Interactomics Basics and Applications

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Lecture – 09
Biomarkers: Harnessing the immune system for early detection of disease - II

We have with us a distinguished faculty Dr. Joshua LeBaer, he is one of the pioneers of the field of protein microarrays especially NAPPA technology; Nucleic Acid Programmable Protein Arrays. Dr. LeBaer also is the key leader of biomarker discovery programs and he also leads one of the program operated by the Early Detection Research Network or EDRM in US for the biomarker discoveries. So, he brings lot of his expertise, his experience of both technology development as well as how it can be applicable for the clinical problems and especially biomarker discoveries.

Imagine that you know, we are working let us say right in an Indian context, I am in Mumbai based and we have the samples coming from Maharashtra from you know different hospitals from Tata memorial and KEM and Hinduja various local hospitals here. Now our population is very restricted, we are talking about people only coming to these hospitals and trying to look at in a given context of a given disease; what kind of proteins are being changed.

And let us imagine that you know that particular protein, a given protein looks pretty interesting which looks very uniform in a given disease context in this population base. But if you think about, can we claim that protein as a biomarker; I think that it may not be the right claim, right. So, how to claim that you have a good biomarker? Of course, the biomarker should be generic, it should be global and it should really work in you know variety of clinical settings.

So, validation is really required; that is the key for making the success of any potential candidates to the right you know biomarker for the clinical and therapeutic interventions.

Today Dr. Joshua LeBaer is going to talk to you about some of the details about biomarker and validation strategies.

So the first thing that most of us as scientists would do, when we do a biomarker is we would observe a difference.

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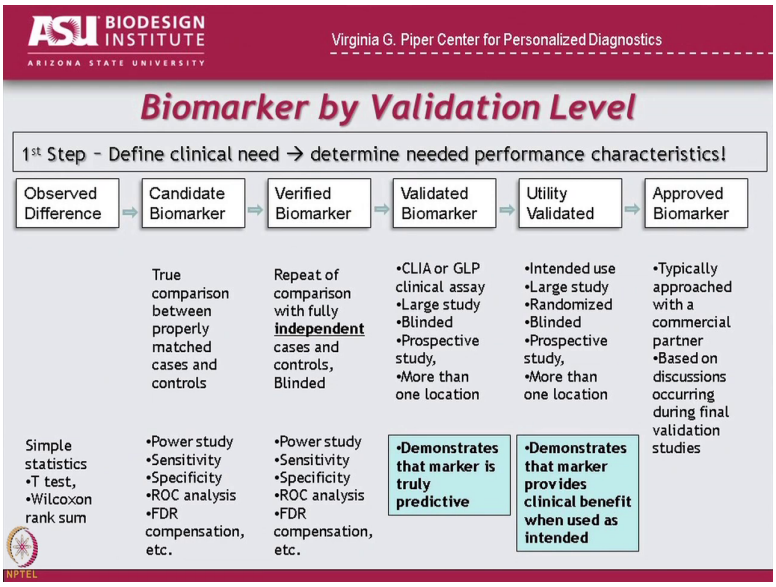

So, you remember those two graphs I showed you, that is the first step; you take a bunch of samples you know cancer samples and healthy samples or early stage and late stage or whatever your comparison is, you measure something and you see that the value of that x is much bigger here than here and there is a difference; you say wow, ok.

And the first thing you have to do say, I do not have a biomarker yet; because you do not have a biomarker yet. But you do have an observed difference and the type of statistics you might

do, our simple statistics; you might do a T test, you might do a Wilcoxon rank test; something simple to confirm that those two values are different, but that is not a marker yet.

So, now, how do you go about getting a marker? So, the next step is you need to say ok, I think I have a biomarker; now I need to do a larger scale comparison, I have to look at more people, right. And so, we would call that a candidate biomarker and we will do a comparison between properly matched cases and controls. So, how do you match the controls to the cases though?

Student: Age.

Age, right.

Student: Gender.

Gender, right; those are the two big ones I would say. Maybe as you pointed out the population, right; so you are not going to take a bunch of people with HIV in Africa and compare them to a bunch of Americans who have no HIV, that would not be a fair comparison; two very different populations. So, you are you need a you know try to stay within the same communities, same age group, same gender group; ideally the best matching of cases and controls would be the same group of people that go to the same hospital, except that this group has the disease and this group does not.

So, that is they are coming from the same population. And then that is we call a matched population. Sometimes like in their specialties, like in the case of a cancer, lung cancer study; you would want to make sure that the cases and the controls had similar smoking histories, right.

Because you do not want to be finding a marker that predicts smoking, you want to find a marker that predicts cancer. So, you have to consider your cases and controls carefully, you

match them. And then the first thing you do is; determine how many cases and controls you need to study, and how do you do that? How do you figure out how many to study?

Student: At least more than 100.

Student: Power analysis.

Power analysis, yeah; you need that you need to get a statistician that help you do what is called the power analysis. And a power analysis is a statistical mathematical study that takes into account; how big a difference in the value you expect to see, how prevalent that the diseases is in the population, you know how narrow the variation is in the measurement that you are making does it vary a lot, does it vary little.

It takes a lot of these things into account; they do some mathematics and they will say you know, what for the difference you are trying to achieve, you need to do this many people a cases and controls. Typically when they say the difference you want to achieve; though the way they will phrase it is, if you want to detect a difference in with 80 percent certainty, this is how many you have to study. And so, you have to, you have to say at what level you are willing to say I might miss it.

So, you will say I am willing to do this; I will do this study if I can get it 85 percent of the time. So, that is what a power analysis is. If you see a study where people are doing biomarkers and they did not do a power analysis; they did not do it right. And I can tell you that 85 percent, 90 percent of what comes to my desk as an editor, they never did a power analysis, right. And so, that is a real problem, alright.

Then you are going to eventually measure sensitivity and specificity and we are going to come back to that; we are also going to talk a little bit about the receiver operating characteristic curve analysis and false discovery rate compensation. But all of these types of mathematics will come into play when you do this first candidate biomarker study, ok.

So, you did your study and you got it looks promising. So, you get a marker and it has let us just say 85 percent sensitivity at 95 percent specificity. So, are you done, can you publish; no, what do you have to do next? So, you did a study, you did the power analysis, you compared the populations, you found a biomarker; it has 85 percent sensitivity, what do you have to do?

Student: We need to check some of other similar markers or else if anyone else has done the same one.

Well you certainly can look for other people who have done the same work; but the simple answer is, you have to repeat the study, because you are going to get markers, right. Typically many of us are going to be studying thousands of variables; if I am on my array right, on the NAPPA arrays we have now maybe 15000 proteins, right. So, let us say the chance of fight, let us say that the if you the P value that people often say is point of 5, right; 5 percent.

So, the chance of finding that value by chance alone is 5 percent; that is what it means when you set a probability a p value of point of 5. So, take 5 percent of 15000, how often am I going to find a biomarker by chance alone; quite a bit, right. Just by chance alone when you study a lot of variables, you are going to get a marker that works, alright. So, the first thing you have to do when you get markers that look promising is, test them again on an other population and that is what is here.

So, you repeat the study, you verify the marker and its important in this case to use a completely different set of patients and controls. And that is important why? Why is it important when you do the second study to use different people?

Student: Prevent redundancy.

Prevent redundancy, ok, maybe expand a little bit. So, you have already shown that marker works for that population; for whatever reason that marker let us assume you did their study carefully, separates cases and controls. The question you are asking in this study is that, a general fact or is that just happen to be a random chance for that one population. So, by doing

it in a different population, you are verifying that in fact, it really is for the disease and not just by chance alone.

So, there is a famous story in proteomics, some of you may remember this. But at the beginning of this century there was a pot; there was a paper published in the lancet, there was a proteomics paper and they developed a blood test for ovarian cancer and it was based on mass spectrometry. And they predict they claimed that had a 100 percent sensitivity and 99 percent specificity, astonishing numbers. Anybody who knows anything about biomarkers; look at that and said bull shit that is not right.

There is no way that you could get a 100 percent sensitivity, biology is not that predictive. Well so, they got a lot of press hold programs were started at the NIH around it, a huge amount of excitement; it was a big deal that, proteomics had solved the detection of ovarian cancer and it all failed. It was a huge miserable failure and it setback proteomics by a decade; because people stopped funding us, because they said that we make claims that we can support.

And one of the fundamental mistakes that they made in that study, was in their validation step they used the same control group; they did use different cases, but they used the same controls. And so, they did not follow the rule that this group has to be different from that group. And consequently for whatever reason that control group had a defined pattern that was definable as control and that is what allowed their biomarker to work; but it was just random chance, it had nothing could do with ovarian cancer.

And so, that was a huge error, so you have to be careful about that. So, that if you get to this point and your marker still holds up; now I think you are ready to publish. At this point you can say I have got a verified biomarker, this is worth telling the world about and then you can send it out for, you send it out for review. I will tell you as an editor for JPR; if I do not see this, I do not even review it, I send it right back to the author. If they do not do a validation study, they are out I just I would not even look at it, alright. So, then.

Student: Take different control to put in a different hospital or a different lab.

I am sorry.

Student: A different controlled room put in a different hospital or a different lab.

It could be as long as they are different people.

Student: Different.

Different people, they can be from the same hospital; they have to be different controls and they have to be different cases, oh there can be no overlap and the people.

Student: With a different disease.

No same disease, but different people.

So, for example, if let us say, you have a 200 people with ovarian cancer at your hospital and you found 200 women with you know there are good controls; you could split them into a 100 cases and 100 controls and do your first study. And take the second hundred and the second hundred in to your verification study; that would be perfectly good design, ok. So, after you get your verification, you still have a long way to go to get a valid marker.

Now, you have to do what is called a validated biomarkers study. These sorts of studies are typically a level past most academic labs. Most of us can do these studies, they have to be done very formally. These studies should be done under what is called either clear or good laboratory practices certification; they should be large studies, they should be blinded studies. Blinded means that, the scientists who are measuring the values do not know who has the disease and who does not, right; and all of that is hidden in the documents. They have to

make their predictions based on what they set back here. Typically there should be a prospective study. What do I mean by prospective study?

Student: Forward predictive study.

Right. So, what does that mean?

Student: where improved patients samples.

That is, right. So, you are now looking at samples that you collected last year; you are collecting samples in the same manner that you would be doing it, if you are treating patients. You collect the sample and you test it and you see whether it predicted properly or not; and then you need to do these in more than one location.

If you get this done, what that tells you is your marker is truly predictive it really does predict the disease; that is great, that is already something to be very proud of. Now you have a marker that predicts disease; are you done? You can tell you are not done, because there is still space left on the slide, right. So, there is still more to do, right.

So, just because the marker tells you that the patient, that can predict the disease; you still do not know if it will be a clinical benefit using that marker. And so, the next step you have to do is what is called a utility study. You have to ask if I use this marker on a population, will it tell me something that reduces mortality or morbidity in that population; because I detected the disease early.

And so, here what you do is the same thing as here; randomized, blinded study, prospective study, but in this case you are doing it as an intended use. You are measuring it, you are predicting an outcome, you are telling the patient and you are acting on the prediction; and you ask the question in those people with whom I use the marker, did they have a better outcome than the people who did not use the marker.

Did the marker save lives, did the marker reduce disease; and this is where a lot of markers fail. So, some of you may be familiar with this marker called CA 125; which is a very good marker for ovarian cancer. There is no doubt that, CA 125 levels correlate with ovarian cancer; that marker is used all the time as a disease progression marker to monitor ovarian cancer, it is quite specific.

The problem is, if you do CA 125 to detect cancer; it you do not see any better outcomes. And the problem appears to be that by the time the CA 125 levels are measurable; the cancer it is already too late, they it does not come up early enough.

And so, it is a predictive marker, so it fits it succeeds here and it fails here. If this works here, then you get an approved marker and now you are in good shape. I can tell you that this whole process is very long, very expensive and has only been successfully done a handful of times, ok.

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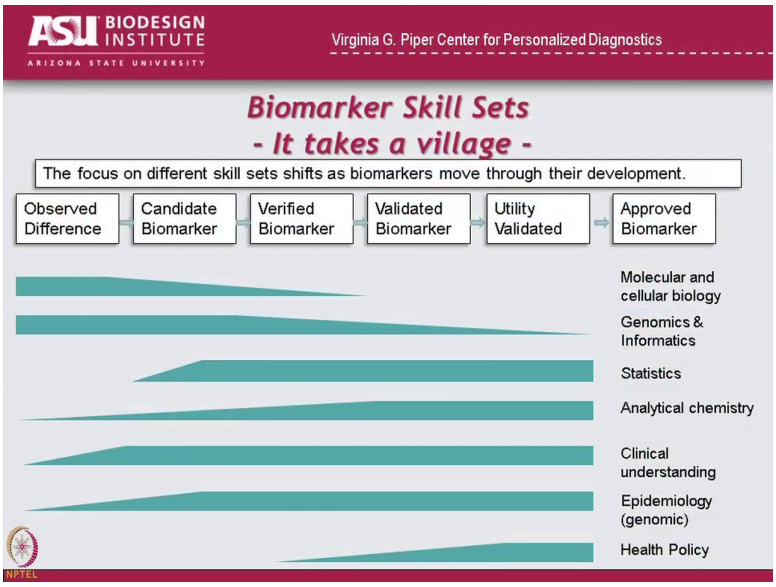

So, what are the skill sets that you need to accomplish all these tasks, right. And so that is what is shown here; and this is just a emphasize that, to get a good marker you need a multidisciplinary team; there is no way around that. So, you need to have early on you need to do these first sort of studies, you need people with molecular and cellular biology experience; throughout the study, but especially at the beginning you will need genomics and informatics.

As you go further into the study, you need good statistics, you need to develop strong robust markers that you can that do in the clinic, you need good analytical chemistry; obviously, you need good clinical understanding and understanding of epidemiology and then when to use these markers depends on looking at health policy. So, at different stages of the game, you are

going to need different experts; but throughout the whole process you are going to need a lot of experts, so.

Student: May I have a question.

Yeah.

Student: Second and third stage power analysis basically holds you.

Well how you do it varies a little bit; it depends the way you do the power analysis depends on the study and what goes into it. So, for example, oftentimes when you are at this phase you might be doing protein arrays or you know next gen sequencing or some kind of large scale, omics scale study; where the number of variables is very large.

And the type of power analysis you have to do with large variable numbers is different than if you are testing doing a power analysis for just one marker that you have as a predictor. In this case you may have to do modeling statistics to get a good predictor, you might have a simple formula you could use over here; but the idea is the same, it is just the execution is different, ok.

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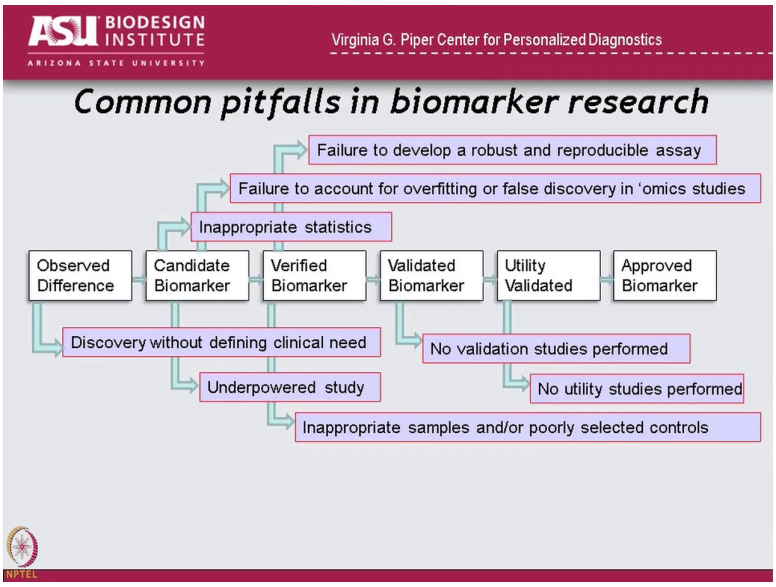

So, where does this go wrong? So, this can go wrong in a lot of places and it does all the time. So, the first mistake is you discover some kind of a difference, but without defining a clinical need; you have not defined the clinical need, your difference may be meaningless or may be useless. People often do inappropriate statistics on these candidate biomarkers, they will look for P values instead of doing proper biomarker statistics.

People do not do and they do what is called an underpowered study. What is an underpowered study?

Student: Sample size is not good as a.

Poor.

Student: The sample size will not be.

The sample size is too small, yeah, exactly the sample size is too small and there is two consequences to that; the first consequence to that, the most common and historical consequence is that, if you your sample size is too small, then you run the risk of missing a good marker because you did not study enough people, you did not you would not have as enough of a chance to find the marker. In the modern era the problem is a little bit different; the modern era the problem is these days we do not study a few variables, we study tens of thousands of variables.

And so, in the modern era an underpowered study usually means that, you are going to find differences that are meaningless; you are going to find random chances that this gene is different from that from in the cases and controls and it is not related to the cancer at all. Because of what is called overfitting? Overfitting is statistically finding something that is not really real, and it is a huge problem in our field.

I can pretty much guarantee you, if you see a paper published and typically they are published in the best journals, science cell nature; you will see a paper published next week or a month from now on a marker that has a 100 percent sensitivity and 99 percent specificity. And if you look carefully they probably over fit, because no markers are ever that good, ok.

So, failure to account for overfitting I just said it; you heard it here first. Using inappropriate samples poorly selected controls. So, people do not carefully match the controls. So, for example, I have seen studies where people used a bunch of cases from one location and then they ordered their controls from a company. And then they compared the two and said I found a marker, I can tell the difference; well they can tell the difference between samples that came from the company and samples that came from this hospital, they did not necessarily find the disease.

In fact, of you know Paul Temps is a proteomics researcher at Sloan Kettering; Paul did a study where he was looking at prostate cancer. He was trying to replicate the kind of approach

in that ovarian cancer study, I mentioned earlier that totally failed; but he was trying to do it right.

And what he found was, he was looking at prostate cancer and he found a marker that was remarkably good at predicting prostate cancer. But you know credits to Paul Temps, because he looked a little harder and what he realized was that; the prostate cancer samples were all drawn in blood samples from men who were about to get biopsies, they were all in the hospital and they were going to get biopsies.

And the samples that came from the controls, all came from the outpatient clinic and it turned out that the two locations used a different manufacturer of the blood tubes. So, the blood tube type was a little bit different and when he did all the analysis what it turned out was; he had found a really good biomarker for blood tube type and nothing to do with the disease at all, it had to do with the types of the tubes that it came with. So, you have to be very careful.

And so, and then people often fail to develop a good robust and reproducible assay. If you are going to do the kind of late stage validation here, you need to have a good assay for that. Some many people forget to do this study here or they do not do this study here. And so, that is sort of summarizes some of the major problems that you can encounter, ok.

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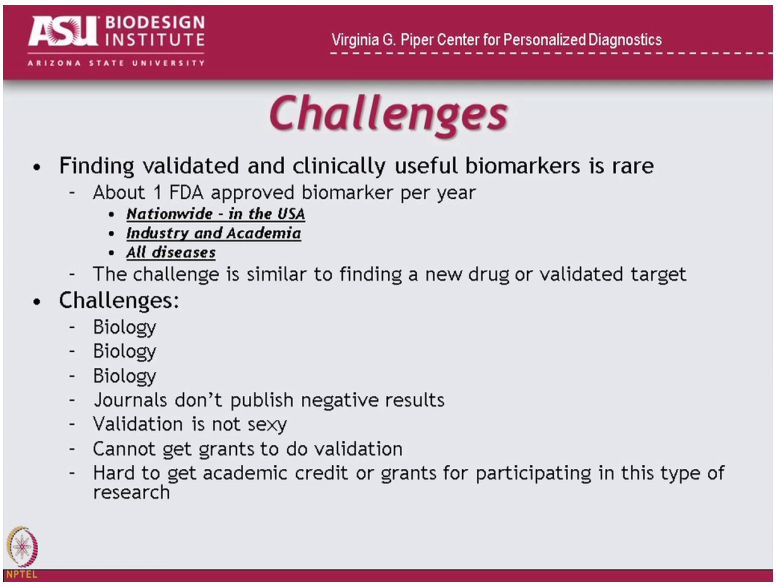

So, lots of challenges; finding a good clinical useful biomarker is very rare. These days in the US on average maybe one to two biomarkers a year will succeed in making it through the FDA. So, this is very very challenging and that is combining all the work of academia and industry; all combined that is all we get. I would argue that the big biggest challenges are the biology itself, it is very hard to find a molecule that specifically can predict the outcome of a patient.

So, you have to look extremely hard to find it; but journals do not publish negative results, and so oftentimes people do not realize when markers are bad. And so, they end up, you know only publishing bad biomarkers. No one likes to do validation in fact, in an NIH at the US; it is very hard to get funded to do a validation study.

So, let us say you do a good biomarker, you have all the best intentions; you do the observations difference, you do the initial study and you need to do verification study. And you say ok, now I want to validate this marker; the response you will get on your grant application almost always is well you have already studied this marker, why do you want to study it again. And you can say, because I want to validate it; they are like no, you already studied it you are done, it is like no I am not done.

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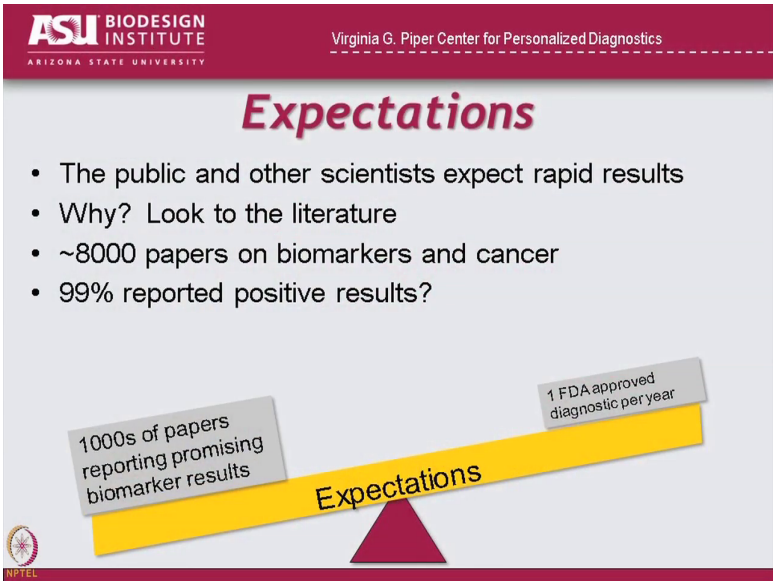

So, that is exactly one of the problems that we face all the time, alright. So, let me move on then. So, nonetheless the public really expects to see these results and that is partly; because there is thousands of papers that report good biomarkers. And there is usually only one good one per year and so, everybody thinks that it is easy; but in fact, it is really hard. So, that is kind of a take home message.

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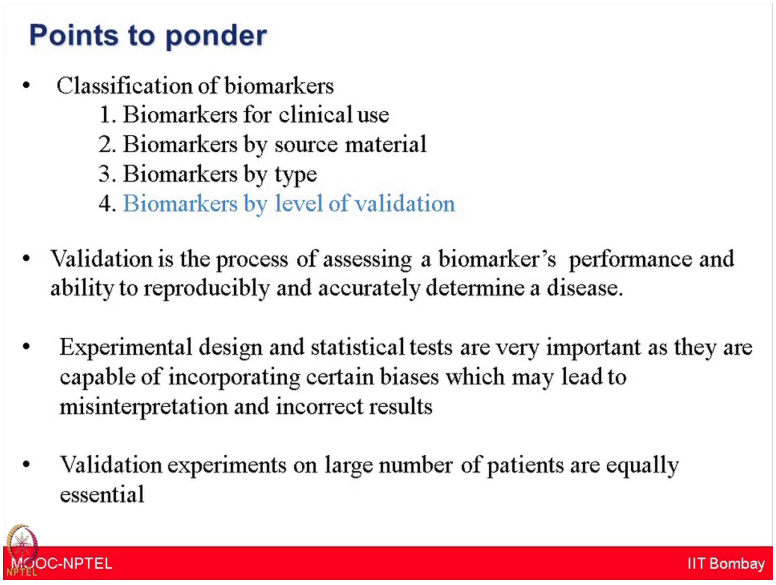

Alright. So, just to conclude Dr. Joshua Lebaer has talked to you about different basic consideration; how you can be confident that a lead which you have identified as a you know potential protein candidate, whether you can term them as a biomarker. What type of test you should do both from the a statistics point of view as well as the right clinical assays in the clinics in the labs which can ensure that the candidate which you are identified that is actually a potential biomarker.

So, these basics are very important for you; even if you are a student or you are a researcher who are planning to be involved in the biomarker based programs. I think you know your strategies thinking about the power calculation, the statistics, looking at the sensitivity and the sensitivity of the biomarkers as well as your plan to do validation of the candidates becomes

very crucial. I hope these basics are really giving you new insights about how to now utilize this understanding, this knowledge for the actual clinical applications.

Thank you very much.