far as we know these cells are not signaling through the cytoplasm. Placing the AR-V7 in context now this is the specific antibodies used, the ultimate result will be a yes or a no it's not a quantitative, so here essentially there is a primary antibody that identifies the V7 on cells. It is defined as detective if the signal intensity exceeds a certain threshold. Again here is an example of a spike cell where you can see that there is AR-V7 present, this is a control cell that is conceivably expressed as the AR-V7. It is nuclear localized and again this would be considered as a positive result, this being a control of cells.

Then we've since gone on to look at clinical testing using this nuclear localized single cell AR-V7 assay, and as I mentioned earlier we focused on the decision points. The typical patient who is progressing on androgen deprivation therapy will typically receive Abiraterone or Enzalutamide as their first treatment, both of these are as I mentioned approved and life prolonging. Very few patients are receiving taxane in this context, it's the second decision that becomes that we feel most critical do it for a patient with Abiraterone, do you change to Enzalutamide, do you give a taxane or similarly for a patient who receive Enzalutamide should you go to Abiraterone to ultimately benefit that patient or a previous taxane. Obviously the disease as it progresses becomes more and more heterogeneous and arguably more difficult to treat. I'll be happy to answer questions related to that, but again as you get to the third and fourth decision point and you see the diversity of cell types it feels like you are treating multiple cancers at the same time.

We use standard criteria to assess the outcome, again these are examples of the post therapy PSA changes and really these fall into three categories; patients who don't respond at all the idea being if you kill cells the PSA should go down. What you'd like to see is a significant drop that is sustained, and then there is a category where we see a drop and then slow rises, I previously called these the drifters but we were actually I think seeing now there is the emergence of resistance of cells that are regressive to know about. Again the patient population was typical for a castration-resistant group, but you see the number of patients who have received; one or two lines of therapy, three lines of therapy, four lines of therapy again we do not prescribe what drug should be given, this was a decision made by the treating physician. The important thing is associations of the outcomes at these different time points; some were chemotherapy naives, others chemotherapy exposed and not uncommonly most patients will have bone metastasis.

These three plots represent the frequency in which we detected the AR-V7 cells, these are the absolute counts in individual patients, the AR-V7 positive cells being in the yellow. You can see that in each time point there is not all of the cells are AR-V7 positive and there is quite a range, both in the number of cells present and the frequency. In the first line setting quite rare increasing in the second and even more so in the third line setting, and if you look at these sort of on average you see a steady increase as the disease progresses. On the top you can see the response to an AR signaling drug namely; Abiraterone or Enzalutamide and virtually all of the non-responders had nuclei

localized AR-V7 present. If one looks at the curve with taxanes you can see that there are a variety, a group of patients who have the AR-V7 present who do respond, so again this does not predict for sensitivity to the drug.

Importantly if you had the AR-V7 present you do not respond even with one cell, but there are a proportion of tumors that did not respond where the AR was not present, therefore it is not the whole story which really is not a surprise. Similarly with taxane you see a relatively balanced distribution so it does not appear to associate with response.

Looking at in summary for the performance estimates, all the PSA responders to the AR directed treatments did not have AR-V7 present and if they did as mentioned they did not respond, you do not see a relationship between the presence of the marker and response to taxane. PSAs can be I don't want to say a sketchy end point, but if you are using a drug that modulates androgens you'd like to see more evidence of benefit, so here we looked at how long the patients were on treatment whether the AR-V7 was present or absent. What was the time to radiographic progression pre-survival and overall survival, and in each case patients who had AR-V7 present did worse.

We then went on to look at a multivariate analysis to support the hypothesis that in fact the presence of AR predicts for an inferior outcome, and if you noticed looking across all samples you don't see any particular balance to one form of treatment or the other. The AR-V7 negative setting no relationship, but in the AR-V7 positive samples there has a ratio for mortality was only, was reduced by almost 75% for use of a taxane rather to an AR directive therapy. Notice the how is the ratio here and again in multivariate analysis this held up, this is hypothesis generating.

Where do we stand, we've shown that the AR-V7 prevalence increases as patients receive more treatments, it's very specific even with one cell present which makes me nervous a little bit as a clinician but it was a consistent result. Predicts for de novo or resistance to the AR drug; shorter time on treatment, shorter PFS and shorter overall survival but importantly it's only part of the story. We did see the treatment specific effect which would suggest that if you see it give a taxane, and importantly these results are available in time in order to form a medical decision. About three weeks ago the tests receives [inaudible 00:33:32] clarification in California, and again I think everyone is aware of the collaboration that is now going on between Epic and Genomic Health as it goes forward and now a prospective validation trial is under development.

I'd like to thank my collaborators at Memorial Sloan Kettering as well as collaborators at Epic Science and I'll be happy to answer questions, thank you.

Julia Beaver: Do we have any questions from the audience? I can start with a quick question obviously cost is a big factor in oncology care, what is the cost of this technology and are you already incorporating it into clinical practice or are folks waiting for the prospective validation studies?

Howard Scher: Well I don't set the cost but it's not something I would set up in my garage if that's what you are asking. Actually having watched this technology developed from the first

prototype which looked like a college physics lab to a robotic system has been quite unique, so it shows that academic should stick to what they can do well with the appropriate resources. How it will be priced obviously is not up to me and how it will be used, again the test is now certified I think it's really, there are a few trials that are starting to address the real question.

Where I'm still a little uncomfortable as a clinician is the single cell component and I think that's going to be very critical. That's part of the reason we're not just doing one slide we actually have ten to twelve slides stored in each patient so you can, and one of the things to be worked out is how many slides do you actually stain to be confident that a zero is a zero or a one is a one. I think that's where the higher numbers I think are relatively easy and I think there will be fairly quick uptake of the test.

Speaker 1: [Inaudible 00:35:39] great presentation Howard but more about the performance of the test. A little bit interesting you never gave us even though I understand that you can only biopsy about 50% of prostate cancers, you still never gave us any concordance between this test in terms of sensitivity and specificity.

Howard Scher: Relative to a biopsy?

Speaker 1: Relative to a biopsy that's the first question.

Howard Scher: I'll start with that, if you start with one autopsy studies where essentially every lesion can be analyzed you would see that there is a wide range of profiles so it does look like there are multiple tumors present. The question if you do when we are doing a metastatic biopsy we look for the site that one, is active on PET scan and number two we think we can get tumor. That may not always be the most informative one for the shall we say ultimate driver mutation. There has been a nice study that was done by [inaudible 00:36:42] group where they looked at PTEN status in tumor and in CTCs concurrently performed, and they are close but not perfect and I don't think that's a total surprise given how we see heterogeneity of response in multiple lesions.

Speaker 1: We're done for the sensitivity or concordance with this test correct?

Howard Scher: This is directly with a biopsy done and a blood sample drawn around the same time.

Speaker 1: You said PTN.

Howard Scher: PTEN status.

Speaker 1: Not AR.

Howard Scher: Sorry?

Speaker 1: Not AR.

Howard Scher: AR is also being done during the test.

Speaker 1: On CTC.

Howard Scher: Yeah.

Speaker 1: What's the ...

Howard Scher: Again we don't have, there is not enough numbers but it does look fairly consistent.

Speaker 1: It's pretty specific given that one it's specificity.

Howard Scher: Again, as I mentioned we don't have enough numbers yet to really go on statistical significance but even the one shouldn't be there if you think about it.

Speaker 1: In a hundred patients do you really routinely detect the CTC, what's the percentage of patients?

Howard Scher: The second part now is to look at which patients do you see cell numbers, so what we have done and are in the process of doing are associating the metastatic pattern with the number of CTCs that are detected. For example we know from previous experience that about 15% to 20% of patients that do not develop bone metastasis have a relatively low frequency of circulating tumor cells. Most of those patients are in bone and I think the ultimate guide book would be or what is the most, what is the optimal test for a patient based on the pattern of his disease in order to get the information you need. Some of it is obviously going to be a biopsy, some we hope it may be a blood base whether it's ...

Speaker 1: Can I ask a more definitive question, if we have a patient progressing in bone resistant to their first line AI, what percentage of patients would the test pick up a result? How many will it be informative in?

Howard Scher: The test will be informative depending on a number of bone amount, so there is a relationship between that. In the later stages you are seeing average of 80% if somebody has one bone that is a lot lower frequency, it's not a simple uniform number.

Speaker 1: Just two last questions you've never done this different kind of CTC detection looking in normal blood and last of all CTC numbers always being prognostic at least in breasts, you didn't have that in your multivariate. How significant is the CTC number and do they interrelate but I know that it didn't count with the taxane, but does it count at all?

Howard Scher: No, the circulating tumor cell number is a prognostic factor that is also being finalized but you would see a similar relationship. The question is do you only count, do you count only traditional CTCs or all, or focusing on all the cells excluding apoptotic cell population and there is a clear relationship between number and survival.

Julia Beaver: Right thank you. It's a great privilege for me to introduce the next speaker my mentor and friend Dr. Ben Park. Dr. Park is a professor of oncology in the breast and ovarian cancer program at Johns Hopkins and the associate director of the Hematology Oncology Fellowship Training Program and associate director for research training and

education. He will be discussing circulating cell free tumor DNA.

Ben Park: Thanks Julia and thank you everyone for coming again on this rather bombay day, I was walking from the ground high at night I got pretty sweaty coming here, and I realized that for those of us losing our hair a bad hair day could be a good thing because it hides the shiny spots. These are my disclosures, again this is all on slides so I'm not going to deliver these points in interest of time as well as the objectives, but this is the overview of my talk today. I'm going to give a little bit of a background on cell free DNA again probably many in these room already know this, but particularly technologies and where we are to detect this particular analyte and really the steps needed to adopt cell free DNA as routine test for a clinical practice. I'm going to focus because of my background and my interest in breast oncology or at least studies we've done at Hopkins and then conclusions and future directions.

Again I think most people know this and forgive the rather busy slide, but I think there has also been confusion as the field has moved forward of the different nomenclature that people have used. What is circulating cell free DNA or circulating tumor DNA or what I like to term as plasma tumor DNA because of its specifics in terms of nomenclature? Most would agree that circulating cell free DNA or ccfDNA or cfDNA that refers to these small DNA fragments that are shed into the circulation. The circulation could be; blood, lymph, urine, saliva etcetera so again I think that's an umbrella terminology of what we are calling cell free DNA. Circulating tumor DNA on the other hand is the same thing except that it's specifically from cancer cells as depicted here in these blue slides, blue cells so you can see that the multicolored DNA molecules and the translocations are specifically circulating tumor DNA.

As we've progressed as a field we've also realized there is differences in analytes even within blood, so plasma versus serum is something that people have done head to head comparisons on including ourselves and clearly plasma tumor DNA in most studies seems to be superior in terms of the DNA integrity. Plasma tumor DNA really again speaks to the plasma component of cell free DNA specifically derived from tumors.

What are the potentially clinical uses, I apologize there is going to be a mistake on here that I'll get into that I saw this morning. One of the things not just for oncology is that this is really a field that started in fact in the maternal-fetal medicine, where the idea that we could actually detect anomalies in the unborn fetus from maternal blood really has now become clinical practice in standard of care in many parts of the world. Instead of doing amniocentesis or chorionic villus sampling, one can simply draw a tube of blood and actually assay for fetal DNA and see if there is actually inborn genetic errors in that unborn child. This is already in clinical use, one of the things that was appreciated the total levels of cell free DNA again this is in many historic studies decades ago, that the total levels of cell free DNA actually increase in acute inflammatory states and sometimes chronic inflammatory states. People have been looking at the total or rise of cell free DNA in various diseases like acute coronary events and autoimmune diseases where inflammation is the surrogate biomarker.

This is my mistake clearly I'm an oncologist when I write the word solid tumor immediately follows, but what I really meant was solid organ rejection based on DNA

differences between donor and recipient. One can envision is that if you have a DNA difference between donor and host which is usually the case then one can exploit that as the solid organ becomes damaged from autoimmune processes then that DNA is going to get liberated, and we can use that as a marker. Believe it or not there have been case reports where solid tumor organ transplantation by accidents and in fact we have a case report from Hopkins a few years ago for that.

The other newer thing that people are looking at in the bone marrow transplant world is could we use this as potential markers for graft versus host disease, currently there are none for acute graft versus host disease. Again it's the same principle that there are differences between donor and host DNA, but finally again what this workshop and what my talk is going to be mostly about is clinical biomarkers for oncology. As Dr. Janne had mentioned earlier there is a myriad of different potential uses in oncology, I think some are more developed than others and we'll talk a little bit about that.

Ultimately it comes down to what are the technical challenges of identifying again in my case this is specifically Pt-DNA, the problem really has become unknown that the fraction of Pt-DNA is really, really small compared to the total plasma DNA and total plasma DNA is quite variable between individuals. The quality of plasma DNA is also highly dependent on how it's processed so when we're talking about pre-analytical variables, things like making sure it's spun quickly if you have a regular tube or using cell stabilizing tubes like the Streck tubes that we had heard about. This is because if you don't do this with time in the tube blood cells will burst, so the white blood cells will actually liberate even more genomic DNA, sometimes orders of magnitude more which makes it difficult to find those rare Pt-DNA molecules.

Again assaying for these rare mutations is something that we often say is like looking for needles in hay stacks, so how do we do this? There are really probably two technologies that so far have really come to the fore front of the field, there are more in development for sure but digital PCR is one of the first things that people looked at and this was really started by Bert Vogelstein oncologist at Johns Hopkins. The first high throughput was called beaming for beads emulsions, amplifications and magnetics oops, droplet digital PCR is a second generation form of beaming and I'll have a little diagram of that. There is also other forms of digital PCR which probably don't actually have enough compartmentalization to really get enough individual DNA molecules for use in field of plasma tumor DNA, but again people have been working on this.

The other competing or I would consider complimentary technology is actually next gen sequencing, this is really when one thinks about an evolution of digital PCR and that is to do single molecule sequencing. This has allowed us to do what we consider ultra deep sequencing so that you can take the same PCR amplicon or locus and really query this to really high levels of depth reads. The first technology is of the things called tagged amplicons, Bert developed something called Safe-SeqS, Larry Loeb's group at University of Washington developed something called duplex sequencing there is other technologies etcetera. We can measure not only just point mutations, but we could even measure translocations as examples for APML a specific type of leukemia which is always hallmarked by the translocation of chromosomes fifteen and seventeen. If one knows where that breakpoint juncture is, that is unique to that patient's cancer and can

be used to track in the blood.

This is the fundamental problem of the needles in the hay stack, this is a slide I often use and there is many familiar faces in the audience so forgive me you've seen this before, but I find this a very effective analogy. If we were to try to just look and pick out what we would call the mutant George in this it would be very hard to see, in traditional analog approaches is that we just take a PCR, we amplify everything in that [inaudible 00:47:10] and we look at it. Of course if we have a rare mutant George you are not going to see it because you are just combining all those little pictures into one.

The digital approaches of both digital PCR and next gen sequencing really separate out individual molecules and then query them massively in parallel. Most of the times you'll just see the regular wild type George, but every once in a while you'll see the mutant George. Again I always have to preface this that I'm not saying if you have a moustache you are a mutant, it's just I's a good cell from November when the prostate group have heir fundraising.

Again beaming was the first kind of real practical application of this and even this is still considered by many in today's centers a very highly specialized technique, because it requires a lot of instrumentation in equipment and again a lot of knowledge in how this works. Suffice it to say plasma DNA is extracted, it's then pre-amplified it's actually then mixed in these water and oil emulsions with magnetic beads and then PCR has actually done again compartmentalizing individual DNA molecules. These are then broken and for lesion hybridization probe specific for mutant or wild type are then added, and then one can put this through a simple flow cytoma to get both a qualitative and quantitative read out of wild type versus mutant DNA molecules. Again this is one of the first technologies that allowed digital PCR to really be done in a high throughput version in clinical samples.

Droplet digital PCR as I mentioned is the second generation, this has allowed labs like ours to really do this in-house, and it's essentially the same idea but rather than having droplets shaking and made I won't to say randomly but in a less uniform fashion. Droplets are made by these micro capillaries so that one can get uniform nanoliters or picoliter droplets depending on the technology and platform. Again the principle is otherwise the same, there are fluorescent pros that are specific to mutant or wild type and then those are actually assayed in a liter which is very keen to the flow cytometer.

Next gen sequencing on the other hand has some advantages rather than querying for a single mutation which is usually what we do for digital PCR, one can actually query a whole locus in the whole nucleotide sequence that are within. There are trade-offs, the more areas one looks at comparing for example a single locus to cancer gene panels whole exome to whole genome the limits of detection become lower and lower, as opposed to if you just do a single region let's say a hot spot region and PIK3CA one can really get hugely in depth levels of coverage, but again you are only assaying for one region. The trade-off is between large panels with less sensitivity versus a single gene or a locus with really high sensitivity. That's a little bit of a challenge however because of artifacts from next gen sequencing damaged DNA as well as PCR, so that can actually lead to false positives and this is a diagram of what different technologies or bar coding

have done for this.

One can develop barcodes called Safe-SeqS from Bert's group where just one end of the DNA molecule was tagged. The idea behind this is if you really have a true mutation you will actually see it in a compilation of all subsequent downstream reads, but there are potential problems with this if you have an early lesion let's say on one side that could potentially be in your final consensus sequence as well as a PCR mistake that happens very early in the cycle. That can happen, with bioinformatics one can still try to get that out of there, but Larry Loeb at the University of Washington as I mentioned created something called duplex sequencing which is a similar idea, but both ends of the DNA molecule are tagged.

When one puts together the consensus reads it has to be on both the sense and the anti sense, and in this way if there was a DNA error that was only on originally on one strand it won't be present on the other consensus sequence, and similarly PCR artifacts will do the same thing, but real mutation should be present on those strands. These are again bioinformatic challenges that can sometimes mitigate and often times mitigate successfully how we actually distinguish true mutations from the noise.

One of the things that I was charged to do is to really say how do we get this into clinical practice, so these are what I consider the steps needed for getting all of these types of test for clinical oncology use. I'm quoting the current ASCO president and friend and mentor Dan Hayes from the University of Michigan where he often says, "A bad test can be just as dangerous as a bad drug." I really do believe that, so I think we need to really take the proper steps to validate and prove utility of these tests if they are going to get reimbursed and they are actually going to be useful for our patients. Pre analytical validation is the first test to biomarker development, some of the questions that we've already talked about but how reproducible, what are the conditions of the analyte, is it something you can actually put in a tube and keep forever versus having to put and exerting conditions.

Clinical validation really is something where we say does in fact the test make a difference in separating out groups, so could this be prognostically useful, does it actually mean anything in terms of saying that if you have this you will do X, if you don't have this you will do Y. Really I think what we are all striving for in clinical oncology this is the last bullet point and that is clinical utility. I will mention very few biomarker tests that have actually met that benchmark, and by that I mean even if you have clinical validation can you actually do something with this that will affect the outcome of the patient in a positive way. For example if you have HER2 positivity in breast cancer we can now give a drug and that will dramatically alter the patient's course for the general population of patients who are HER2 positive. Again that's a really high bar, very few biomarker tests have actually met that, but I think that's all we should be striving for.

In terms of some of our own work with pre-analytical analytical validation we are one of the first groups to really use these Streck tubes and these Streck tubes are again cell stabilizing tubes that prevent lysis of the white blood cells, and therefore will not contaminate artifactually the analyte when it's in the lab. This was a simple study where we were just comparing if we took two different cell stabilizing tubes as well as

EDTA as our benchmark at day zero, extracted it digital PCR for genomic equivalence what did it look like compared to the cell stabilizing tubes drawn at the same time, held at the bench for room temperature, shaken up a little to simulate transport conditions and then extracted and then run all of them at the same time.

I think what you can see here these are all positive controlled droplet digital PCR bar graphs, these has been published so you can look it up. One of the cell stabilizing tubes we were using didn't actually work consistently as you can see in patient seven and patient eight. Compared to its day zero tube there was a high liberation of genome equivalence representing again artificial white blood cell bursting and contamination by the genome DNA. On the other hand the streck where BCT DNA tubes actually had a much better track record if you will of preserving DNA or cellular membrane and therefore plasma DNA integrity. I don't have any contracts for structure so you know.

One of the earliest studies that we did probably in the mid 2000s of looking at analytic validation and in terms of concordance, so we did both retrospective and prospective studies just looking for PIK3CA mutations, the hotspot mutations in metastatic breast cancer patients. Very simple design if you get a tumor tissue and you get a blood compare the two and what do you find. This was used with beaming and in cooperation with our colleagues at Inostics, and what we found is timing really does matter, for the most part and again this has all been published it's old data. The concordance was pretty high but if you actually had a tissue sample that was three years or more of those, a much higher likelihood that you could get discordant results between tissue and blood. On the other hand when we did the retrospective study on patients and the prospective studies where we actually tissue and blood within three years, their concordance was nearly 100%.

I think that at the time this is we wrote the paper back in 2009, 2010 that was a little bit surprising to us. I think most would recognize now and this is a seminal paper in the Newman Journal from [inaudible 00:55:10] which really showed in this renal cancer patient not only were the different sites and the test disease have unique genetic alterations, but even within the primary tumor depending on when you biopsy that you could get different mutational profiles. For sure there are ubiquitous or shared mutations amongst all the clones, we would expect that those might be the common hotspot driver genes those of that particular cancer. The genetic heterogeneity that we are facing in metastatic diseases is really I think highlighted here by these private mutations that are found in metastatic disease.

That to me speaks even more of the fact that Pt-DNA as a liquid biopsy could be extremely helpful in metastatic disease. We know that we can reliably detect these mutations, tumor heterogeneity has become a really big problem particularly with drug resistance. We and others have now published now that for example mutations in the estrogen receptor or ESR1 can be very different between tissue and blood depending on what you biopsy. In fact we can find multiple mutations, different mutations within the same patient so again I think this further supports the clinical importance and utility of liquid biopsies for this detection.

Then metastatic versus early stage, so this is work that now we are trying to pioneer and

lead into how do we actually use this to make clinical decisions in early stage breast cancer. For that I need to give a little bit of background probably most in this audience recognize that in metastatic disease we can't cure breast cancer, but in early stage we actually can and that's what we call adjuvant therapy. As a medical oncologist we give additional chemo therapies to patients to try to eradicate micro metastatic disease that might come back.

To put this in perspective about a hundred and fifty thousand women in this country alone every year are candidates for these adjuvant therapies. Since we can't follow disease by definition is micro metastatic, the only way we know this works are from these large clinical trials with control arms and poly chemotherapy given after surgery which clearly show that there is a benefit in the group that received chemotherapy. Now, it's not a lot and this is the problem with our business right now and the biggest unmet need I think for early stage breast cancer is that we are over treating. That is who is actually cured after surgery, we don't know right now so we end up treating all these patients but these patients and the green bag don't need it. Then also even if they do need it, who is actually going to benefit and who is not going to benefit.

I would argue that if we had good markers of residual disease we could actually define who should get therapies after surgery and who shouldn't, and then we'd also know who is cured and who is at risk for recurrence and we could also make clinical trials better by enrolling only those patients who have the highest likelihood of recurrence. That is again the group that are still positive after all standard care of therapies. This is our fly in the sky dream that after surgery we could draw a tube of blood and if the patient is negative then that patient is going to be cured, and if that patient is positive not only can we give that patient additional therapies but we could even track and monitor to see whether or not the therapies are working.

This was a study led by Dr. Beaver to my left here at Hopkins and was a proof of principle study looking at patients in early stage disease. This is all breast cancer patients; twenty nine women with newly staged breast cancer, newly diagnosed breast cancer. We got bloods on all twenty nine of them preoperatively, we got tumors for thirty patients because one patient actually had two tumors and then we weren't that successful at getting all the bloods, but we did get seventeen bloods postoperatively.

We only looked for PIK3CA mutations because again this was a proof of principal, but this is again all been published, suffice it to say that we were successful in detecting in the preop blood 93.3% of the time that matched with their tumor. Again 93.3% sensitivity, we had 100% specificity meaning that we didn't actually detect any false positives, probably more interesting in the proof of principle is that there were five patients post operatively where we actually still detected residual plasma tumor DNA. In one of those patients that actually didn't in fact predict for recurrence she had a very aggressive type of breast cancer, metaplastic triple negative breast cancer and within two years she ultimately succumbed to her disease.

I think one can start realizing or thinking about the potential impact, this would really allow for a rapid non evasive assay of residual disease and responsive therapy. My hope is that we could avoid needless therapies and over treatment, we could optimally

quantify the residual risk and the likelihood of cure. I'm really actually also hopeful that this will truly allow for precision medicine rather than treating everyone with all the therapies in early stage breast cancer. We could really say let's just give you X, Y and Z; surgery, radiation but no chemo or combination thereof and I'm hoping this will serve as a useful intermediate end points for clinical trials.

This has led to years in the making our prospective validation and maybe even utility trial if one thinks about it, it's in the neoadjuvant setting and literally this opened at Hopkins yesterday. It will be opening hopefully to a cancer center site near you by the end of the year, it's going to involve ten to fourteen sites. Again the idea here is there are a subset of patients who are after getting neoadjuvant therapy actually have no disease in their breasts and lymph nodes at the time of the surgery, we could call that a pathologic complete response. We are going to try to see with this trial if a patient clears her blood after her neoadjuvant chemotherapy, does that predict for pathologic complete response with the idea in the future that perhaps those are women who could forego surgery if our marker is good enough.

Probably the more useful and more exciting end point for me in the long term is after all standard care of therapies, can we actually define a woman who will still have residual disease and are those women actually going to ultimately recur. Those would be patients I would argue that we should really enroll for clinical trials.

In conclusions I hope I've convinced you that Pt-DNA detection can really change the paradigm and the rationale of how we are going to administer therapies that we can avoid over and under treatment. This really does allow for individualized precision medicine so we can treat patients with exactly what they need; not too little, not too much. I think to get there we are going to require the same high level bar of evidence that we require from drugs and that is prospective trials that really proof clinical validity and clinical utility. Again, I have to thank a lot of people but in particular Heather Persons and Julie Beaver who really led these as efforts as fellows in my lab. Thank you.

Julia Beaver: Thank you Ben, do we have questions from the audience or on the webcast? There is one over here.

Speaker 2: [inaudible 01:01:39] I'm very interested in your notion of over treatment which is something you face in all detects and I mean we do over diagnosis and subsequently over treatment. How would this approach these Pt-DNA would be useful for minimizing over diagnosing in screening setting rather than in treatment setting?

Ben Park: Yeah, that's a great question for those who didn't it the question is how could this be useful for minimizing over diagnosis, and I think that's a tougher question to grapple. There are a number of groups and even private entities that are looking at whether this could be used as a general screen for primary prevention. I got to tell you I'm staying away from that because I think that's a really hard thing to do, if we screen enough patients or asymptomatic individuals I'm sure we will find mutations, but I'm not sure what we will do with that information. Ultimately getting back to clinical utility, it would really take decades to proof that this would actually be worth doing. Not to mention the psychological and perhaps financial constraints to patients and their families.

I do think where we have an opportunity and we are starting to do this now as other institutions, are for patients who are getting let's say diagnostic imaging or screening imaging I should say, can we use this as adjuvant test to determine with higher or less likelihood is that really a malignant lesion. Even if it has a mutation could we follow this if it has pretty blunt characteristics and see whether the quantitative nature could help us decide whether or not this is something to worry about. Right now we are looking at studies for example with our radiology colleagues if a patient has a mammogram and she has a high suspicion, something we call BI-RADS 4 or 5, what is the likelihood of detecting a mutation when we know the likelihood of having a tumor with a BI-RADS 4 or 5 is anywhere from 30%-50%. I think that would help us avoid needless biopsies and would also help this hopefully over diagnose.

Speaker 3: Could I ask you some questions about your study?

Ben Park: Sure.

Speaker 3: I really have some concerns ...

Ben Park: Could you speak up in the microphone a little please?

Speaker 3: Sorry, it was echoing before but I really have some concerns of the technology maybe ahead of the question. You go and you do a panel on this primary tissue determine which mutation you are going to follow in the blood, is P53 in the panel?

Ben Park: Yes.

Speaker 3: P53 a cosine CHIP, causing normal's how do you know it's really you're not over treating if you find a P53 at the end of the surgery.

Ben Park: We are always are using the diagnostic biopsy, so we know it's in the tumor tissue so then we will take a blood pre-surgery or pre-therapy.

Speaker 3: It will be there too.

Ben Park: Well we validate it first, so we are always taking ...

Speaker 3: Do you understand what CHIP is?

Ben Park: CHIP you mean CHIP assays?

Speaker 3: No, clonal hematopoiesis of indeterminate origin of which a huge number of patients have it and p53 is one of the mutations. They have a high frequency of evolution to leukemia and they are recently at ASCO has even reported that people who receive chemotherapy there were breast mutations in the blood.

Ben Park: We have actually presented a case report which was unusual where a patient actually had both a primary lung cancer as well as a breast cancer, so I do think that that is a

caviar we will have to deal with. I don't actually think it's that common the CHIP, I'm sorry I wasn't familiar with that term, those tend to have really low allelic frequencies in the blood. What we have seen for example and we have a case report out there is patients who have actually had JAK2 mutations or DNMT3 mutations which are not related to their tumor, but they have them and they are otherwise asymptomatic but when one looks at the quantity of mutation ...

Speaker 3: What would you call a lower allele frequency?

Ben Park: In the tissue it would probably be 5% or less, in the blood it's probably about less than point 0.1% but it could be higher.

Speaker 3: These CHIPs easily have high allele frequencies.

Ben Park: Again my point is that one can look at the landscape in the blood and make a determination based upon what you find in the tumor and the two should be pretty concordant in terms of allelic frequency, so that gives you the idea. I'm not saying what you are saying is not, we can't consider that, but I don't think that's actually such a ...

Speaker 3: I don't know how you would interpret it that's you know.

Ben Park: We've seen this where we can different levels of allelic frequency, but I think if you are going after the Cornell driving mutations and they match in terms of equality for mutation allelic frequency with the tumor tissue, that one is pretty safe.

Julia Beaver: Thank you, I think we have to move on, I'm sorry.

Speaker 3: What about just one other question, what about PI3K which is in normal tissues.

Ben Park: Why don't we discuss offline, I appreciate your comments thank you.

Julia Beaver: Thanks so much. Our next speaker is a Dr. Tewari who is an associate professor in the department of internal medicine and biomedical engineering at the University of Michigan Comprehensive Cancer Center. He is going to be discussing extracellular RNA.

Dr. Tewari: Great thank you, so I will just flip forward here, I'll have to use this let's see I'll adjust the slides here?

Julia Beaver: I think they are loading.

Speaker 4: You can hit the button.

Dr. Tewari: Okay, there we go, that's just my disclosure that I advise for a company named as Miraculous. You've heard so far about circulating tumor cells and then mutant DNA for cancer as some of the various types of liquid biopsy approaches. The ones you've heard so far are ones which are further along the developmental pipeline, although still very contemporary and topical circulating tumor cells were perhaps amongst the first ones

now circulating tumor DNA. Also having reached at least in some cases clinical utility and approval I'm going to talk to you about extracellular RNA. This is really the more forward looking talk of the session, I'm going to attempt to review the state of the science in the extracellular RNA field which is essentially on the horizon as perhaps a next stage technology for liquid biopsy, but again it's all really in the research phase right now.

Just to give you an overview of the talk I'm going to spend probably the bulk of the time going over data, specifically talking about circulating microRNAs. These are extracellular or cell-free microRNAs and in fact I say here plasma serum as opposed to circulating tumor DNA. Although there are some differences between plasma and serum, they aren't really drastic drawbacks to serum as is the case with ctDNA. Then I'll spend the rest of the talk looking even more forward into some sort of developing technologies for supporting extracellular RNAs beyond microRNAs, and then in the end really give maybe a few minutes to something even more futuristic and that is what's the missing element for all of these different like of biopsy biomarkers, but especially extracellular RNA ones to really be 'transformative' for health care.

I will start with the circulating microRNA story, and then again as I mentioned in the other proceeding side I'll take you through a little bit of the history where we are right now and where are things going. If you looked back in 2005 the idea of actually looking at RNAs and plasma or serums as cell free biomarkers seemed a little unlikely, and for various reasons although there were some papers about RNA as a biomarker from some decades prior. The big concern was really that there was a lot of ribonuclease activity in blood, in plasma in particular and the idea was that RNA was generally believed to be unstable in the circulation.

Now microRNAs around that time had just been discovered a few years ago in humans and just as a very brief primer microRNAs are short RNAs, they are about twenty two nucleotides long that are processed from longer forms. They function in gene regulation by inhibiting messenger RNA activity for really tens of thousands of genes and there is over a thousand of these microRNAs that are known. Because these microRNAs around that time were starting to be found as pretty important in regulating multiple properties of cancer cells and also have been found to be pretty good tissue biomarkers, we and others actually became interested in this question of whether or not this microRNAs might be released from the cancer cells and might end up in the blood. Again there were many advantages potentially, the fact that they were already tissue biomarkers, lots of copies per cell and of course the fact that they were nucleic acid so we could detect them sensitively.

To make a very long story short, we and others in the field asked these sorts of questions of whether you could find these microRNAs in the blood, whether they were stable to degradation and then ultimately whether cancer drug biomarkers could be found there as well. I'm going to summarize because of the brief bit of time, basically the answers to all of these questions were, yes you can get more details of this paper if you like and I'll go through a little bit of this data. Perhaps the most surprising and striking results is that we found the microRNAs were really stable at least once they were in plasma, so when these are basically three microRNAs just from a healthy

individual from plasma incubated at room temperature up to twenty four hours and as you look at this, there is really no degradation in it, you can see this in multiple individuals.

The other thing that we found over time is that if you profile in this case these are patients with advanced really as a castrate-resistant prostate cancer. If you profile the microRNAs there are hundreds of microRNAs each dot has a microRNA, and looked at the abundance on average of individual microRNAs in patients versus controls. In fact there were a number of microRNAs that were at least ten fold sometimes forty or fifty fold on average higher in the cases compared to the controls. We spent several years trying to understand what these mean not just are these biomarkers but what are they really telling us.

It turns out that three of three microRNAs turn out to be microRNAs that actually function in suppressing epithelial-mesenchymal transitions. Really what they do at least one of their functions is to enforce the epithelial cell state, and this was as a proof of concept really made sense since prostate cancer is largely an epithelial cancer. What this was telling us was that while these patients have evidence of some sort of epithelial process in the serum and that was great, but again just a proof of concept because we already know that prostate cancer is epithelial.

There was another one and I'm illustrating this just really as examples of what you can learn potentially from extracellular RNA, but the second one was that I like to talk about was mir-210. This turns out to be very interesting because it's a microRNA that's induced specifically by hypoxia, and as you can see down here at least in cell culture experiments it's the induction is very dramatic fold induction with hypoxia. In fact if you look in the media of cells with a little bit of a time delay you actually start seeing it released in some way into the media.

Because tumor hypoxia has been known to be associated with treatment resistance and actually multiple kinds of treatment resistance radiation as well as chemotherapy and other treatments, we asked a question of whether if we took this group of patients and here you are just saying the average. If you took the group of patients needs separated them according to whether they were responding to whatever therapy they are on at the time their blood was drawn verses not responding, how does the mir-210 level segregate. As you can see here this is the group that was responding, this group that was not respondent this is actually a log ten scale, and we saw a pretty a substantial difference between the two sets of patients.

Now this is not, this is sort of early stage study of course it needs prospective validation of that. I make the point here mainly to give the example of a microRNA in this case which potentially reflects the environment or the micro environment that the tumor cells or either the tumor cells themselves or their micro environment is experiencing, and we are able to potentially see this in the circulation.

The conclusion this far then is that of course we can detect these microRNAs, they exist, they reach the circulation. I think an interesting important point here is what they can be informative regarding, and the two examples I have given you is one of epithelial

differentiation so the cell type tissue of origin and the other is the cell state potentially, and that's this case the hypoxia pathway activation example. There are other examples now in the literature especially related to cell tissue of origin. I'll just take a brief moment to contrast extracellular RNA versus extracellular DNA or cell-free DNA or however you want to, whatever term one would like to use. In my view I think they give, perhaps somewhat overlapping but also different kinds of information whereas the circulating tumor DNA can be very specific for the cancer with a mutant DNA.

It's at least within one cell it generally hard wired unless there is a tumor evolution happening which does happen over longer time frames, so the idea is that it may be less dynamic. Then for a particular locus it might only be one or two copies. I think the big difference with RNA is where the specificity can be less potentially only relative based on expression pattern. We have this potential at least to look at more dynamic events, and the example that I gave you is the one related to hypoxia, so this is again an overview.

I'll take just a few minutes to go back to this observation where the DNA, sorry the RNA microRNA in plasma was found to be really stable. We spent several years thinking about this question and really then doing experiments to figure out why this is the case, because it was really an unexpected finding. To make a long story short, what we found is that most of the microRNA in the plasma about 98% is stable because it's protected by proteins, so it's not naked floating around in the plasma but in fact it is protected by a protein class known as the argonaute proteins. Then there is a minority however, a real minority but maybe about 2% that seems to be associated with extracellular vesicles of one class which is exosomes and I'll talk about this a bit later.

The field of extracellular microRNA research is really expanded, sorry the font somehow is turning out to be very small here. There is over three thousand papers with lots of different applications both in cancer as well as in monitoring drug toxicity, even forensics now I'm just waiting for it to appear on CSI but I haven't that yet. Just to give you a very brief overview, so there have been a lots pre-analytic and analytic challenges now identified and overcome, one of the first ones was that blood cell lysis was releasing lot's of microRNAs and they were also platelet contamination that was a cult.

A series of papers have addressed this, at least now I think in general at least we understand the common, the confounding factors and ultimately day to day variation was an issue, whereas real time PCR is otherwise used with standards if you have DNA targets. With RNA it gives too much variation and digital PCRs you heard about before has also been sort of validated now as being able to overcome this. When I look at where is this field at right now, I don't know if you've seen this, this Gartner Innovation Cycle. It's actually a cycle that repeats over and over it starts with the technology trigger, then there is the pick of inflated expectations and there is the deep trough of disillusionment and then hopefully the slope of enlightenment by which time everybody has forgotten about the technology and then it actually starts working right.

I venture to say that in fact perhaps circulating microRNAs are at the slope of enlightenment now at least to the extent that we are starting, we understand some of the key variables. If you look at it from this other direction which is developing

biomarkers and aligning the biology with the technology, with the unmet clinical need which is the analytical, clinical and validations and the utility, I think analytical validation, clinical validation have been at least in principal accomplished. Of course they have to be accomplished for every specific test that would be developed and the place that the field has not reached yet, but perhaps in the next five years we will start seeing some examples of this is clinical utility in fact lining up the biomarker and the technology with a very specific unmet need.

Now I would like to spend a few minutes talking about extracellular RNA beyond microRNAs, and there are two things I would like to talk about, one is this idea of exosomes and extracellular vesicles and RNA that might be associated with them. Then just a couple of slides on how next generation sequencing is likely to expand the space of at least biomarkers that are discovered and then need to go forward in the next few years.

If you come back to this one initial conclusion might be well most of the microRNAs are not appearing to be associated with this exosomes or these vesicles, so maybe these are not great sources of biomarkers. In fact I think that would be an over simplification and the conclusion that really is not necessarily so, just because there are very few microRNA or least the over abundance is lower in the exosome fraction compared to the rest, that's not necessarily true. The case and it has to be really be determined on a case by case basis, because in some cases one example is in colorectal cancer at least there are some publications indicating that the performance of the exosome associated to microRNAs is higher.

Even aside from microRNAs this was a paper from several years ago that actually was sort of the basis of a company called Exosome Diagnostic now that's in existence. In this case messenger RNA was detectable in serum exosomes or extra cellular vesicles as I will talk about, so basically this is a developing area. There has been quite a bit of excitement, confusion, discussion about exosomes and how RNA has been associated with exosomes could be developed as biomarkers. I'd just like to take a few minutes to at least state some under appreciated facts, and I think where the field is right now again it's my opinion and what kinds of questions need to be answered to bring this forward.

One of the biggest I think under appreciated things is that people talk about exosomes as these membrane bound vesicles that are released by cells, but in fact they are one of multiple classes of vesicles. It's really a cell biology where do they originate question, but it can big implications for what kinds of RNAs might be associated there. Exosomes actually start by endocytosis ultimately forming these intercellular bodies that then butt off even intra-luminary to make these exosomes that are eventually then released. Also vesicles can be released right from the plasma membrane as well as by apoptosis.

In fact if you look at even the best methods these are vesicles produced by ultracentrifugation, differential centrifugation very fairly clean method. You can see a big diversity of the types of vesicles and these large ones here for instance, these are five hundred and nanometers are definitely not exosomes and the other ones are. In fact all the methods that are used really copurify these in some of the simplest of the

methods, not only copurify these but also copurify protein complexes. That's probably why they are actually quite a bit of this bit of confusion and conflicting results in the literature on this topic.

On the flip side, on the positive side I think we are, the field is starting to come out of this. There are lots of efforts now underway to try to tease out this biology, to try to separate the different types of vesicles and their organizations like the International Society for Extracellular Vesicles that's setting benchmarks and standards and a reporting criteria. The NIH has multiple, it has programs that are addressing this. I think this is going to ultimately lead to improve reproducibility, and ultimately also really understanding when it comes to the biomarker and to these vesicles what are we in fact dealing with.

The other thing I'd just like to spend of couple of slides on is next gen sequencing and how this is impacting extracellular RNA discovery, now next gen sequencing I think everyone is aware of this and certainly from the prior talk. On the surface it seems like this is an easy thing, sort of a well established thing in the field and I think for DNA in fact it is. When it comes to extracellular RNA in fact is a much more challenging at this point in our a research problem. Some of you may be aware of this program, so the National Institutes of Health through the Common Fund Program which is sort of like trans NIH program, has now funded a program called Extracellular RNA Communication a broad program just really to study extracellular RNA. In fact there are multiple groups some of which are listed here that are studying extracellular RNAs by next gen sequencing.

Ultimately what we have found in our lab is part of this consortium is that the standard methods right now are not comprehensive, have a lot of bias and we are actually in the process of studying the reproducibility. The goal of this is to characterize baseline profiles from bio fluids from healthy individuals, and there are other groups in the consortium then trying to develop these as biomarkers. Really where we are at with this is that there some technology challenges which are the extremely low RNA input, extremely degraded RNA as well as cloning bias on the positive side I don't have data I can show you yet, but at least internally I think there is progress on all of these fronts now, so I expect this to move forward. This is why I say that in the coming years the space of RNAs that are extracellular as biomarkers is expected to grow probably in the next two to five years I would imagine.

When it comes to circulating extracellular RNA beyond microRNA, I would say that we are still, the field is still at a point where all of these segments are really understudied but are expected to expand soon. Take home points overall that there are extracellular RNA is a real entity. It may be complementary to ctDNA as a biomarker for precision medicine and I think the key at least hypothesis right now, I would have for that is that it has the potential for providing more dynamic information. Ultimately though as I've mentioned this is really a frontier, and more biological knowledge technology development and further progress towards the clinical utility is where things are moving now.

I would like to take just a couple of minutes to now look even more into future and

maybe step out of extracellular RNAs in particular, but really about liquid biopsy biomarkers in general. This actually kind of relates to a course I took as a post doc, did a post doc in systems biology and I took a course on complexity science by Yaneer Bar-Yam this was his text book. I think as people are probably aware of, humans are complex systems and overall although we have of course shining examples where we can make really great predictions. In complex systems in general real prediction especially a long term prediction is pretty much a fantasy, and short term predictions on the other hand can be more accurate and weather forecast is a perfect example, but this requires ongoing frequent monitoring.

I think this idea has implications for disease biomarkers, so if this is disease activity this could be tumor burden or cancer growth or whatever you want to call it over time. There is certain simple model which will say this just to progress this lineally, and then if you actually believe this is a complex system you usually see at least a sigmoidal or at least an exponential phase to this curve. This has some real implications because what this says is that for any clinical decision you are going to make there is likely to be a window of opportunity that has some shape that not linear. This window of opportunity is when signal is high enough that it can be detectable above the noise, but it's not so high that it's too late to make that decision. A good example is HIV post-exposure prophylaxis it's a window of opportunity for that.

Our current approach is to biomarkers largely seem to ignore in my view the time they mention or at least the time is measured sparsely over time. In many cases at least and again I speak especially for RNA type markers or other type of even protein markers let's say, it's a reference range to which a sample and a data point in a patient compared. The alternative of course is to measure markers within the patient overtime, not a new idea or anything like that but perhaps one that isn't a very commonly implemented. Of course this ultimately really honors and is aligned with this non linear nature of disease progression. Again looking further into the future maybe five, ten years or so at least it's my conviction that this is a direction in which biomarkers are going to need to go. Obviously lots of regulatory practical cost or other questions, so one of the big efforts in my lab is actually trying to figure out how we could practically make this happen.

A missing element I think time and ultimately why are we all here, because we don't want to do the same thing over and over again and try to get the same reality but we want a new one. I would like to just acknowledge my collaborators and funding sources here, thank you for your attention.

Julia Beaver: Do we have questions from the audience or the webcast? I can ask a question, how are you seeing extracellular RNA incorporated into clinical trials or what do you see going forward for determining both clinical validity and utility.

Dr. Tewari: Yeah, so in terms of, I think as I sort went over extracellular RNA is a very broad field from microRNAs to other types of RNAs and so forth. The earliest work that I'm aware of is microRNA is being in a predictive sense actually some of my colleagues at University of Washington are looking at microRNAs in prostate cancer patients predicting seven month PSA, so they are starting to get into at least as it correlates to science for the trials. I think the process is going to be the same for any other markers

just a step wise progression.

Julia Beaver: Okay yes.

Speaker 5: I might be pre-empting the panel discussion a little bit, so when you and Dr. Scher get together for lunch I might imagine that you'd talk about, so how do you do extracellular RNA analysis for AR-V7? Is that something that you can do and if so how, and if not why not?

Dr. Tewari: Yes, so the extracellular RNA analysis for AR-V7 we don't know yet if that's possible, but we are hoping that it will be or at least there are reasons to think that it maybe. That relates to the next gen sequencing part of this, which is and that's I think that sort of the whole, the idea is to actually ask that question. If we can get to the point where from plasma RNA we can sequence RNA then we can actually ask the question of how does that relate to the circulating tumor cells, and the abundance of this and the circulating tumor cells verses not. We've done that for microRNA and it's interesting that it's usually about a hundred fold higher amount of cancer associated microRNAs in the plasma compared to circulating tumors cells. We don't know that for AR-V7, if you know something more about that.

Howard Scher: The first question I'm going to ask you at lunch is when do you decide a particular technology is ready to commit your clinical specimens to so people can see AR-V7 in exosomes, but the question is how consistently. What you presented is an outstanding hope for, but with all the assays that are going when do you make the decision to go to your clinicians and say, "You should be using this now in your practice and start the clinical validity portion."

Dr. Tewari: Right, so I think as Dr. Scher has mentioned, I think it's still that part of the work is sort is beyond microRNAs is still in the analytic even the validity section as well as clinical validity.

Julia Beaver: Thank you and I should have said this from the beginning, but if you could introduce yourself and just briefly where you are from as you ask questions.

Speaker 6: I'll go with that, [inaudible 01:31:57] with a company called Biometric. Actually my question is along the same lines, do you really have to pick the technology and the biomarker ahead of time to move forward, because cancer is a complex disease so we hear about new biomarkers all the time. Isn't there some approaches being thought where you don't have to pick the biomarker ahead of time and look at liquid biopsy as a whole?

Dr. Tewari: Yeah, I can speak about the extracellular RNA but I think a similar thing probably applies for tumor DNA. I think that's where the if I understand correctly I think that's where the sequencing is going to be more comprehensive to not be able to pick at least a specific one for that patient overtime. Fair enough, you guys would like to add to that.

Howard Scher: The question is a very good one, but I think one thing to consider is if you can determine how a specimen should be collected and appropriately stored and understand that in

fact you can use it, let's say three or four years if it's appropriately stabilized you can then do the retrospective, prospective trial. I can tell you that our program is spending a considerable amount of money on storage samples that we've gone through are very extensive collection process to make sure they are useful, and that's part of the reason we have been able to do some of the work we have done with APIC.

They don't necessary have to be drawn in real time, they do it to be processed and fixed but I think just we approach a biomarker like a drug phase one, phase two, phase three. If you don't answer the simple question, some of the things you mentioned like it's the information everybody wants to know but nobody wants to pay for. That's the time when you can say, "Okay, this is ready and working closely with my clinical laboratory colleagues unless the assay performs and goes through a six month validation, we don't touch it."

Julia Beaver: Last question.

Speaker 7: Two questions, two comments, one just that we heard about the biomarkers just like a drug and they have to go through the process of systematic validation in NCI via a program for early detection it's called EDRM we are a five phase criteria

Dr. Tewari: Someone asked about technology and assay.

Speaker 7: Yes, these need to be validated before they are using clinical setting. The point I wanted to make is that I beg to differ with you about that as if the microRNA technology stabilized, case an example you look into the literature. People leading the same samples and this is to do with the discovery, where there is a lack of robustness and also what we call rigor. People leading the same samples discovering RNA, different RNA, how one would somehow justify this that having they all say stage one are polyps but currently the different microRNA. I think that technology and also the rigor of the study must be some sort of standardized and make it robust enough so that at least there is some consistency among various lab. This is one of the major concern that we are facing in the field.

Dr. Tewari: Yeah, and I think the exact question that you are talking about was addressed in some of those papers in terms of putting analytic and analytic validity. One of those papers that I've mentioned, in fact looked at all the literature that was published at least at that time this is three or four years ago on circulating microRNAs. It turns out that about 70% of those biomarkers are not coming from the cancer cells, they are coming from blood cells contaminants and so forth. I think a lot of the discrepancies of seeing different markers and different patients are due to haemolysis, but more likely the platelet contamination that was not taken care of. In the last couple of may be three years or so right, I mean all of these issues have now been brought to light I think to the extent that they will be adopted and these best practices we adopted going forward. My expectation would be that you see a lot more consistency, but a lot of lack of consistency I think was just for these reasons.

Julia Beaver: Great, thanks so much to all of our speakers. Let's give them a big hand and then we'll be taking a break.

