**Interactomics Basics and Applications**

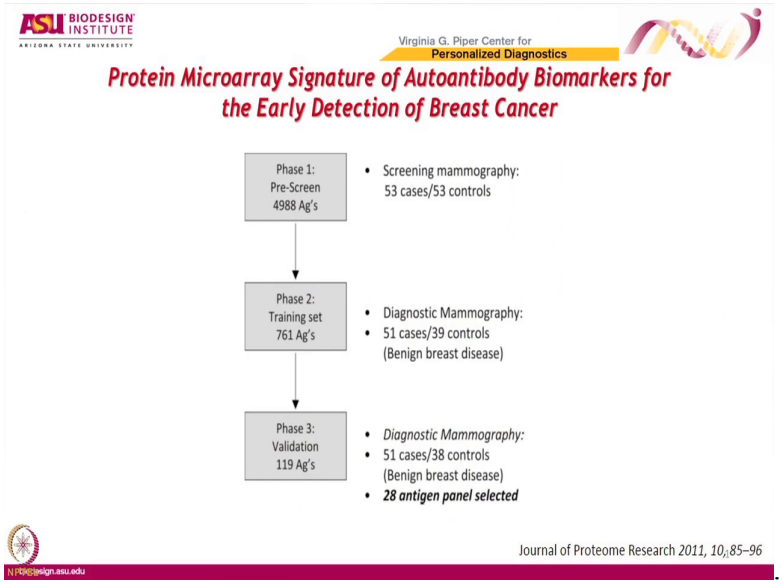
**Prof. Sanjeeva Srivastava**
**Dr. Joshua LaBaer**
**Department of Biosciences and Bioengineering**
**Arizona State University, USA**
**Indian Institute of Technology, Bombay**

## Lecture-12
## NAPPA and its applications in study of antibody immune response in disease and in drug screening-II

In the last lecture Dr. Joshua LaBaer provided his expert opinion and comments not only the researcher, but also as the clinician who works in the areas of breast cancer. In today's lecture Professor Joshua will continue his lecture about a studying auto antibody signatures using NAPPA technologies, which could be used for early detection of breast cancer.

So, let me welcome Joshua again for his lecture on auto antibody detection for breast cancer using NAPPA arrays. So, I talked to you a little bit before about good study design and so when we went to do our breast cancer study, that is what we did.

(Refer Slide Time: 01:09)



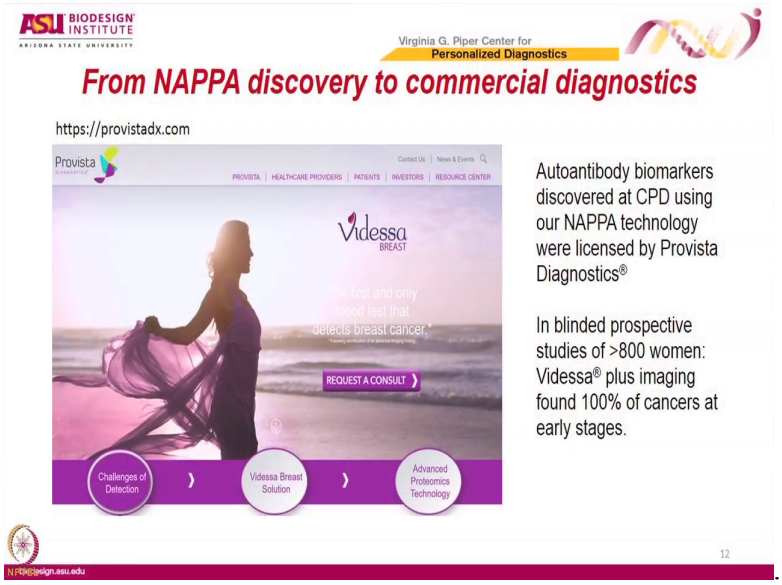

I think I may mentioned this already, but we had around 5000 antigens that we studied; we sent in 50 cases and 50 controls. These are women with breast cancer and these were women who were going to a clinic for routine mammography who did not have breast cancer, ok. So, these were healthy women in the population. We are, from there we identified around 760 antigens, possibly different between the cases and controls.

So, at this stage of the game from here to here, we did not want to be overly selective; any possibility alive, we will just want to exclude everything that was not even possible. So, even though we knew that there would never be any where near 760, we already eliminated well more than enough 4000 and that was already good for us. Then for this set we printed a new array, duplicates on the array and we compared 50 cases to 39 controls; in this case where the controls had benign breast disease.

So, they had cysts and other things in their breasts, but no cancer. And so, that allows us to distinguish being cancer and controls. I will tell you that it is a little odd when you look at it that we had 50 and 39. You would have thought that we would had 50 and 50, right. It turns out that, we did originally have 50, but about 11 of them turned out to have cancer after all and so we had to take them out of the study. So, that kind of thing can happen you have to be prepared for that. And then we did a third set; these women were different from these women and these women were different from both of these women, and this was the final validation, so three phases of validation.

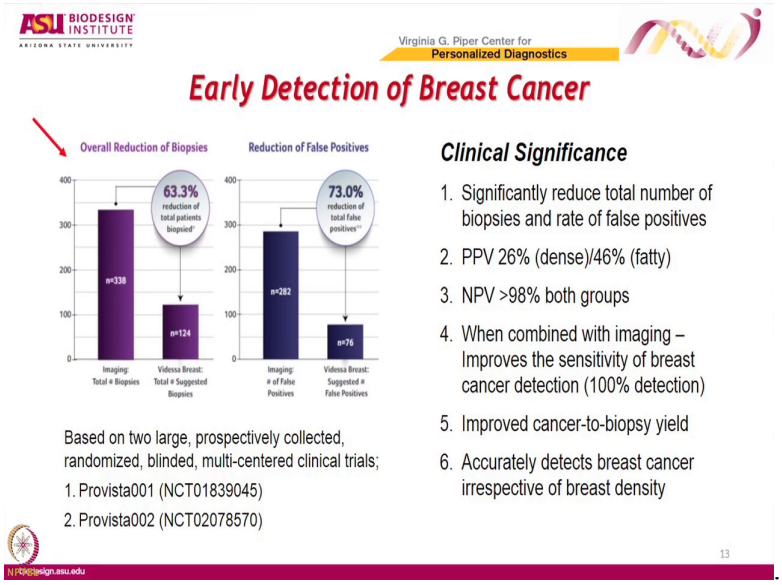
In this case we did it blinded, so we did not know who was what and then we identified 28 antigens; that even blinded were predictive of breast cancer, ok. And I think in the end what worked for us was the fact that, we were so careful and going through all of these phases; that what we got in the end really did make a difference and that is why it was licensed by this company to make this Videssa which is a blood test that is based on our biomarkers and a couple of other markers they added to detect breast cancer.

(Refer Slide Time: 03:27)




And this is now been through a couple of clinical trials; I think I mentioned that earlier.

(Refer Slide Time: 03:31)




You can see that in this clinical trial and this clinical trial they have reduced false positives dramatically; while at the same time detecting all of the cancers. So, the combination of the marker plus mammography found every single cancer and at the same time reduced a lot of biopsies women who did not need it, ok. So, that I think I told you a little bit of that story before; any questions of this part, so far before I go on.

Student: Commercialize the rapid Eliza.

No we have not commercialized the rapid Eliza, we have a core facility that will do it, we well help you do it if you want; yeah it is not hard to do, an academic lab can do it and we have written up the protocols.

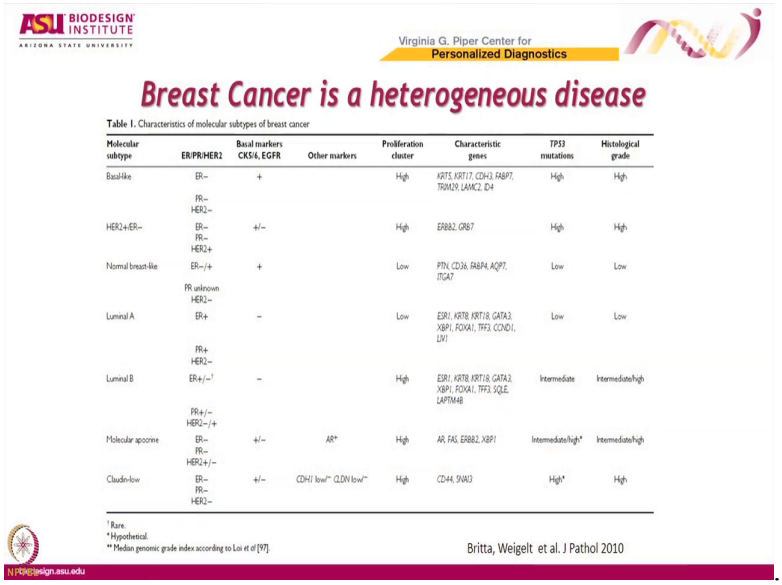
Student: What are the working criteria for affinities that are required for this device to work?

You know I think they are kind of anywhere between certainly anywhere up to I would say 100 nanomolar maybe; if you use SPR, you can get up to micromolar affinities, but anything below that any tighter infinity then micromolar, you can detect on the arrays.

Student: We can.

You can, yes. So, micro molar is a little iffy, certainly anything sub micromolar you can detect yeah, all right. So, we moved on and I mentioned to you before that, we know that breast cancer is a heterogeneous disease; that means, that there is multiple subtypes.

(Refer Slide Time: 05:05)

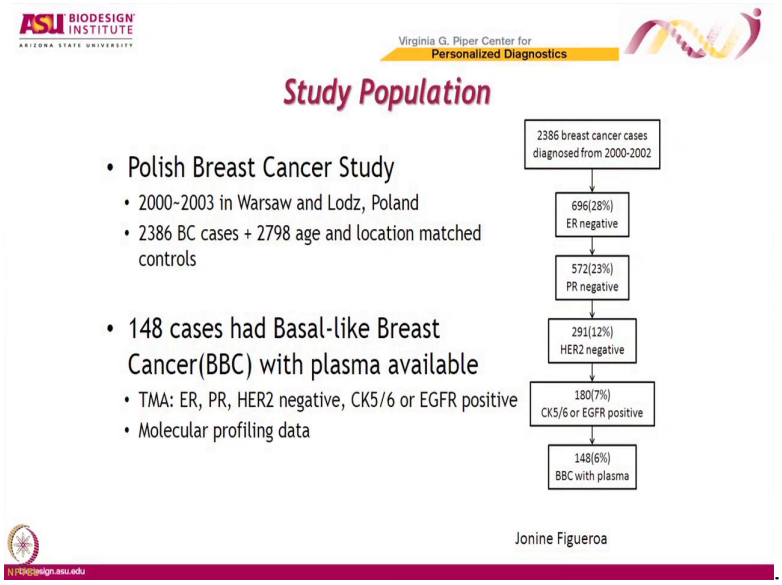


But there is the basal-like subtype, the HER 2 subtype, luminal A, luminal B, Claudin like low. So, these are all various subtypes of breast cancer. And we were especially interested in this one here; the basal-like breast cancer, because it is estrogen receptor negative, progesterone receptor negative and HER 2 negative. It also tends to have either EGF 4 or cytokeratin positive disease.

And this particular type of breast cancer is very lethal, it tends to occur in younger women, tends to occur in women of color and it is often not detected well by mammography. And so, all those features make it a disease that would benefit from early detection.

Because it does not have estrogen receptor and it does not have HER 2 receptor. A lot of the drugs that we have today like Herceptin and the different inhibitors will not would like to mock to on the natural thing; will not work on that disease; because it is it the companion diagnostic is negative, right. So, we thought would not it be good if we could get some markers for that disease. So, to do that we collaborated with Jonine Figueroa; she was at NIH at that time and she was running this large polish health study.

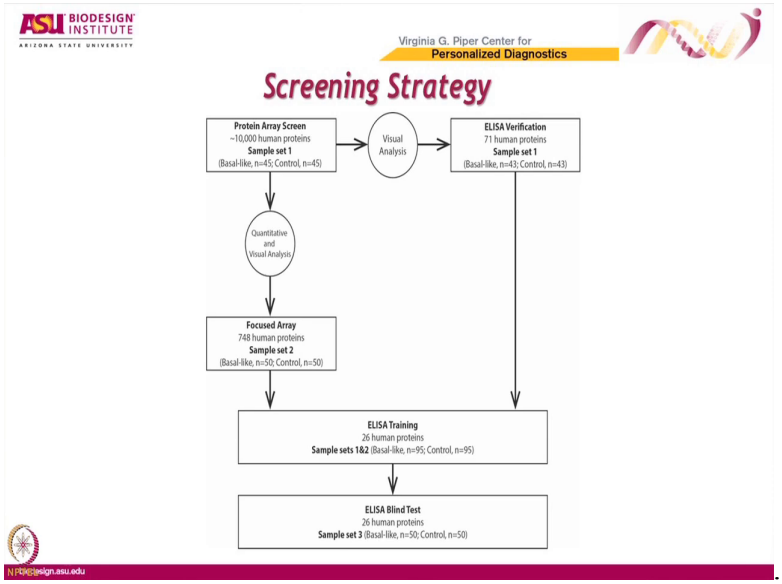
(Refer Slide Time: 06:31)




This study had roughly 2400 breast cancer cases in Poland along with around 28 hundred age and location matched controls. So, we took her study and we sorted it for women who are estrogen receptor negative, progesterone receptor negative, HER 2 negative and who had either CK positive or EGFR positive. And so, from all of these we got down to around 148 cases of true basal-like breast cancer.

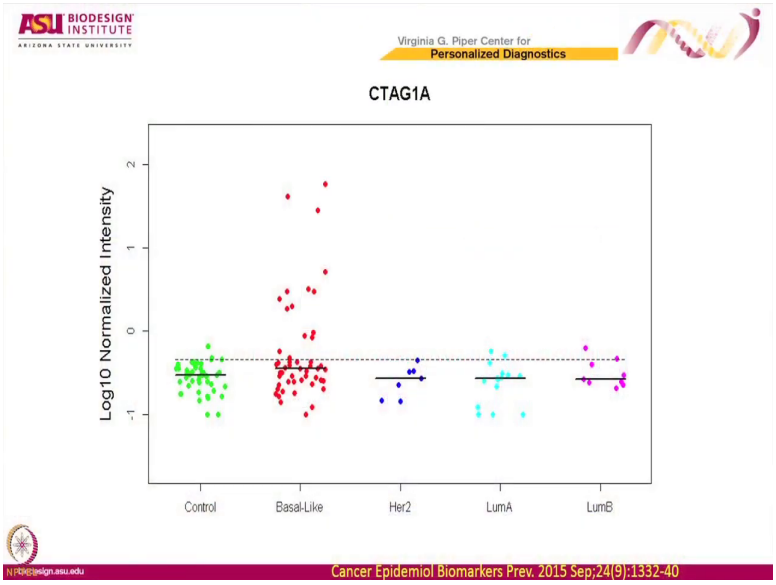
And then she was able to get us 150 age and age-matched controls, ok. So, I will not go through the details of the study design here; but I will point out that we did very much the same that we did last time.

(Refer Slide Time: 07:22)



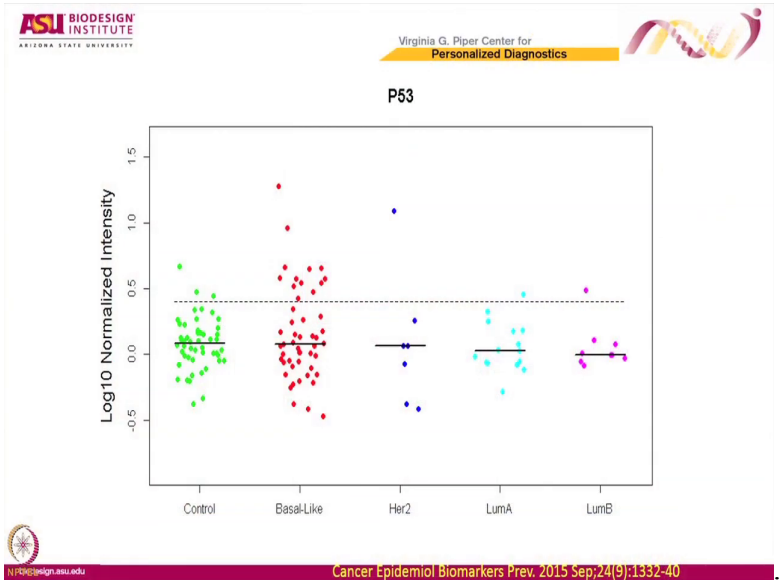

So, we had this sort of multi-phase study, where we you know did basically did two stages of discovery and then verification. And then did a third level of validation where we tested a set of 26 proteins on a sample set that had never been seen before, completely independent samples and we did that blinded. And it turned out that we did not get a lot of markers that held up all the way through, but we did get a couple.

(Refer Slide Time: 07:04)



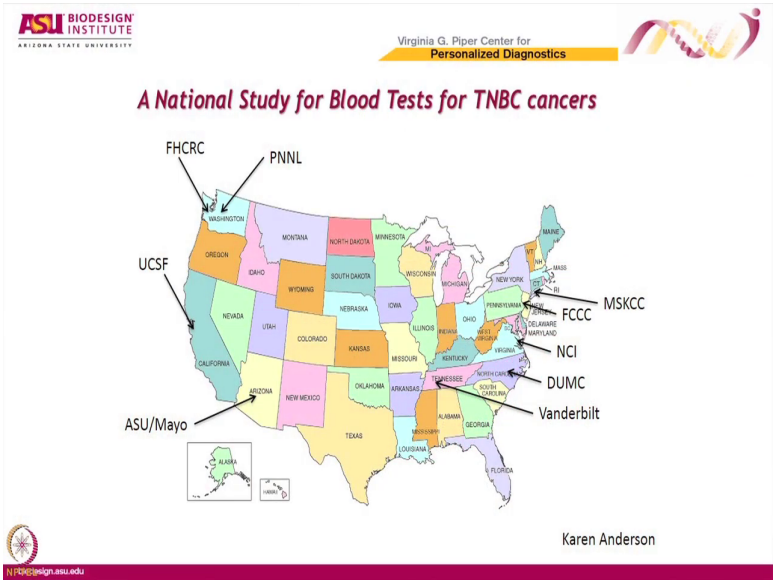

This is CTAG1A and you can see these are different types of breast cancer luminal B, luminal A, HER 2 these are control women who are healthy. This is the cutoff value for the test and you can see that at least for the basal-like there are definitely a number of responders here. Now, not everybody is a responder, but all of these were responders, right.

(Refer Slide Time: 08:17)



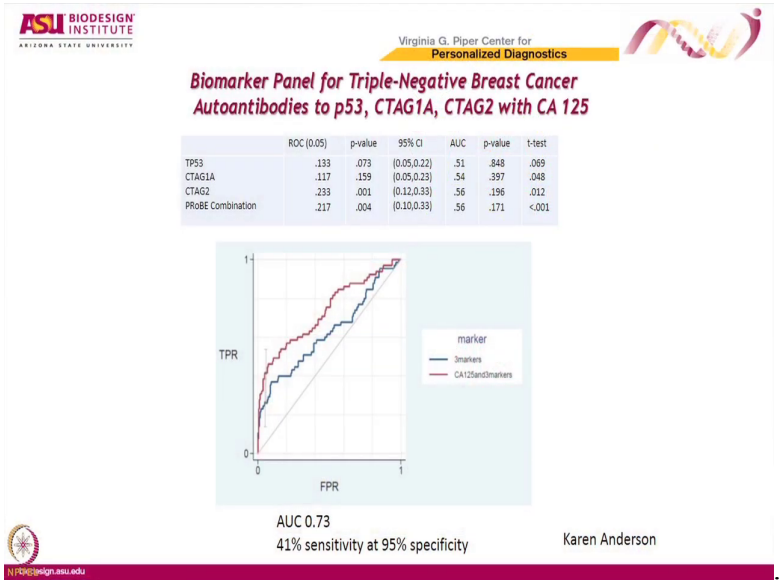

Here is another protein you may have heard of P 53 before, it turns out that it is a good auto antibody protein and again it was particularly positive in these basal-like breast cancer cases, ok.

(Refer Slide Time: 08:29)



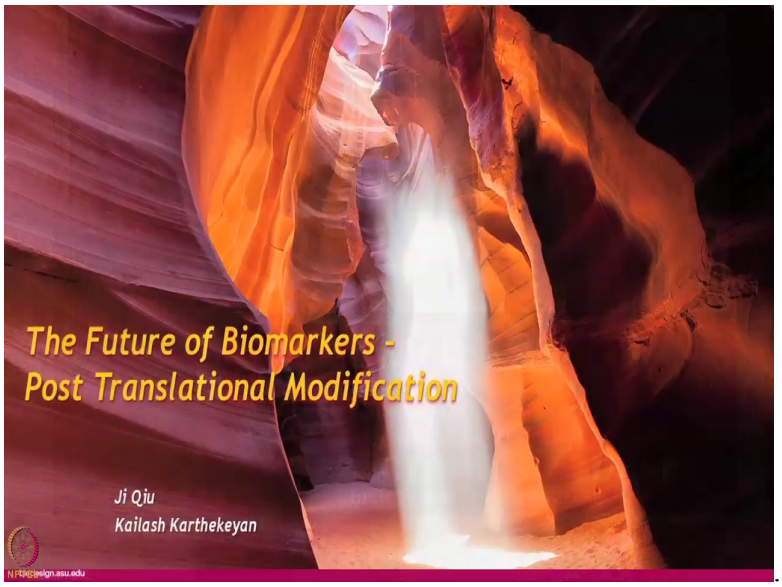

So, we actually participated in national study to see how these markers held up against other markers for triple negative breast cancer. This is through the early induction research network at the national cancer institute in US. There were I think 14 different laboratories have participated in this study; it was fully blinded study, so that all the participants were given samples that were unmarked and asked to test them and predict cancer for them. There were over 80 markers tested and after all of that and all the studies that were done; the only markers that held up were our markers, the antibody markers I just showed you and those markers well there they are.

(Refer Slide Time: 09:14)



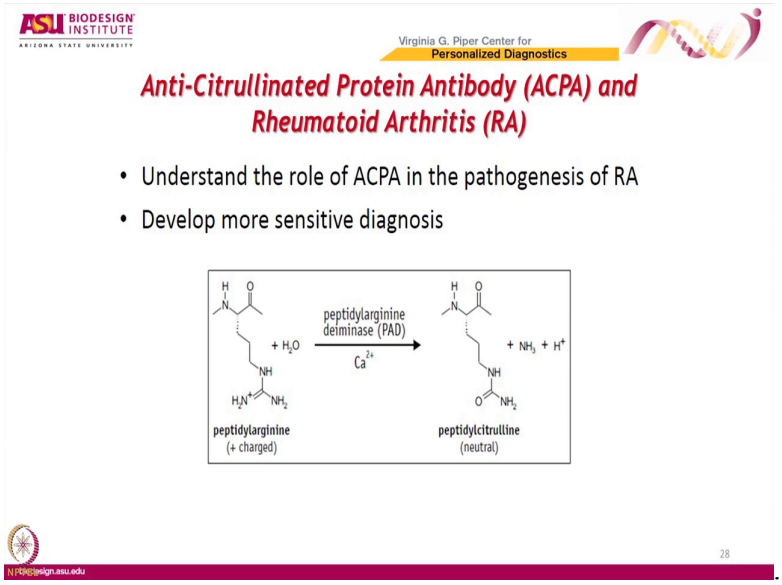

So, p 53, CTAGA and B; here is the AUC curve, we did not really talk about AUC curve I think you are going to get that soon when you get your statistics lecture, but this gives you some flavor of that. And then we added that together with CA 125 you get a slightly better performance here in red. And so, this right now I think is the best blood test for basil cancer, ok. I am gonna any more question any questions on that, ok. So, I am gonna enter the last section of the talk.

(Refer Slide Time: 09:49)



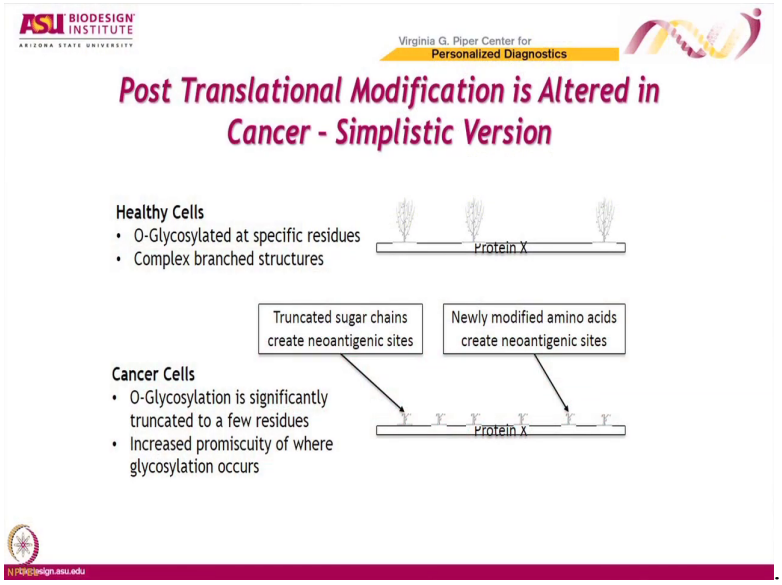

I am going to talk a little bit about where we are headed with breast cancer studies and we think that the future of biomarkers is looking at post translational modification.

(Refer Slide Time: 09:59)




So, here is one form of post translational modification what is going to come back later. This is called citrullination it is when you take an arginine and you treat it with this enzyme peptidylarginine deam deiminase and it converts the pep the arginine into this thing called citrulline and that this is the citrulline molecule here. So, you get this ketone here.

(Refer Slide Time: 10:26)




So what we mostly have been interested in is glycosylation. So, you guys are all familiar with glycosylation, right. So, what fraction of proteins in the human do you think are glycosylated. I heard someone say 70; certainly more than 50 anyway, I do not know if it is as high as 70 it could be, but certainly more than 50 to 60 percent of proteins have sugars on them. So, it is very common and you know that like glycosylation occurs N linked and O linked. And today I am only going to be talking about O linked glycosylation; mostly because most of the studies done in cancer have been done on O linked glycosylation.

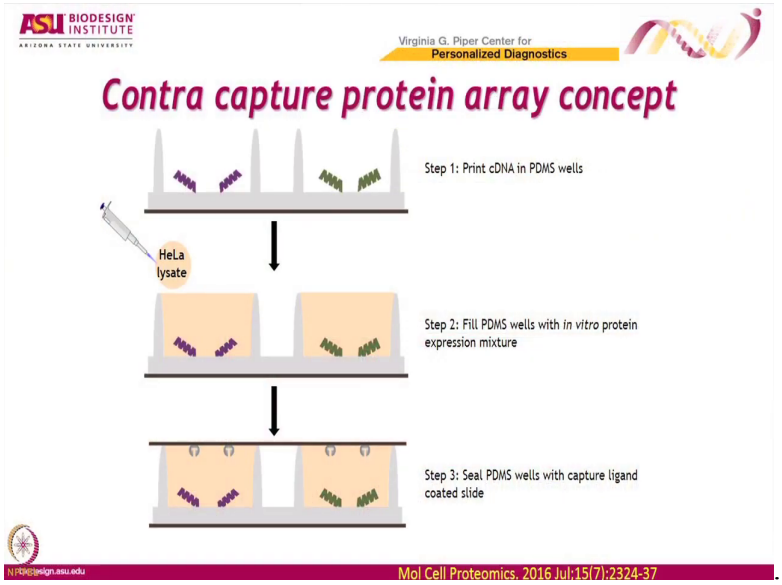
So, typically what happens with like glycosylation is you have these sugars attached to specific amino acids and they form these very complicated branch structures, right. So, they have this you know many many many sugars stacked upon one another that is pretty typical in normal cells. In cancer two things happen; the first thing happens is that you get truncated sugar structures. They get truncated, because there is an enzyme called cosmic that is missing

and that is the one that only adds the sort of fourth level sugar. So, you get three levels and then it sort of stops.

So, it is like getting a crew-cut, it gets very very short hair, right. The other thing that happens in cancer is it gets more promiscuous. And so, instead of only a couple of places on the protein, you start adding sugar at multiple places on the protein. So, to someone like me that looks like an opportunity right; because there are two things now that are different about these modifications. They create two new shapes that could induce an immune response; the first is that you get these short sugars and so the immune system might see that as different from normal, and the second is that you now have amino acids that are modified, they did not use to be modified.

So, both of these are potentially inducers of an immune response in it and that would mean that for me, they are an opportunity to look for new auto antibodies. So, to get our platform to work well with looking at post translational modification, we had to clean up the platform a little bit.

(Refer Slide Time: 12:51)




And so, consequently, we developed this method here which is called contra capture. What we do is, instead of printing on a our DNA on a glass slide like the way you are doing it right; we print our DNA in these soft wells of PDMS membrane. And then if we put the DNA in these wells, we add the HeLa the lysate to make the protein and then we cover the well with a glass slide that has the capture agent on it.

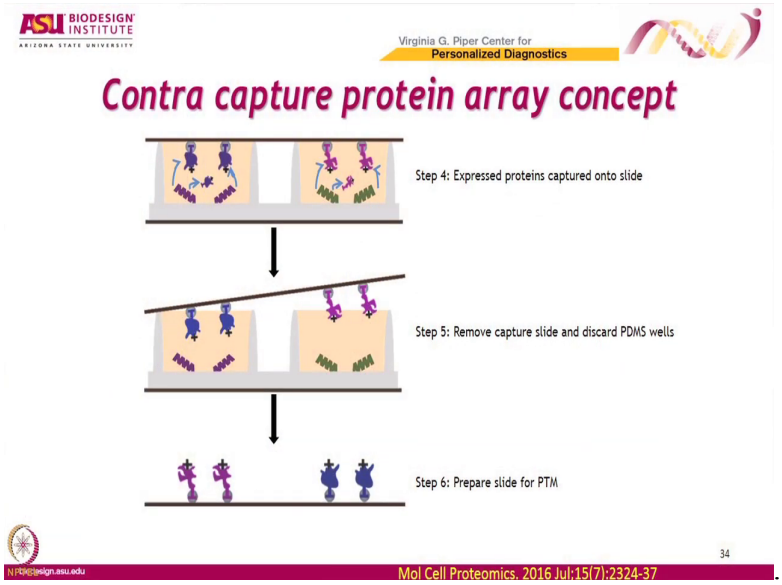
So, we separate where the capture agent is from where the DNA is; you know you normally at NAPPA you print the capture agent and the DNA at the same place. Here they are on opposite ends of the sandwich. The other thing that we are doing here is, we are not using anti GST anymore we are using the halo tag. Are you guys familiar with halo tag, how many people know what halo tag is? Not so many. So, halo tag is it is an enzymatic tag that it is an enzyme that normally is supposed to bind to halo alkanes that is you know alkanes are constructed of

halo alkanes have a chloride at the end or a bromide at the end and the enzyme in it is normal function binds to that and removes the bromine or the chlorine.

In the case of the halo tag, the enzyme has been mutated, so that it binds to the chloride; but it cannot remove it. And because it binds to the chloride in a covalent attachment, it now becomes stuck, permanently stuck to the chloride. So, an essentially way of causing a protein to form a covalent linkage with a specific tag, the reaction is very specific. And so, you can selectively pull one protein out of a mixture of millions by having halo alkane on the end of it.

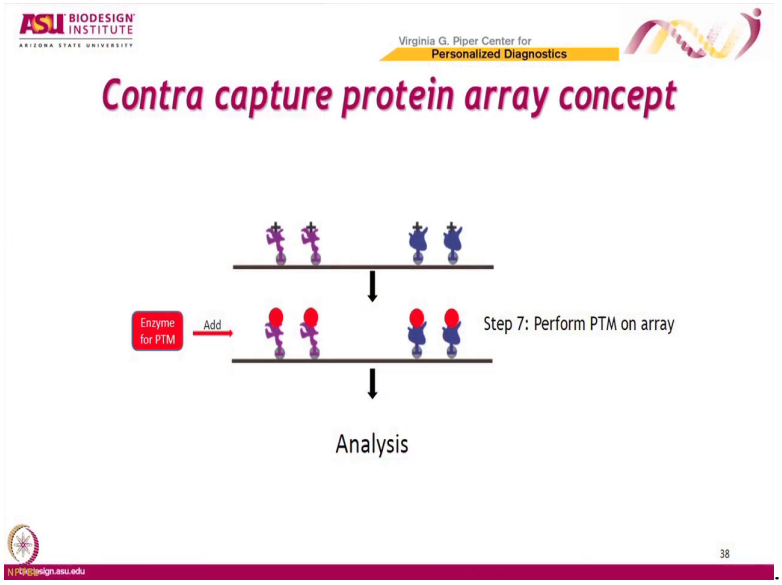
So, we put the halo ligand on this glass slide and the proteins that we are producing have the halo tag. And so, when they see the glass slide, they form a covalent attachment to the glass slide. What we like about a covalent attachment; of course, is it is much more permanent, you can watch it really hard and will stay stuck, all right.

(Refer Slide Time: 15:18)



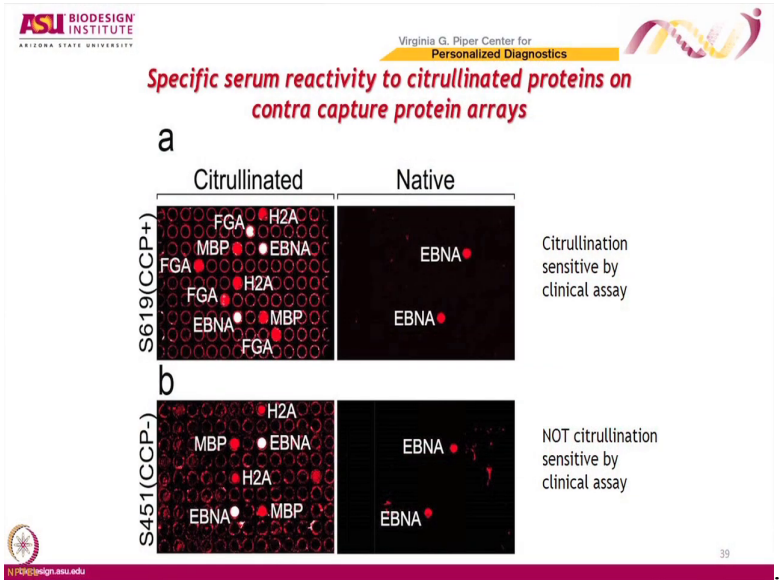

So, then we expressed the protein; the protein goes up it links up into the halo tag up there where it forms a covalent linkage, we lift the cover slip off the well, right. And now we can throw this stuff away and we are left with a glass slide that has these proteins covalently attached to them. And they are absolutely clean; there is nothing on this slide, but the tag and the protein. So, now, we can come in with an enzyme.

(Refer Slide Time: 15:49)




And we can add a phosphate group, a sugar group whatever we want to add and we can modify the proteins and then study them.

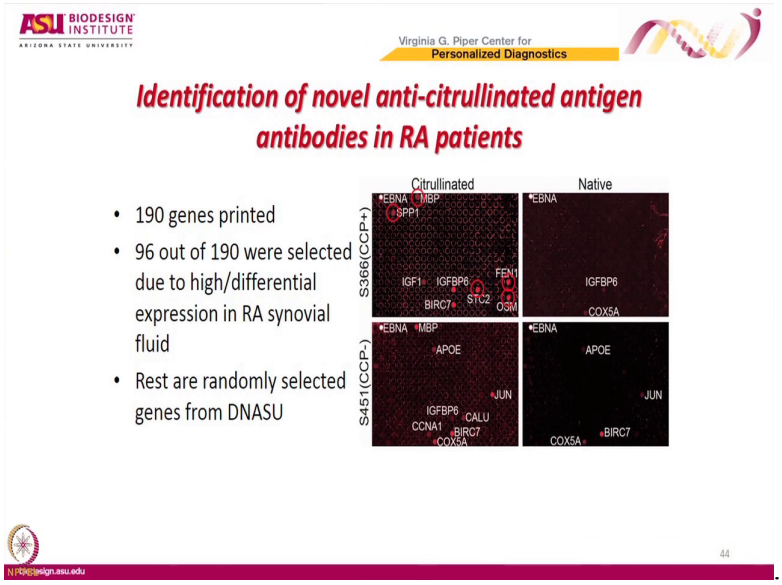
(Refer Slide Time: 15:59)




And I will just show you a couple of examples; here is some NAPPA arrays, here remember I mentioned the citrullination, here we either citrullinated the array or we did not citrullinate the array. And here is a patient blood, this is a patient who happens to have rheumatoid arthritis. In the case of rheumatoid arthritis patients make antibodies against citrulline. And you can see that this patient has all these antibodies to citrullinated proteins; but does not recognize the proteins when they are not citrullinated. You will notice that we have this EBNA protein that is our positive control.

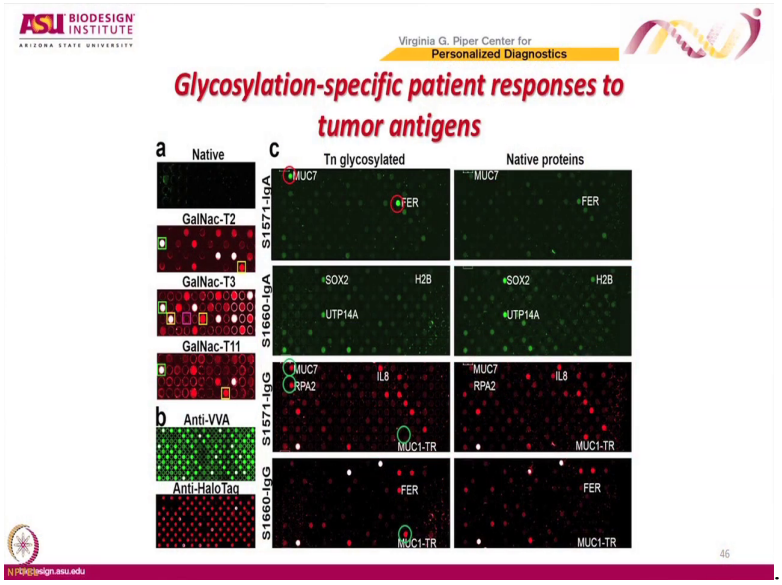
So, that is a different antibody and not socially need rated right related and yeah; it shows us that the this the assay was working, but not taking all these other proteins. Here is a bunch of others this other patient same idea and again only signal are present in a citrullinated array.

(Refer Slide Time: 16:52)




Identifying new interactors based on the citrullination; here you can see some responses here, they were not previously known and again their citrullination positive or specific.

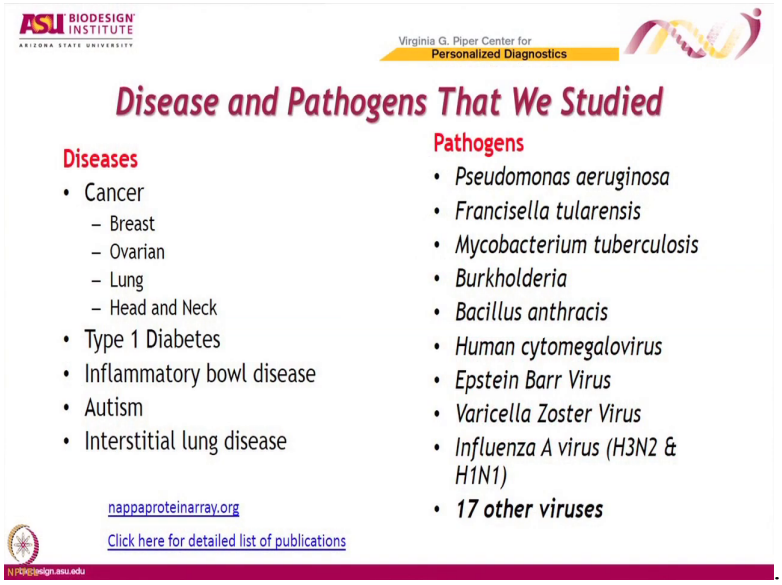
(Refer Slide Time: 17:13)




This is the same general approach, but now we are doing with glycosylation. So, here we have added some sugars to the protein. And again these are different patient's cyra. And if you look carefully, you see a strong response when it is like glycosylated, but not when it is not.

Here is a couple of responses that you see on there unlike glycosylation, they are not present when they are not. And so, and here is another one down here. So, the hope is that these responses will give us a new opportunity to find biomarkers for specific disease; because of the profound differences in glycosylation that occur in cancer, ok. So, these are some of the diseases that we have studied using the NAPPA.

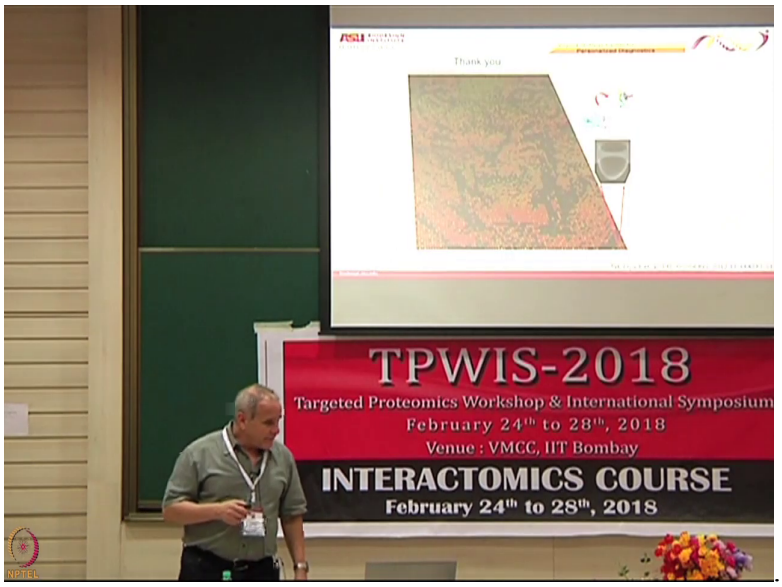
(Refer Slide Time: 17:55)




I have talked a lot about breast, we have also done ovarian and lung and also head and neck, all of these that we have identified some markers that have been published over the years.

Type 1 diabetes is an autoimmune disease and so, we have looked at that. We have looked at inflammatory bowl disease; we recently published a paper there. We are looking at autism that is early stage and also interstitial lung disease. And then we have looked at a series of different pathogens over the years, this one we just published earlier this year and actually now we are increasing the list of viruses dramatically.

(Refer Slide Time: 18:32)




So, with that I am going to stop for today, I can take questions.

Student: In your list couple of your earlier lecture questions probably related on (Refer Slide Time: 18:45). So, when you are taking your protein on site, how to you prevent your protein that is translated from the it gets translated from one catalyst from going and binding to the anti GST with another.

Right, so how do we avoid diffusion? So, I my last lecture I will probably talk about that a little bit more. You do get some diffusion. At the spacing that we do on the arrays and the size of the features that we create, the amount of diffusion is pretty limited; it is about we actually measured it, it is about a couple percent compared to the spot itself, so that immediate neighbors will have around 2 percent of what you would have a signal.

So, we thought that was low enough that we could get away with it, where it becomes an issue is if you try to make the arrays at a much higher density and move the spots closer together, then you start to see more diffusion to neighboring spots. And so, when we get to that stage, we have induced a different technology that involves creating Nano wells.

Student: With so many markers that are found for breast cancer, is there a reason why breast cancer is more prevalent in US compared to for example, in India.

That is a good question. Well first of all I do not know the numbers here; certainly there is there could be a number of factors certainly there could be risk factors that are different in that population than this population. It is also possible that it is detected more often in the US, possibly because of more aggressive screening programs; which may mean that like I do not know what the overall per capita mortality is from breast cancer in India versus the US. Does anybody know that?

Student: Kind off happens more there as compared to here.

Yeah, well it is different from mortality or just the disease. Because one of the issues that people raise a lot with breast cancer not unlike prostate cancer, is are we detecting disease that does not need treatment. So, as you know in prostate cancer that is a big issue; because of the use of the prostate specific antigen, we were detecting a lot of men with prostate cancer, who will die with prostate cancer, but not because of prostate cancer. And so, they end up getting treated when they probably did not need to be treated.

And so, that may be true, people have argued that is true with breast cancer as well; that we may be over diagnosing it in the US and we do not really know yet. So, I yeah I have to look at the statistics; but I do not know offhand that any major differences, but I could imagine a number of them.

Student: Sir, can we walk back from biomarkers as to why this happens and?

I do not think the markers would help us in that particularly. Mostly in part because these markers were developed in the US, right. So, they were based on a population in the US. I do not know if that would necessarily tell us about breast cancer here; they might, but we just do not know, yeah.

Student: One more question about cell free.

With what, yeah?

Student: Cell free.

Yeah.

Student: Why is the that they used wheat germ, the rabbit cell free, the reticulocyte they are not very stable, they do there own folding issues?

Oh.

Student: Why is there not an extremophile cell free expression system?

Oh interesting question well, yeah. So, I couple of points there; first of all I should just make the point that we do not use wheat germ, we use human cell free. We do that because we are looking to make human proteins and we think better chance of getting good folding. That said all of the cell free lysate tend to be a little finicky, they definitely are. You they, if when you think about what they are doing, they are very complex; within that tube you have everything to do both transcription and translation.

So, you are asking a lot; first you are making up you have a promoter binding, transcription factor to produce RNA, then you have to have ribosomes bind to the RNA, tRNA is recruited, amino acids added and then you have to have an energy generating system. Because as you

know translation requires ATP usage and then you have to have chaperone proteins present to fold the proteins in the natural folding.

So, you are asking a lot, and to get all those components in a single concentrate that works; it does not entirely surprise me that it is temperature sensitive and that it is fragile. That said believe it or not, you can lie awful lies the cell free lysate from bacteria. So, you make it a powder, you can add water and make protein from it. So, that is pretty stable.

Student: But the efficiency is very low.

Efficiencies is low and it is not good for large proteins. So, would not be my favorite choice by far; but it I think it does not surprise me that such a lysate would be a little bit sensitive. It would be cool to get extremophile to do that, I do not know any one is tried.

Student: Breast cancer that grows on; breast cancer is growing in India; but the problem is that it occurs decade earlier in Indian women compared to most of western country.

Ok.

Student: Do not know whether it is (Refer Slide Time: 24:31).

I yeah I do not know.

Student: Same the same Leukemia, chronic myloid leukemia; we are getting I am doctor especially I am working with the leukemia (Refer Slide Time: 24:43). So, I know that there are very young cancer patients who are coming with you know chomic myloid leukemia that is the picture is different in US coming at the early (Refer Slide Time: 24:56)

Perfect CML.

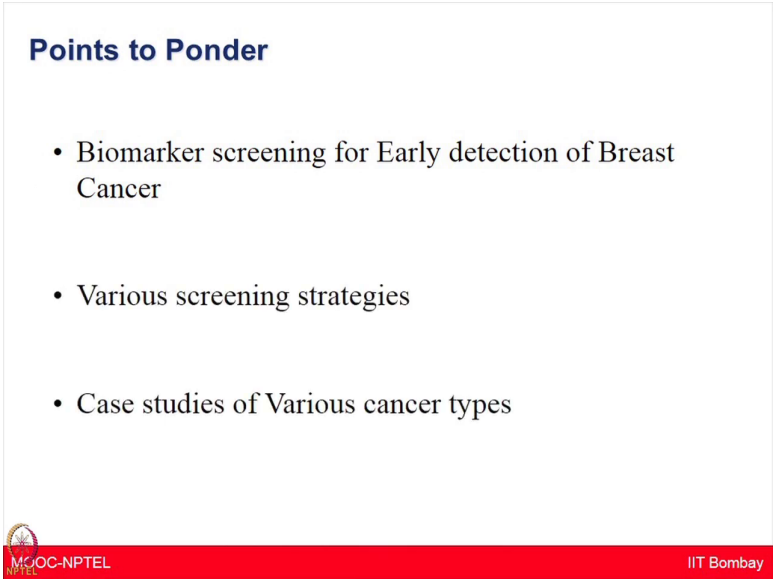
Student: CML

Yeah that is interesting about, I do not know, I mean usually when you get cancers in young people that is a sign of either translocate genetic translocation.

Student: Abnormalities.

Yeah chromosomal abnormalities, because that is a pretty young age to have just sporadic mutation, ok; I think we are done.

(Refer Slide Time: 25:25)




I am sure you have enjoyed both these lectures delivered by Dr. Joshua LaBaer. Talking to you about utility of a technology especially NAPPA technology platforms; the insights which

are required by performing these assays, your details for testing the reproducibility, thinking about accelerator design and then finally, the outcome which one could obtain from these experiments can be so remarkable which could be utilized for the patient care.

You must have understood the clinical significance of a rejection of biomarkers; you also studied about the biology of cancer in some detail and the tests that are now being used in the clinical trials. You are introduced to the concepts of contra capture protein arrays which can be utilized for studying post translational modifications. And you also got a glimpse of how various diseases could be studied using NAPPA technologies.

In the next lecture well continue our discussion about use of novel technology platforms for various biological applications. And you will have series of interesting examples and illustrations to convey the utility of these technologies, at the same time what entails to obtain the success from these experiments which is your careful experiment, your quality control checks, your data analysis and your insight and understanding about how to make a meaningful biological experiment. We will continue these in the next lecture, till then.

Thank you.