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# CBER SCIENCE SYMPOSIUM

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P R O C E E D I N G S

(9:31 a.m.)

DR. ATREYA: Good morning, everyone. Welcome to the third and final day of the symposium. This morning, we have four speakers, and the session is Methods and Biomarker Discovery for Product Safety and Quality.

My name is CD Atreya. I am from the Office of Blood Research. Today, the session has -- each speaker has -- the first two speakers have 35 minutes and the others have a little bit less time. So the first speaker today in the morning is --

So the first speaker of this morning is Dr. Dan Huh. He's a Professor in the Department of Bioengineering at the University of Pennsylvania. He's a pioneer of organ-on-a-chip technology. And his research group at Penn focuses on developing micro-engineered models of human physiological systems for biomedical applications.

Dr. Huh won several honors and awards.

The details are in the book that we are distributing. To name a few, he has the NIH Director's New Innovator Award and also the Distinguished Achievement Award from the University of Michigan.

And the second speaker is the session's keynote speaker, Dr. Sathy Balu-Iyer. He is a professor in the Department of Pharmaceutical Sciences at SUNY University at Buffalo, and he is an Associate Dean for Research School of Pharmacy and Pharmaceutical Sciences.

He's an elected fellow of the American Association of Pharmaceutical Scientists. His interdisciplinary research involves biophysical, immunological, and pharmacokinetic and dynamic approaches to rationally developing immunotherapy modalities.

And he has over 100 publications. And he's an inventor on patent on 30 applications. And that is what it is. So with no further ado, the first speaker, Dr. Dan Huh, welcome.

DR. HUH: All right. So let's see. I

see over 200 people online, so I just want to make sure that I share my screen. It's on the slide share mode.

Okay, awesome. Good morning, everyone. So, I'd like to start by thanking the organizers for the invitation to speak about our work today. And I'd like also to congratulate them on the great turnout of this meeting.

And so I have a lot to cover this morning, so I'll just get right to it. At Penn, I lead a research group called Biolines. Here's a photo of my lab. I have about 14 people working in the lab, equally split between grad students and post-docs.

I would say we are problem and question-drive lab, but we identify ourselves as a group of bioengineers working on a technology known as organ-on-a-chip.

So, I'm assuming many of you are familiar with this term, but just to get everyone on the same page, what we do in my lab basically revolves around this idea of creating

microfabricated chips for cell culture, but we also use these systems to provide the cells with the type of environment that they would like. They would feel at home, if you will, so that they do what they're supposed to do.

So one way to think about it is that we try to trick the cells into thinking that they're sitting in the body so that they build themselves into complex tissues and also recapitulate their native functional capacity.

So our goal is oftentimes to engineer these realistic human tissues that can be used for a variety of applications. So in this approach, my lab at Penn has developed a variety of human tissue model systems over the last 11 years or so, some of which are shown here.

So for this talk, two quick things to point out. The first thing is, 85 percent of what you're going to see today has not been published. So these are very new results I'm going to be actually presenting and also represents what's happening at the very forefront of research work

happening in my lab, which I think it's going to be good.

And I would like to organize the discussion around these three questions we've been thinking about, we've been asking ourselves a lot over the last five years.

So the first question is, as you can see there, how do we make these organ chip model systems more realistic, more representative, of their deeper counterparts.

The second question is, what kinds of information can we really get from these model systems, and what's their value? And can we improve the capacity of these model systems as data generators?

And lastly, I'm going to be talking about how we can make this technology practically more useful.

So let me get to the first question. So I have to say, the complexity of the realism of the model is always dependent upon the question or problem you're trying to address.

But as those problems and questions we try to deal with in biomedical research become more complex, I definitely see an increasing need for more realistic complex model systems. How do we achieve those?

So, if you're new to this technology, one of the first questions you could ask is, how do we actually make these model systems? How do we go about designing and creating these organ chip systems?

So let me just spend a couple of minutes, just maybe one minute, talking about the general design principle we as organ chip engineers follow.

So obviously it's not the poor organ represented by these chip systems. It's really the functional elements that we try to recreate. So once you have your -- once you have your target organ, identify its functional units, then you go in and take a closer look at the system to figure out what type of cells there are, how they are organized with each other, and most importantly,

what kinds of environment they are sitting in.

And in the last step, you would actually create a chip that would align to mimic those essential features.

It's easy to say this but this is a step where engineering expertise and ingenuity actually really come into play.

So just to give you a quick example, so let's say, let me make this up. So you are interested in developing an eye model, eye-on-a-chip, to test some therapeutics, ophthalmic drugs.

So in that case, your target organ would be the eye, and the functional element of the eye would be the ocular surface, as all of you know, that consists of cornea at the center surrounded by the conjunctiva.

Then we take a closer look at the structures and cell types. And so the corneal tissue, let me just use that laser pointer here, consists of this stratified epithelium supported by collagen rich stroma containing these codocytes

and conjunctival epithelium contains these goblet cells that produce a mucus, which is one of the key components of tears.

What about the environment? So what I'm showing here is eye blinking, which I think is a very unique environment for these cells and tissues, which important for spreading the tears to form this tear film that serves to hydrate the surface and also forms a smooth reflective surface for light transmission.

So to mimic those features, what my lab has done in the past, actually, I'm showing this an example, we created this in a device and use this 3-D scaffold that is highly porous that looks like contact lens to inject a mixture of collagen gel and codocytes in to the cavities of the scaffold.

We also came up with a way to deposit these two different cell types to mimic the concentric tissue pattern.

And we can also grow these tissues and differentiate them under physiological conditions,

for example, exposing the cells to air, and as a result of differentiation, they become stratified, form tight layers, and they form these beautiful microstructures that are almost identical to the features of these microscopic features seen on the surface of the human eye.

And once you engineer those systems, you can create a system like this where this very soft elastic ray is actuated by this motor to mimic blinking.

So using this blinking actuation, we can form a very thin tear film, as is shown here in this OCT image, and in this paper we also use the system and turn it into a disease model, dry eye disease model, and we work with pharmaceutical companies to test their investigational compound.

So my apologies for going through these very quickly, but there's a reason to do that. So I will say we now consider this as a very traditional approach to creating organ chip systems.

What I mean by that is, even before you

create this model, you have a very good idea of what type of cells they are going to be and where they're going to be.

So it's pretty deterministic in that sense. But as we start thinking more and more about creating more realistic complex model systems over the last five years, we actually are now exploring a new approach where we use embryogenesis development tissue and organ development as a source of inspiration.

So what I'm showing here is respiratory system development happening during embryogenesis of [INDISCERNIBLE 00:09:42] embryo. And it's amazing how these stem cells and presenter cells can do this through this process of self-organization and face specification and so on.

So can we at least try to mimic certain aspects of these very dynamic processes happening in people?

So the first example I'm going to actually present today is a model of human marrow,

more specifically hematopoietic vascular niche in the marrow.

This is a new paper coming out in Cell Stem Cell. So in the interest of time, I'm going to skip the introduction, but as many of you know, bone marrow is one of the organs that develop the last during embryogenesis.

And it's known that these hematopoietic stem cells arise from hemogenic sites. And they move around a lot through different anatomical sites receiving signals needed for their expansion and maturation.

But eventually, they migrate into the medullary cavity of the bone and they colonize this compartment. But it's also known that this process happens internally with the development of these assigned soto blood vessels in the same compartment.

So the question was simple. Learning about this, we ask ourselves, would it be possible to mimic this concurrent process of HSC colonization in vasculogenesis in an engineered

system?

So the first thing we had to do was to figure out ways to engineer realistic, sign soto blood vessels. And so long story short, this is actually one of the platforms and devices that we use.

This is a cutaway view of this device. It's got three compartments and there are these protrusions from the ceiling that partially actually separates these three compartments.

In this design, one interesting thing we can do is to inject liquid into this air filled channel, and because of surface tension, liquid is pinned along the center lane, if you will, due to surface tension.

It's a pretty well known physics phenomenon called capillary pinning effect, as demonstrated in this movie. So using this technique, we would inject a mixture of ECM precursor solution, hydrogel solution, with fibroblast and vascular endothelial cells.

We would actually gel it and fill the

side chambers with media, feed the cells, and we would also feed the side chambers with endothelial cells. Seeing this is a 3-D culture condition, something really interesting happens.

So all these endothelial cells in the gel, in the presence of all these supporting factors and ECM produced by surrounding fibroblasts, they self-assemble into these 3-D vessels that open to the side channels.

So let me show you a movie. I hope the movie is not too choppy on your end. This is actually a sped-up movie that happens, a birds eye view of the device.

So these are RFP expressing endothelial cells cultured in fibrin scaffold in the device, and as you can tell, over a period of five days or so, they've developed this vascular network in this device.

As a result, we can engineer very realistic blood vessels that are very similar to human micro-vessels. I'm just going to slide through these.

And in this image, actually, we used GFP expressing in those in the side chambers. And what you can see at this interphase is that the endothelial cells in the side channel actually form endothelial sprouts into the gel, and they end up connecting with the red blood vessels in the gel. And as a result of this anastomosis, the entire vascular network becomes accessible and profusable by using these two side channels.

We can also see a flow of blood cells of different kinds. These are actually really fun movies to watch.

So going back to this marrow model, we actually used this approach. And what we did was we added CD 34 positive human hematopoietic stem and presenter cells into the mixture. We let them kind of self-organize.

It took us about six months to figure this out, but under the right conditions, they self-organize into these very complex tissues that kind of resemble hematopoietic vascular niche, or the environment, in the marrow.

So just to kind of demonstrate this organ specific function, depending on media conditions, these HSPCs can generate erythrocytes. So we see the formation of these restored blastic [PH 00:09:46] islands, device affluent contained in these cells that look like red blood cells.

And also, we are able to induce the formation of myeloid cells, granular sites. We got to really locate it close to these blood vessels. And we found out that many of these cells are functionally mature neutrophils in the device.

But very importantly, we are also able to maintain a very small fraction of those stem and presenter cell population with colony forming activities actually in this system.

So, one of the things we can do is to harvest these tissues as a whole, living or fixed tissues after the experiments for further analysis.

So, we actually use this capability to do single-cell sequencing to really dive into

cellular heterogeneitian complexity. We would actually start with just three or four different cell types, but as a result of HSPC differentiation into these different lineages, we would get more than 10 different cell types.

And we can also look at cell/cell interactions through by performing ligand receptor interaction analysis and so on. In the interest of time, I'm going to actually have to skip this, but I'm happy to tell you more about these results offline if you're interested.

So when these granular sites are generated under myelopoietic conditions, they actually look -- they are situated in the vicinity of the vessels. But when they are stimulated with pleural and vent OIN cytokines or drugs, they have the ability to intravasate and flow out of the device through the vasculature.

So we actually use this capability to build a multi-organ system that contained marrow connected to an area on a chip. So this is actually a photo of the device. And the area on a

chip contains three compartments, epithelial compartment with the lining of epithelial cells that are fully ciliated in mucus producing cells.

The middle compartment was filled with the fibroblast containing hydrogel. And the bottom compartment was lined with vascular endothelial cells to mimic the vasculature.

So one set of experiments, what we did was to infect the epithelium to mimic airway infection. We used pseudomonas. So as you can tell, we saw compromised epithelial barrier. But very importantly, these cells produce a lot of cytokines and chemokines. And because those two devices were fluidly connected, all these factors floating to the marrow, and actually they induced massive release of cells actually from the marrow chip.

And many of these cells, vast majority of these cells, were mature neutrophils that are CD 60 positive. And we also saw the number of cells going in was much larger than the number of cells coming out.

So we thought something was going on in the device. And so we went in and used the scanning electron microscopy to look at the events.

So as you can see here, on the vascular side, these neutrophils that stick to the vascular endothelium, and then they undergo transmigration. On the epithelial surface, they come up and then they move toward the bacteria and they kill them. We saw mitosis as well as neutrophil swarming happening in this engineered system. So I have to point out the fact that scientifically, there's nothing new. We've known about these things for many decades, but being able to mimic this complex cascade of events in this engineered system without, that was something.

And in collaboration with GSK, we actually succeeded in scaling up this model using a robotic system. I'll actually talk about this toward the end of this presentation to look at myelotoxicity and myelosuppression of commonly used anti-cancer drugs.

So I'm happy to tell you more about the results. So this actually, the project started as part of the Tissue Chips in Space program. On the way up in our first launch, the flow regulator was short circuited. So it was an epic failure. But thanks to this project, we were able to build this really nice model that we are now using for a variety of other applications.

This idea of creating more realistic complex model systems would not be complete in my mind actually without talking about, thinking about organoids.

So about five years ago, I was asked by a senior editor in science actually to write a review or prospective article to talk about how organ chip technology can be used to advance the field of organoids.

So, long story short, this paper talks about how the tightly controlled micro-environment of these micro-engineered devices could be used to build more functionally mature actually and more in vivo like organoid-driven tissues, and with a

higher predictive capacity.

So, I don't think I have a lot of time actually today, but OCTOPUS, this is actually a platform we demonstrated a couple of years ago to kind of highlight the promise of this approach.

And so, this work was motivated by a lot of failures and difficulties we as engineers faced when we were actually getting our foot in the door. So, as you can see here, this is one of the ways in which intestinal organoids can be aerated by embedding stem cells in Matrigel drop.

So we did it, and we tried this method and we saw these beautiful mini organs forming in the Matrigel scaffolds. But over time, they die off. And it starts at the core, and it propagates through the entire gel drop.

So this is the reason why we learned the hard way. People stop at day seven or day six and they passage them into new drops. But by doing that, if you think about it, the flip side is actually you have to constantly perturb their process of development.

So they really want to be sitting in the same gel maturing and growing, actually. So we thought this is a consensual representation. Wouldn't it be nice to eliminate this hypoxic core? You're left with this shell without any divisional limitations. You would then radially segment the shell to lay flat to minimize division limitations.

So this is actually the device that we came up with to achieve this goal. So basically, it's actually, it was designed as a tissue culture insert with a sensor access port and with eight radially open chambers.

And just to demonstrate the ease of use, let me play this movie. So all you have to do is, you don't have to make any changes to the workflow or the materials that you use. All you have to do is to inject the mixture of cells and gel into these inserts and you fill the wells. And that's it.

So the question is, does it work? So it does, actually. So in Matrigel drops, these

organisms would die off over time, but in OCTOPUS, they would continue to grow. And we were able to form these huge, actually, interoids, human intestinal organioids. This is a size comparison to help you better understand how big we are able to grow them.

It's not about the enlargement size. The tissues actually become actually more mature. So as evidenced by these cell text specific markers, we also did glucose stimulated calcium sicnolene as a functional assay.

And we were able to show that we get better cellular complexity heterogeneity by using the OCTOPUS device. And it's also possible to use patient derived cells to use these diseased organoids. They spontaneously recapitulate their normal features, like compromised barrier function in a normal epithelial morphology insulin and so on.

And then, this is actually the last figure of the paper where we introduce this organoid vascularization platform that can be used

for a variety of actions. In this example, we used intestinal organoids. We can form these vessels around these organoids and profuse the entire construct.

So, this is one example, but now we have actually other organoid models. For example, these are liver organoids, IPS 2 derived liver organoids that we're using to study heart failure-induced liver fibrosis.

And we are also working with Deborah Cotton's lab at BU to grow these long organoids where we form more phenological special conditions to make these cystic, spherical, long organoids look more like they're in vivo counterparts and so on, okay?

All right. So, just checking time here. Need to speed things up here. So the second question is, how do we actually increase their capacity as a data generator? What kinds of information can we really get? And how can we use actually this information?

So this example I'm going to be actually

talking about briefly is a new paper coming out in Nature Biotechnology. So, where we use our platform to model CAR-T immunotherapy. We actually use a very similar platform in this study. The only difference is we actually introduced the patient x-points, tumor x-points.

We vascularized these tumor x-points in our device. We also have an open-top configuration where we would actually form the vessels first and then when these tissues become available, we go pick them up, open the device, and we drop them in there to vascularize.

But at the end of the day, what we can do is to form these fully profusable, accessible, vascularized human solid tumors. So we're now using this actually to investigate CAR-T therapy of solid tumors, which is a huge clinical challenge, apparently.

So we are now using this platform to investigate three essential steps of CAR-T cell trafficking, which is first, they would have to recognize the tumor cell, tumor-associated

antigens, and then they would have to get out of the vessels and move toward tumors. And then in there, they would have to exert antigen-directed cytotoxicity for a certain amount of time.

So can we actually really model these essential steps and investigate those essential steps? So just to show a snapshot, this is actually CD X tumor engineered over express, mesothelin tumor antigen.

These CAR-T cells are designed to target mesothelin. So this is two hours after infusion, as you can tell. Many of these CAR-T cells are stuck to the tumor associated vessels. They show activated morphology. They move around. And what's really nice about this model is we can view the entire process of extravasation, directional migration towards the tumor, and then effective functions.

So within a couple days, many of these cells are found within the tumors. Unfortunately, this is not representative of what happens in clinical patients. We actually did this study to

really demonstrate the proof of concept.

But, so as a result, we can actually also track these tumors to measure their size and phenotype and so on.

And to talk about information, we really try to actually really diversify our methods of analysis and get as many different kinds of information as possible. So in one example, we can get tumors or CAR-T cells from the tumors and differentiate or actually distinguish them from those in the surrounding stroma. And as a result, we are able to use, for example, photometry to really identify and also track and really look deeper into the phenotype of these CAR-T cells that can get into the solid tumors.

We did similar things using single cell sequencing. We have a lot of data here. But I'm going to have to skip many of these data today. But one important thing here is actually, we conducted using sequencing data a ligand receptor interaction analysis to find out this well known CXCL/CXCR three signaling, which CXCL 10, CXCL 11,

secreted by those endothelial cells and the tissue, they bind to CXCL 3 receptor on CAR-T cells or T cells, and that's actually, that contributes to, that's known to contribute to CAR-T cell trafficking or T-cell trafficking in general.

But we found out that this enzyme, DPP 4, was interacting by truncating these chemokines. And so the question was simple. Can we actually inhibit the activity of DPP 4 as a way to increase CAR-T cell trafficking?

So long story short, it was very effective. And so we use this FDA-approved drug actually that's currently used for diabetics to inhibit the activity. And as a result, we were able to dramatically increase the CAR-T cell trafficking.

The second question was can we go further and use mesotheliomics actually to maybe try to identify biomarkers, potential biomarkers, that would be indicative of efficacy of this combinatorial approach.

So in collaboration with Josh Rabinowitz's lab at Princeton, we actually did unbiased double mixed analysis of affluent samples collected from our devices. And as a result, we were able to suggest a panel of biomarkers that could be used for potential clinical indicators.

I don't have time to talk about all this, but this is a new paper coming out in Nature of BME where we actually use on-a-chip to really rebuild something very new and interesting about sensitivity of asthmatic tissues to mechanical forces exerted by bronchoconstriction, and implantation on a chip where we actually can study directional migration of embryo cells into maternal tissues to find out what factors really contribute to this process and so on.

I'll skip this. So the last topic, if you can give me just a couple minutes, five years ago, actually had I given this talk five years ago, this is where I would have finished my talk, but we've had actually, we have a field, we've had a lot of success, I have to say.

A lot of papers, a lot of patents coming out. But if you think about like the real-world impact this technology has made over the last 10 years, we're trying to get there but it's questionable.

So this is what I have been talking about as inconvenient truth to at least our organ chip models that we build in our system. I don't mean disrespect to the field.

So one of the major issues is, if you think about it, the reason why people are upset about this technology at the beginning was it allows us to mimic complex things happening the body in these engineered manmade systems.

But the flip side is, it's also the complexity that makes this technology, it makes it really difficult to translate this technology into industry practice, research practice.

So one of the key challenges, my personal opinion, is the scalability. So simply speaking, it becomes really difficult to conduct many, many experiments at the same time. So it

really limits our ability to get sufficiently large amounts of data that we need to make accurate prediction or to better understand complex duties, processes happening in trackable disease and so on.

And another kind of well-known fact is many of these model systems are not very reproducible in terms of fabrication, in terms of experimentation. So, after a seminar like this, I would actually, a line of people actually asking and wanting to test some of those models in their labs.

And I would say, many times we actually think this is our experience as a lab, many of these experiments fail when they try to do it in other labs. So it's not very reproducible.

So we now try to actually, this gets us to the last question I wanted to address, how do we actually make this technology practically more useful?

So, to resolve that scale issue, we now walk away from these single channel, single

experiment devices, and we and other labs commonly use these plate devices, each of which contains tens of replicates, but these are the most recent events.

This is a six-inch wafer size device containing over 200 individually addressable self chambers. In other words, on one device, it becomes possible to conduct 256 independent experiments. It's fairly easy to stack five of these devices, which makes the number of concurrent experiments over 1,000, which I think is orders of magnitude higher than what is currently possible in my lab, what used to be possible in my lab.

And this was actually by a phenomena engineer, my former grad student, Andrei Georgescu, who came up with this very sophisticated, elegant floating circuit design.

Long story short, using this floating circuit, these rectangular chambers are blood forming devices, actually you saw earlier. It becomes possible to precisely control what goes

into each of these chambers, which I think is very exciting.

We also view humans as a source of irreproducibility in errors. And so we try to remove humans completely from the entire workflow. So to do that, we build robots.

So these automated systems are making our lives easier, but what we are most excited about is that we now are able to think about drastically increasing the amount of data, or the type of data we can get from these human cell based, supposedly much better in vitro model systems.

And so, I have a couple examples, but in the interest of time, I'm going to skip that today.

So based on this vision and technology, we started this company called Vivodyne three years ago. Andrei Georgescu, who is my former grad student who developed this platform, is now leading this effort as the CEO. The company is intending to offer and is currently offering very

realistic tissue without harming scalability and complexity.

So we actually can generate AI scaling data using these fully automated systems, actually. So this is the main workhorse that we use in the lab. Our business model is not to sell these systems, but to generate data. It's really the data that our business model is based on.

Really, it's entering automation. So we automate the entire process of tissue engineering analysis. So all you have to do is put a vial of cells and media bottles in the industrial robotic arm, combined with many other systems, take care of the entire workflow.

So we're gaining traction and currently working with many pharmaceutical companies to really demonstrate and also verify the potential promise of this technology, but what's really important here is actually, what's really exciting is that we can now think about generating large scale, large amounts of human relevant data before clinical trials. So that's actually what we're

most excited about now.

Okay, with that, I'd like to thank my group, collaborators, and funding sources. Thank you very much for your attention.

DR. ATREYA: Now the floor is open for questions.

UNKNOWN FEMALE: Okay, good morning. Thank you for that beautiful talk. I guess I can say I experienced the first microfluidic microprocessor being presented.

My question to you, as I have many questions, but what I would like to ask is, now that you are thinking about this scaling up and repeatability, are you thinking about incorporating the human diversity, genetic diversity?

Because one of the things that microchips organoids, is we only have one donor cells, right? And how can we incorporate that?

DR. HUH: Yes, that's a good question. We are in the process of designing some case studies or representative studies actually to

address the question you just mentioned.

And so, when a troop is low, actually the variability oftentimes in our experience has been a source of frustrations. And sometimes it works, sometimes it doesn't, sometimes it shows phenotype A but sometimes it would show phenotype B.

But when you have high enough perplexity, the variability and also diversity actually, these things actually become very interesting topics to investigate.

And so, we're now thinking about actually conducting more systematic experiments to look at specifically at genetic diversity and also patient variability.

It would also obviously would have to depend upon the availability of these tissues. And so right now many of these studies, the platform, I think it's fairly ready, but the tissue sourcing, cell sourcing, sometimes becomes problematic or sometimes becomes a bottleneck.

But we're in the process of sorting

these issues out. I hope that answers your question.

MR. NORCROSS: Yes, hi, Mike Norcross. Hi, Dan, that was really impressive. I mean, you got any more systems to talk about?

So, I want to know on several things, one is on the bone marrow, do you see platelet maturation, megakaryocytes, in bone marrow cultures?

DR. HUH: We do see the formation of megakaryocytes, but in very small numbers, I have to say. So, it would be fantastic if we can go down that route and then start producing platelets. But we haven't. So what I can say is there is a small number of megakaryocytes that form in the device.

But we don't know what happens to them over time. But, yes, that's the answer I can give you now.

MR. NORCROSS: Okay, and the other thing is about lymph nodes or immune responses. You briefly touched on that, but have you made any

more progress on kind of getting an immune response to happen on the chip? You got T-cells, antibody forming cells?

DR. HUH: Right.

MR. NORCROSS: How you doing on that?

DR. HUH: Yes, so, the focus of that study was on innate immune cells or innate immune systems. So, neutrophils are of course responders actually responding to infection. We do see formation of small numbers of lymphocytes in this system, but again, like, for this proof of concept study, we didn't think it would make sense for us to really put a lot of emphasis on that, given the fact that they would be --they would form there, but in a maturation and subsequent process would require other organs and tissue systems.

And so, we haven't done it yet, but there's a lot of interest. And so, in terms of modeling immunity, this is pretty much the only system that we currently have, to be completely honest. But, I mean, we've been getting a lot of inquiries.

But we do not have any further studies based off of this work yet.

MR. NORCROSS: And then on the CAR-T cells, did you do any correlations with the patient responses? These are tumors from patients. Are they going to get the same CAR-T cells? Do you see any relevance of what you see in vitro versus in patients?

DR. HUH: Right. So, the data that these CAR-T cells crawling out of the vessels, moving toward the tumor, was again a very artificial system, I have to say, where we overexpressed the mesothelian inputs.

In tumor cells, we inject into mice to form the CDX tumors. What we try to do to transplant, put in patient meso, what is it? What type of actually, the skin tumor, actually?

And then we did the same experiment using CAR-T, mesothelian targeting CAR-T cells. We were able to show their trafficking there. But again, as I said, this is not representative of what happens in most patients, right?

So one of our ongoing studies is to look at T-cell exclusion exhaustion. And I think it's too early to talk about that. But we're getting there. Yes.

MR. NORCROSS: One thing I was just going to point out to you, DPP 4 effect on chemokines.

DR. HUH: Yes.

MR. NORCROSS: We were the first ones to publish that many years ago in JM where we looked at different chemokines and could see some of them were inactivated but others are actually activated and changed specificity for receptors.

DR. HUH: So was it done in the context of CAR-T therapy?

MR. NORCROSS: No.

DR. HUH: Yes.

MR. NORCROSS: This is way before all that.

DR. HUH: Okay. Okay.

MR. NORCROSS: I mean, this is back in the dark days. But we found DPP 4 chemokines. It

was in with HIV. We were studying HIV.

DR. HUH: I see.

MR. NORCROSS: David remembers years ago.

DR. HUH: Thanks for pointing that out. It's good to have -- good to know that, yes. All right.

MR. VILLA: Hi, Carlos in the Office of Blood here at FDA. Really fascinating work. Have you looked at using whole blood as the profusate in some of these systems where you can look at the function of actually the blood that's going into and interacting with the organ?

DR. HUH: We have not, actually. So the only, maybe the closest thing I can think of is actually RBC profusion in the context of transfusion-induced endothelial injury.

So I didn't talk about any of that today, but we actually showed in the paper that stored RBC can harm endothelium, actually, in the transfusion settings.

But whole blood in general, we don't use

actually whole blood as profusate. We use just defined media that's actually, I mean, that's actually one of the major challenges in developing multi-organ systems, when you have multiple cell types across these different devices. How do we make all of them happy, right?

So, in the body, we have whole blood. But as far as I know, whole blood actually in those in vitro settings doesn't work for those purposes. Yes. So that's actually -- yes.

DR. RAGUPATHY: Excellent presentation. This is Viswanath Ragupathy from Office of Blood. All these micro fluidity platforms, this sort of kind of simply is a major role. So did you consider any kind of modifications with the surface chemistry for different cell types, thereby you could able to complete all these cells on a chip?

DR. HUH: Yes, so it's definitely possible to do that. In our case, in many of our model systems, we use either naturally -- almost all of these model systems, we use natural ECM as

coating materials.

And what's also important to keep in mind is that over time, we grow these cells in these devices for at least two to three weeks. And so over time, they kind of deposit their own ECM to remodel their surrounding matrices.

And so that's kind of -- that's been the approach we've relied on in many of these studies. But, yes, I certainly acknowledge the possibility of adding more complexity to the cell surface and interactions. Yes.

DR. ELKINS: Whoops. We have lots of online interest. So several of them revolve around the overall question of how you validate each of the systems, individually or collectively, against things like animal models, traditional in vitro culture systems, or even human systems. Could you talk a little bit about maybe the overall strategies there?

DR. HUH: Sure. So our strategies, actually, yes, I didn't talk about any of that. But in the eye model, for example, that could be a

good example, we would actually use a variety of techniques like histology and expression of differentiation markers and production of secretive factors.

In many cases, in vivo data are available in the literature that we can use to compare our data against. And whenever possible, we also try to have access to gain access to human data, like of any kinds.

In many of those situations, the challenge is the huge difference in scale. So measuring cytokines, secretory factor levels in the blood could be a good set of data. But if you were to compare what's happening at the cellular tissue levels to what happens at the whole organism, whole body levels, that becomes a huge challenge.

But we try to actually gain and get human data that can be compared to our in vitro data. So for that, we look at the structure. We look at functional markers. And with the availability of the old mixed data, multi old

mixed data, like single cell sequencing atlas data, papers, we try to maximize the utility of these available data into literature for verification validation purposes.

DR. ELKINS: Thank you. Some of the more specific ones. In the OCTOPUS and microfluidic chambers, how do you overcome cell aggregation when you're feeding that could limit the media flow and organoid growth?

DR. HUH: Yes, so that's actually one of the things we're now investigating. Like, how far apart do these cells want to be? But in the first study, we didn't really care about that too much. So we just made sure that they were nicely suspended.

And also, this radial geometry seems to help. And so it's equal distribution into these eight radial chambers, and that seems to help in terms of spreading the cells uniformly across the gel.

DR. ELKINS: Next, is it possible to use the 3-D culture systems to isolate exosomes or

secreted components of signaling proteins?

DR. HUH: Certainly. That's a very hot topic now, and now as these model systems or model development techniques or technologies become more mature, many of us actually try to kind of leverage our capabilities for other purposes.

And so one of them I think it's definitely, I see on the horizon where this is actually turning out to be a very active field, active topic of investigation.

So it's actually readily possible to get affluent samples or cells from these devices for further analysis. And so, it's fairly straightforward and easy to isolate exosomes from these in vitro bioengineered tissues.

And we have one example, but I don't think we have time to talk about that. But it is definitely possible, yes.

DR. ELKINS: The next one could be lengthy, too, but we'll try it. In in vitro systems that serve as disease model systems, can you comment on the timeline of changes in

relationship to the disease onset?

DR. HUH: Yes, so, I mean, there are really, in my mind, two questions. And so, do we want to mimic acute responses and processes versus chronic responses and processes?

And so, for modeling acute responses, you can start off with normal cells and stimulate them as needed, as long as the experiments you're doing are relevant to the real life situations.

But oftentimes, it's really the chronic inherent indigenous features we want to drill down, or look deeper into.

So in those cases, commonly we use -- we try to get diseased cells from patient samples. And these days, many of these vendors like Lanza, they carry a wide variety of actually primary human cells isolated from diseased tissues.

So we would actually order these cells from reputable commercial vendors or we try to work with our collaborators in medical school to get patient explants and harvest and isolate cells from those samples.

DR. ELKINS: Next, going to the other end of scalability, have you used platforms to evaluate the feasibility of individualized approaches, personalized medicine approaches?

DR. HUH: Not yet, but certainly, that's one of the main directions we are headed. Yes.

DR. ELKINS: And then there are questions about the immune system that we have already covered. What steps do you need to take to ensure interactions between biological surfaces and physical surfaces that don't alter the physiological

conditions?

DR. HUH: Yes, so we think about these questions a lot. But at the end of the day, it's actually, it's kind of, they're in a collective behavior and collective properties that matter to us. So we would use those, define end points.

I briefly mentioned earlier actually to really see whether or not they're exhibiting the properties they are supposed to exhibit.

And so to achieve that, you could

engineer the surfaces emitted from waves or biological surfaces and physical surfaces. I'm not sure what that means exactly in the context of model development.

But again, it's really their properties and functional endpoints, phenotypes, that we monitor and we care about. Yes.

DR. ELKINS: One specific one. In certain 2-D culture systems, like lung epithelial, the cells survive for months to years with maintenance, how long can cells survive in the multi-channel microfluidic plates?

DR. HUH: Yes. So, the longest we've done in lung models, some of the lung models we have was 2 1/2 months, and we didn't have a good reason to continue. But, yes, those experiments or duration of experiments really depends on the goal or the question you're asking.

So if the question involves lengthy culture, typically, somewhere between one and two months is considered pretty good, and pretty good long-term culture.

But going beyond that can be challenging. That's another half-hour lecture discussion.

DR. ELKINS: And for the final half-hour lecture potential, I'll bundle together several questions that are interested in your thoughts on the prospects for a whole body system, putting it all together.

DR. HUH: Yes. So the whole body body-on-a-chip as many of you know, actually, there are pioneers actually in this area. It's still an active area of investigation.

Again, just to limit my answer to what we've done in my lab, we haven't had -- I mean, there are many, many interesting questions we could investigate using whole body on a chip systems, but our interests so far have focused mainly on kind of single organ tissue systems.

And the questions involving single tissue types or single organ systems. And so, but, just know that it can actually build multi-organ systems connecting fluid. It could be

connecting or somehow you can figure out ways to allow these chips to communicate with each other so you can actually build these separate organ chips fluidically or somehow connect them to whole body physiology, which will be very, very important for drug testing studies and so on.

Yes. But there are people actively working on that idea in the field. Yes.

DR. ELKINS: Thank you very much.

DR. HUH: All right. Thank you.

DR. ATREYA: Without further ado, please invite the keynote speaker today, Dr. Balu-Iyer.

DR. BALU-IYER: Thank you. So I get five more minutes? So thank you very much for this opportunity. It's indeed an honor to be here. So can you hear me? Can you hear me now if I move out of the podium? Don't me if I work for a cell phone company or something.

So, what I'm going to share with you today are the lessons -- is it turned on or something? Oh, okay.

Okay, thank you very much for this

opportunity. What I am going to share with you today are the lessons we are learning about immunogenicity of biologics. In particular, prediction and mitigation of immunogenicity of protein based therapies.

So, just a disclaimer. So I am going to talk for about 40 minutes. Now you know exactly when the start of my talk would end, that, too, after the impressive talk of the first speaker.

So if I don't stop, I'm sure CD and Dan will come to your rescue.

Immunogenicity in the context of protein based therapeutics refers to unwanted immune responses. In the clinic, it manifests as anti-drug antibody response, such as ADA.

For example, 1/3 of severe hemophilic patients who received Factor VIII develop anti-Factor VIII antibody. And this kind of antibody response can impact efficacy, both directly and indirectly, directly to the formation of neutralizing antibodies.

These neutralizing antibodies are the

antibodies that recognize the regions and the protein that have critical for biological activity, so impacting directly pharmacodynamics and efficacy.

For example, ADA response against the antigen binding region of the molecular based drugs can inhibit its binding to the target antigen, impacting efficacy directly.

ADA can impact efficacy indirectly as well by through the formation of binding antibodies. These binding antibodies, R3PK, generally, they increase the clearance and reduce the drug exposure.

So, immunogenicity and ADA response should be considered in the context of clinical pharmacology and pharmacokinetics and pharmacodynamics aspects as well.

Sorry about this. So a couple of -- a couple of examples of how ADA response can impact pharmacokinetics and pharmacodynamics. So rheumatoid arthritis patients who receive adalimumab developed anti-rheum antibody. In the

ADA positive patients, the efficacy is lost as measured by the pain scale.

So the next example is anti-PCSK antibody. During the clinical trials, several patients developed antibody against this drug that increased the clearance of the protein and reduced the drug exposure.

So, this immunogenicity can be a significant issue that can lead to a major contributor of discontinuing drug development process. That too, if it happens in the later stages of the clinical trial, the clinical sort of financial impact could be very significant.

So addressing the issue of immunogenicity during drug discovery and drug design and pre-clinical trials should be very useful in cost effective manner.

So, you know the best in this world that the incidence of immunogenicity is reported under Section 6.2 of the package insert.

In 2016, analysis by Food and Drug Administration scientists, they looked at the

post-marketing analysis of 121 clinically used therapeutic proteins, and they found out that 108 of them reported incidents of immunogenicity in their package insert.

And many of them impacted efficacy in PK. So overall, the ADA impacts safety and efficacy of significant number of therapeutic proteins.

So, the mechanism of immune response against therapeutic proteins, this is the schematic that shows that, in a piece of dependent process, upon administration of therapeutic proteins, antigen presenting cells suggest cells take up the protein that process them and process them into bits and pieces as we all know, epitoles, and present it to the T-cells in the context of major still compatibility complex of human leukocyte antigen.

And with the help of [inaudible 57:33] these cells get activated and activated T cells differentiate the B cells into memory B cells and plasma cells and plasma cells secrete the antibody

against the administered drug.

There are several factors that can influence immunogenicity of therapeutic proteins, and they are classified as patient related, product related, and treatment related characteristics.

So first is patient related characteristics. This HLA, it's a human leukocyte antigen, is a highly polymeric protein, different [inaudible 58:13]. So depending on their HLA genotype, individuals vary in variability to mount an immune response.

The second patient related factor is the immune status of the patient. So autoimmune patients due to their overactive immune system respond more than the immunocompromised patients.

There are several product related factors impact immunogenicity of therapeutic proteins. So primary sequences containing epitoles that are recognized by HLA increases the incidence of immunogenicity.

As we all know, proteins are expressed

in subculture systems, so expressing systems based in purity generally increase the incidence of immune response.

And a related topic is the glycosylation, which is a post-translational modification. And glycosylation has been linked to several plasma survival and immunogenicity of therapeutic proteins.

In this particular case, lack of glycosylation increased the incidence of the immunogenicity compared to the glycosylated version of the drug.

The next one is speculation, which is a covert modification that's attaching the peg to the therapeutic protein to see benefits in the PK properties, and there is a link between pegylation and immunogenicity.

One of the common, most important, most important product related factor that impacts the immunogenicity is aggregation. The link between aggregation and immunogenicity have long been known. However, there are different types of

aggregates that have been recognized, and they vary in their size, confirmation, morphology, et cetera, and our understanding of type and amount of aggregates present in the protein formulation impacting immunogenicity is expanding.

The next factors influencing immunogenicity is treatment related factors. So for example, prolonged treatment increases the immunogenicity incidents. For example, in this case, long term use of adalimumab in RA patients increased the incidence of immunogenicity to about 28 percent of the patients. This was really a use of two years.

And the next important treatment related factor is route of administration. So based on extensive experience with vaccines, subcutaneous route of administration is expected to be more immunogenic than intravenous route.

Due to current significant interest on this topic, I am going to expand on subcutaneous route of administration and immunogenicity of therapeutic proteins.

So subcutaneous route of administration is desired over intravenous route because patients can self-administer at home avoiding a trip to healthcare set up. So it is cost effective and convenient. Because of this, compliance is very high.

Depending on specific protein examples, it can also have additional benefit that is switching from IV to subcue administration can also have additional benefits.

However, the subcutaneous route of administration is challenging due to three interrelated pharmaceutical issues that has formulation of pharmacokinetics and immunogenicity.

For example, in formulation, due to the limited injection volume because of the subcutaneous delivery and also hype therapeutic formulations, for example, molecular antibodies, leads to some stability issues and also some physical chemical properties of the protein solution.

So upon administration of therapeutic proteins, generally it leads to incomplete bioavailability. That is a pharmacokinetics issue. And then third is immunogenicity, as I mentioned to you before, that based on extensive experience with vaccines, that subcutaneous route of administration is expected to be more immunogenic than IV route of administration.

So, head to head comparison of, in a clinical trial, of clinical ADA response following subcutaneous and IV route of administration was performed for Orencia, which is an (inaudible) CTLA where they found out that the efficacy and immunogenicity are comparable between these two routes of administration.

There are few pre-clinical studies have also support the notion that subcutaneous route of administration does not increase immunogenicity.

This brings us to an important question. Is that the generalization that subcutaneous route is more immunogenic than IV route is still universally valid if the therapeutic proteins is

given in the absence of (inaudible).

So if you flip the other side of the coin, there are several pre-clinical and clinical observations that support the notion that SC route of administration is more immunogenic.

For example, in the table we compiled for the clinical trials comparing ADA incidence between subcue and IV route of administration, the subcue route of administration did increase the incidence of immunogenicity.

This brings us to a conundrum, that both pre-clinical, the clinical and pre-clinical studies both support and refute the notion that subcue route of administration is more immunogenic.

Irrespective of whether subcutaneous route of administration is more immunogenic or not, understanding what drives the subcutaneous immunogenicity is useful to design safer biologics.

From this, we need to have a mechanistic understanding of what drives subcutaneous

immunogenicity. However, this topic is not complete. So what we did was, we attempted to propose a model similar in molecule model, what drives the subcutaneous immunogenicity of therapeutic proteins using (inaudible) for using vaccine (inaudible) where the control experiments were done in the absence of (inaudible), and our own studies, and also others in this process.

We tried to formulate a mechanism of what drives subcutaneous immunogenicity. First question is asked of ourselves, is there any difference in the antigen presenting cells of the protein of product process of the protein, is there any difference between subcutaneous route and the IV route of administration?

So some elegant studies done in late '90s and 2000, they used the molecular antibodies that binds to MXC (inaudible) and MXC2 complex. What it allowed them to do is to track the fate of the antigens and the cells that produce them following different route of administration.

Following intravenous route of

administration, antigen and specific B cells rapidly take up the protein and present it in the spleen within four hours of administration.

This is followed by presentation by dendritic cells after 24 hours. After this, it's just a numbers game, because antigen and specific B cells outnumber the dendritic cells in the spleen, so it is safe to assume that antigen and specific B cells in the spleen are the primary antigen presenting cells following intravenous route of administration.

In contrast, dendritic cells produce the protein antigen to the T cells in the lymph node following subcue route of administration. So, the dendritic cell, we all know is the primary initiator of T cells. So early on, the subcutaneous immunogenicity may be driven by neurological exposure to very active dendritic cells.

The anatomy of the skin and subcue space contribute to this immunological exposure. So, just a primer on the anatomy of the subcue space.

So the most superficial layer is epidermis, and the layer beneath this is dermis, and the layer below that is hypodermis, or subcutaneous space.

So the similar component of the subcutaneous space is made of (inaudible) fibroblasts, and macrophages, mostly. And then the fibroblasts intersects the competence of extracellular matrix that's collagen and hyaluronic acid.

We all know this extracellular matrix is a barrier to the subcutaneous delivery of therapeutic proteins.

In terms of immune cells that constitute the subcutaneous space, landerhand cells, dendritic cells, a type of dendritic cells, resides in the epidermis, and then there is a dermis resident dendritic cell that is found in the next layer.

So then you might wonder, the passing of the protein in the subcue space, how does it increase the immunological exposure to these

dendritic cells? Because this exposure is important for the intensity of the adapt to response because it has to present it to the T cells in the local lymph nodes.

So under the set of experiments that was done using (inaudible) antigen, then we could follow peptide and MXC2 complexes, like monoclonal antibodies approach what they used before, they found out that two waves of antigen presentation and processing of first following subcutaneous route of administration.

The first wave of presentation that the protein presented itself after depositing it here, it exposes itself to the lymph node resident dendritic cells. So how did they find their way from here to here? It's PK, pharmacokinetics and biodistribution.

We all know the monocular wave dependent of PK and lymphatic uptake is well known. And this kind of presentation, this kind of presentation occurs within a few hours of therapeutic protein administration.

And then second wave of presentation is mediated by the migration of these cutaneous dendritic cells. They migrate from this space to this space and then carry the protein to the local lymph node.

The cellular and molecular mechanism of this particular migration of cutaneous dendritic cells is well characterized, and I would like to highlight three steps in this particular process.

The first step is that upon administration of the therapeutic protein, it increases the phemokyne receptor expulsion, such as CXCR 4 on the dendritic cell.

The second step upon administration of therapeutic protein, it triggers the production of pro inflammatory cytokines, and that increases, or regulates the production that in turn increases the number of lymphatic vessels.

And then third step in this process, these lymphatic vessels secrete ligands for these chemokine receptors.

So basically, what drives the migration

of the dendritic cell is nothing but the receptor ligand interaction that drives the migration of this dendritic cell.

So overall, what drives the subcutaneous route of immunogenicity of subcutaneous route of administration is that immunological exposure to dendritic cells and their migration.

This particular cartoon that shows the two waves of presentation, for example, upon therapeutic protein administration, they find that they are rare and present it to the local lymph nodes that is driven by biodistribution of the therapeutic proteins.

And the second wave is that upon administration of the therapeutic protein in a subcue space, these dendritic cells migrate and then they travel and present it to the local lymph nodes and then to the T cells in the local lymph nodes.

So overall, what drives the subcutaneous route of administration, immunogenicity of this, is the immunological exposure to dendritic cell

and its ability to migrate.

So this mechanistic understanding, we used it for two purposes. The first one, we used it to develop an actual risk assessment tool to predict clinical immunogenicity, and the second one is the rational development of innovative mitigation strategies.

So could these two purposes be used this mechanistic understanding? The first one is risk assessment tool of clinical immunogenicity.

Some might ask, why do we need an actual clinical immunogenicity prediction? So, for example, if it is a high to good screening, and validated method, it could be useful in a high throughput manner, to screen for compounds and develop less immunogenic, safer version of the protein drugs.

And then the second one, it can also align with FDA Modernization Act that it can act as an animal trial alternate for rapid screening of this.

And then, third reason, as I mentioned

to you before, percent of the clinically used therapeutic protein showed

incidence of immunogenicity -- reported incidents of immunogenicity in the package insert.

That raises an important question. Is there any gap in our understanding of pre-clinical screening, of pre-clinical development, and a clinical correlation? Is there any gap that because it's not sufficient enough to predict clinical immunogenicity? So what are all of our current tools to predict a screen for this compound during the pre-clinical stages? It's the in silico tools.

There are routine screenings for lenience epitoles. But when it comes to the confirmational epitoles, they did not predict well.

The second unnatural amino acids containing peptide and protein drugs, and chemical instabilities, and also it doesn't have any impurities in the formulation that can be predicted by in silico methods.

So there are some issues with that. And then any diesel based acids require a prolonged culturing conditions and a very high concentration of the protein to make a meaningful conclusion.

And then they used the skin models and skin explants. For example, the major problem with these things is that it doesn't have an immune cell that's irrelevant. So immune response, you measure with not relevant immune cells.

Second thing is maintaining the integrity of the skin explant is very difficult in an explant system, so it maintains its prediction by these methods may be limited.

So, then use of animal models, that's generally done. That is generally done because, for example, the relative immunogenicity of many therapeutic proteins, you can compare, they can provide. So use of most models are very useful because it's cheaper to do it, you can manipulate them to get transgenic mice, and also, to say, very controlled background is raise so that the varied routine response will be too tight. Then

it may be beneficial to it.

However, when it comes to predicting the incidence of immunogenicity, that too for new biologics, there are limitations. It cannot predict very well.

Because evolutionary pressure, evolutionary pressure on a species that sniffs the ground and a vertical species like us, there's so much of difference.

Mice are not humans. We do not have a tail. So humans are not mice, but humanization of mice is very successful.

But humanization of rodents could go the other way, too. So irrespective of whether its ability to predict, ability to predict immune responses is limited.

So we use this current understanding of what drives the subcutaneous immunogenicity, what drives the (inaudible). We boil that down into three immunological readouts.

So the first readout is the migratory phenotype, this expression of CXCR 4 on dendritic

cells. Combined that with an activation phenotype that's percentage of (inaudible) producing dendritic cells. And the third one, we combined this with a direct migratory potential that is number of dendritic cells migrating using a transfer assay.

So this transfer assay is that outer chamber. We put the therapeutic protein in the presence of chemokine ligands. And in that chamber, we grow dendritic cells from donors that can capture patient readability in a number of cells migrating from inner chamber to the outer chamber used to calculate percentage migration. And then that is extended to migration index.

And we combined these immune readouts to validate an approach whether these three readouts can predict the clinical immunogenicity incidents. As you go from top to bottom, it increases the incidence of clinical immunogenicity.

So what we found out was that CXCR 4 is very sensitive to the immunogenic potential, and then that matches with the expression of CD 40 and

interlocking to all expression.

But this is not enough to correlate pre-clinical and clinical immunogenicity. However, when they combined that with the migration potential and number of cells migrated, the correlation between pre-clinical immunogenicity and clinical immunogenicity, the correlation was very high.

So as you can see that it predicted immunogenicity of this therapeutic proteins very well from low, moderate, and high immunogenic response.

So the next topic, so the advantages of this ex vivo model is that it is mechanism based and sensitive, that can capture early steps in the antigen presentation and processing, and also it can give you an innate and adaptable connectivity that is missing in somewhat in all these pre-clinical tools.

Then it could be done in a 96-volt plate, so it's high proof and cost effective. And importantly, it's not influenced by the mechanism

drug action, because there's no direct exposure. So anti (inaudible) drugs and T-cell engages that immunogenicity could be predicted.

It captures patient variability. It can screen for impurities and aggregates. And also, it can be combined with other screening tools as I mentioned before to get a complete picture of what drives immunogenicity of therapeutic protein in some of our outstanding studies that came from FDA will be incredibly useful in this regard.

So the next application, what basic understanding of what drives the subcutaneous immunogenicity, we use for the purpose of mitigation strategy.

As you know, an important step in this process is migration of dendritic cells. So any process that reversed the migration would be an immunogenic contributor. So avoiding that would be helpful.

So any inflammation that causes this, the cleaning of the product free of impurities, aggregates, and (inaudible) contaminates would

reduce the migration.

At the molecular level, this migration is accompanied by similar -- sorry, molecular infraction between receptor and the ligand.

So any process that interferes with this process, take a small molecule formulation excipient, that binds to CXCR 4, that prevents the migration of the dendritic cells would be a good approach.

But our approach has been using the challenge as an opportunity. The challenge of immunological exposure to dendritic cell and migration has also presented us with an opportunity.

For example, if you expose the therapeutic protein to the mature dendritic cells in a tolerogenic context, it can convert this cell into a tolerogenic phenotype and then it can produce regulatory T-cells that can reduce the immunogenicity of therapeutic proteins, whereas if you present them in immunogenetic context, if will increase the effective T-cell population.

So, for this purpose, we want to harness the biological properties of lipids. Why the focus on lipids? We think that lipids are superheroes. So to this structure of lipids, we add the Superman costume so that it can save the world.

It did, right? In the case of COVID vaccine, this lipid nanoparticle delivered the modern vaccine for COVID vaccine that saved millions of lives. Because of that, this is our lab logo.

So when harnessing the biological function of phospholipids, it gave us tips. So the word tips means different things to different people.

For the restaurant employee who is waiting at the table, tips mean something to him. A laboratory scientist, bench scientist, tips mean something else to her. We always use helpful tips to even solve all our day-to-day tasks.

But for the next few minutes, tips means tolerance inducing phosphorite seeding. This

harnesses the biological function, novel biological function, of phosphorite seeding in that it can convert an immunogenetic tolerogen.

In harnessing this property, we double up immunotherapy tolerance platform with broad clinical potential. And in this talk, I want to talk about to prevent a reduced immunogenicity of therapeutic proteins.

So what is? So phosphatidylserine is an anionic phospholipid that is present in the inner leaf flap of a living cell. When the cell dies, it flips to the outer leaf flap. This externalization of PS sends an "eat me" signal to the phagocytic cells.

The phagocytic cells take up this cell debris exposing phosphoric seeding and clear them with no immunological consequence.

So generally, a (inaudible) process was always thought to be an immunologically silent event where the externalization of PS mediates that the immune system to ignore the cell debris to prevent immune responses and a wide

autoimmunity.

However, the lessons we are learning by using phosphatidylserine to reduce immunogenicity is that it is not an immunologically silent process.

If PS get exposed and it is taken up by the immune cells, it teaches the immune cells to tolerate the antigen. So it is not an immunological inherence, where it actively teaches the immune system to tolerate it.

So based on this, our hypothesis was refined so that PS is not just a clean up crew, but a well-meaning teacher.

So, if phosphatidylserine induced tolerance to therapeutic protein, pre-exposure of the protein in the presence of phosphatidylserine will desensitize the immune system.

When you rechallenge them with a free form protein, it should not note a response. So this experimental design was based on this one.

So we pre-exposed the therapeutic proteins in the hemophilia mice with the Factor

VIII and the Factor VIII in the presence of various phospholipids.

For example, phosphatidylserine and a charge matched to phosphoryl glycerol as a control. We also added another treatment group that is Factor VIII dexamethasone.

So as you then, after pre-exposure, two weeks wash up period, and all these animals were re-challenged with pre forma Factor VIII. And two weeks later, we collected the blood and measured the anti Factor VIII neutralizing antibody titers.

As you can see, the animals that were pre-exposed to Factor VIII PS show significantly lower antibody types compared to another treatment groups.

I would like to bring up the comparison between Factor VIII PS and Factor VIII dexamethasone group. Both of them show the reduction in the titers during the pre-exposure period, but only PS treated group still remembered the antigen building, the re-challenge period, not to mount the response, whereas dexamethasone did.

So this pilot shows that the PS is not just an immune suppression but it actively teaches the immune system to tolerate Factor VIII. Immunological ignorance cannot explain this.

So this is the first to our knowledge, the first direct evidence that phosphatidylserine has the ability to induce tolerance towards an antigen.

So, this PS media to the fact, we found to be antigen specific. When Factor VIII treated mice were re-challenged with non-crossed reactive antigen truvalvomin, the animals responded normally.

And we believe that this antigen specificity could be coming from regulatory T-cells. Three different experiments supported this role of regulatory T-cells in this process. For example, adaptive plants of regulatory T-cells from unimmunized mice to mice. When this recipient mice was re-challenged with Factor VIII, these animals also showed hyporesponsiveness.

At the molecular level, we found out

this antigen is a (inaudible) is driven by TFG beta and one of the PS receptor called TIM-4 receptor.

So at pre-administration of an anti-TIM-4 function blocking antibody reversed the PS media to the fact, clearly showing that the role of TIM-4 antibody in this process.

So overall, if you expose any antigen to phosphatidylserine, in the presence of phosphatidylserine to mature dendritic cell, it innovates this maturation, and then with the help of TGF beta, these tolerogenic dendritic cells produce regulatory T-cells and that can block the B cells.

So harnessing this particular -- harnessing this particular understanding of how PS work, we tried to develop an immunotherapy platform, tolerance platform.

So, just a couple of highlights about TIM-4 receptor. So out of 13 different PS receptors, TIM-4 is specifically expressed on antigen-presenting cells, engaged in the integrity

pathway for their intracellular signaling to induce tolerance.

And an additional interesting study that showed that TIM-4's receptor is sensitive to the PS surface charge density.

That means TIM-4 receptor, this tolerance inducing receptor, engages only involve if the cell debris exposed phosphatidylserine above a threshold limit.

So based on that, our working hypothesis became that increasing surface charge density will increase the tolerogenic potential by engaging the TIM-4 receptor.

For this to design a platform, an antiparticle platform, or a T-reg adjuvant, then we need to understand the structure/function relationship of phosphatidylserine.

So what is the structure of phosphatidylserine? It has a polar head group that is O phosphatidylserine. It is connected to the acyl chain through a glycerol backbone. And structural variations can include two or one acyl

chain. If it's one acyl chain, it's called lisophosphatidylserine. And the number of carbon atoms in the acyl chain, a number of double bonds in the acyl chain, would give structural variation.

In the interest of time, I'm going to talk only about O phosphatidylserine, the head group of phosphatidylserine that is the minimum structural requirement for engaging the TIM-4 receptor that will affect the T-reg adjuvant.

So we investigated the use of OPLS in reducing the immunogenicity gains to several molecular antibodies in the presence of volume expanders, like hyaluronidase.

As I mentioned, the hyaluronidase break down the hyaluronic acid. It allows larger volume of therapeutic protein could be injected. But it recently showed that it increased the incidence of immunogenicity with molecular antibodies. So we wanted to check whether it can reduce the immunogenicity for cantuzumab, altumomab, and rituximab in the presence and the absence of

hyaluronidase.

As you can see, the OPLS treated animals showed significantly lower ADA titers against the protein and also reduced against the volume expanders as well.

That correlated with a reduction in the plasma cells and also showed increase in the effective T-cells and also -- but surprisingly, it did not increase the exhaustion marker, but did increase the production of regulatory T-cells such as lag-positive T-cells that correlated with (inaudible) experiments that showed that increase in the regulatory T-cells.

So mechanism is that if you inject the protein in the presence of O phosphatidylserine it can expose it to therapeutic protein to the dendritic cells in a tolerate context that can produce regulatory T-cells that can reduce the antibody levels.

So summarizing my talk, sorry, probably I went a little bit overboard, but that immunogenicity in the context of protein

therapeutics is unwanted immune response that manifests as ADA response that impacts efficacy.

There are several factors that influence the immunogenicity and subcutaneous route of administration is an important treatment related factors, and readout of the proposed mechanistic understanding of mechanism of what drives subcutaneous immunogenicity.

And we used it for two purposes, to develop risk assessment, somewhat validate it. Still it's a process, ongoing process. And rational development of innovative strategies.

And then with this, I would like to thank funding agency, National Institute of Health, supporting this research, and taxpayers like you, and the hardworking students at facilities, collaborators, and mentors. This is my disclosure statement.

Thank you. I will be happy to answer any questions you have.

DR. MAZOR: Hi, I'm Ronit Mazor from the Office of Gene Therapy. Thank you for the

presentation. It was very interesting. Can you hear me?

DR. BALU-IYER: Yes, I can hear you.

DR. MAZOR: Okay. So I was very intrigued by the mechanism of how the phosphatidylserine induces tolerance. I'm thinking about a lot of therapeutic proteins whose mechanism of action is a apoptosis and those are still immunogenic, thinking about a lot of cells that are undergoing apoptosis and we're still seeing immunogenicity when we have other proteins in there.

So can you elaborate about the mechanism and why you're convinced it's that and not the nanoparticle that have tropism to immune cells?

DR. BALU-IYER: Okay, that's a great question. Because the other things, they don't have phosphatidylserine in them. So I strongly believe in the phospholipid. It's a superhero.

I think it is adding phosphatidylserine using antigen specificity. Antigen themselves may not be able to achieve this. People tried

exosomes and exosomes also expose phosphoryl feeding that could be used.

So apoptosis exposure to phosphatidylserine that too in the apoptotic context is only tolerance inducing. There are other ways of PS exposure happen in many other ways.

That's where we started doing the structure/function relationship. And then there are certain biophysical characteristics and structural requirements for an antigen specific tolerance.

If you look at the -- there are 13 different receptors. So if there is not PS exposure at setting threshold limit, other 12 receptors that take the PS exposing cell debris or anything, and they clear them with no immunological consequence.

But coming back to a question, that's an interesting approach that induce apoptosis with therapeutic proteins. And then if therapeutic proteins found its way to the cell memories,

exposing phosphatidylserine, ensure that that will also induce tolerance.

In my opinion, they are not becoming part of the cell debris. That's my understanding. As I told you, most of the time, I'm wrong.

DR. MAZOR: Thank you. So if I understand your thought, you would assume that anti-cancer therapeutic agents that work through apoptosis should be less immunogenic than one that works in other mechanisms of action?

DR. BALU-IYER: It should be. But problem with getting this kind of disease situation that they are used for a very short time, and also getting immunogenicity for a chronic condition is very nicely explored.

But for shorter duration, it's very challenging. But it could, as I told you. It becomes a part of apoptotic bodies or exosomes by some means, it could produce tolerance.

But already, the patients are immune compromised. Normally, they tend to respond less. So these are all multiple things. Probably, I did

not answer your question. I'm sorry if I did not.

DR. MAZOR: Thank you.

DR. BALU-IYER: Thank you.

DR. ATREYA: Thank you for a wonderful talk, as always. I was looking at the model, in your model, one of the things that's missing for subcutaneous is the stroma cells, right? How do those figure in and how during periods that might trigger the stroma models, how do you take those into consideration?

DR. BALU-IYER: That's a fantastic question. Because we need an immunological trigger in this model. That's one that triggers it.

Now as you look at it, the system is very, very simple. It lacks many components of subcue space. So we created what was lacking in the subcue space. That's one use of immunological triggers, stroma cells.

Let me add a little bit of a trigger mechanism, like LPS in the system, so that it is not enough to mature the dendritic cells but it is

enough to produce the trigger.

DR. ATREYA: That would make it -- so we know that different KR ligands or OPR ligands can synergize. So how does that figure into your model if you're already spiking it with LPS? You may be exaggerating some of the --

DR. BALU-IYER: Yes, that's the way I think it requires more validation. That's the correct point. I do agree.

But we did so much of LPS concentration optimization. That's what the advantage is. Last one, I did not mention, is the disadvantage. There's quite a lot of validation. But we did this.

So majority of the time, it did not increase the immunogenic potential of it, but it requests more validation. That's what we are expanding, or getting more therapeutic proteins that showed clinical immunogenicity and cross validate it.

But it can build into some kind of immunogenicity database. That's what we are

trying to do.

DR. ATREYA: Thank you. So another question that I have, that other people have too, is that you centered on proteins and not peptides. Have you looked at peptides and whether your model can predict immunogenicity with those?

DR. BALU-IYER: That's a great question, and some of these things we just started. It's not ready for prime time.

That is unnatural amino acids containing peptides. We are working on it in collaboration with some pharma companies and the Center for Protein Therapeutics.

And it does predict, but we have to repeat it a few times to make sure it is kind of reliable.

DR. ATREYA: Okay, and one last question. So, in your model, you think that's it PS that in? And then I was thinking back to the GLP1 simulators and the ones that are least immunogenic have fatty acids but not PS. So how much impact of fatty acid in your tolerance, do

you think? Because you think it's more the head, right?

DR. BALU-IYER: That's an interesting question, and I can talk for another 30 minutes on it. But the thing is, what happens is, if you look at this, we did some bioengineering type of thing.

If you look at the assay, it is very important. That's what I carefully mentioned, that it is the minimal structural requirement for receptor binding.

But if you look at the phosphatidylserine shape, when you reduce them from two acyl chain to one acyl chain, the shape changes from a cylinder to a cone shaped structure.

So when you have a cylinder, how you pack the matches in a matchbox, they pack really well. But if you put a cone in a PC bilayer, a phosphocholine bilayer that is cylindrical, they don't pack very well. They just increase the curvature.

The curvaturing flips. More and more PS comes out from the inner leaf flap to the outer leaf flap, and that's where we achieve the PS exposure above a threshold limit.

So to answer your question, acyl chain one, acyl chain and one and saturation does the trick. The others did not. I can share the data with you.

It need to be in writing, manuscript, but I'll be happy to share the data with you.

DR. ATREYA: Thank you very much.

DR. BALU-IYER: Thank you.

DR. ZHOVMER: Hello, Alex. I'd like to extend the question that Ronit asked about tumor cells. I think of it about tumor cells. They are making the cells (inaudible) against the tumor new antigens and interfering with tumor cells may actually stimulate renewed response.

DR. BALU-IYER: That is a good one. I forgot to answer a question. Nice that you brought it up. So we looked at how tumor evades the immune system, okay?

The same thing, parasites, how they evade the immune system. They all do it by exposing or releasing exosomes containing self-antigens.

One commonality of all these things is the exposure of PS. So PS, phosphatidylserine, and then this phosphatidylserine based immune tolerance, like how parasites evade the immune system.

And we published a paper on tumor, how tumor evades the immune system by giving out exosomes, exposing phosphatidylserine containing tumor antigens. That's how they evade the immune system.

Because of that data set, regular tumor enrollment happened in the microvicinity of the tumor cells. That's just an answer to your question, too.

So it does. It can happen. So in order to do that, we flip the other side of this technology. What we have is called Exoblock (phonetic). So we have an anti-PS antibodies in

small molecules to block the PS in the tumor vicinity to have -- it's like developing that as an amino therapy.

DR. ZHOVNER: Thank you.

DR. ELKINS: In the interest of time, one online question. Factor VIII find phosphatidylserine on platelets, is the phosphatidylserine plus Factor VIII still functional?

DR. BALU-IYER: Yes, it is functional. It's a very specific question, yes. It does. And we did the activity assay PTT and then intercell that is in vivo assay with the hemophilia mice and hemophilia dogs. Everywhere, it's found to be very active.

But the interesting thing is that phosphatidylserine stabilizes the activated form of Factor VIII, which is thrombin, which is three times more potent than the regular Factor VIII.

So because of that, it not only pursue the activity, it increases the potency of Factor VIII. And it also works with other therapeutics.

We have shown for several other therapeutic proteins that phosphatidylserine need not bind to the therapeutic protein to see this effect.

DR. ELKINS: That will have to be the last word.

DR. BALU-IYER: Thank you very much. Thank you very much for this opportunity. It is an honor to be here. Thank you.

DR. LAGASSE: Okay, for the next part of our session, we have two speakers from CBER. We'll introduce both of them first and then we'll have the question and answer period after both speakers give their presentations.

So the first speaker is Dr. Zhaohui Ye. He's a Principal Investigator and Chief of Gene Transfer and Immunogenicity Branch in the Office of Therapeutic Proteins here at CBER.

He's responsible for CMC review and policy development and cell and gene therapy products, and he has an active research program on stem cell engineering.

And following Dr. Ye, we'll have Dr.

Robert Daniels. He's a Principal Investigator in the Laboratory of Pediatric and Respiratory Viral Diseases in the Division of Viral Products at FDA.

And his primary research focuses to increase the breadth and efficacy of annual influenza vaccines by developing approaches to introduce neuraminidase antigens into influenza vaccines.

So please join me in welcoming Dr. Zhaohui Ye and Dr. Robert Daniels. Thank you.

DR. YE: Okay, thank you. So I know most of you are here for the flash talks, I guess, so I'll do my own version of flash talk and tell you the story what we did here in our lab.

So as Daniel said, I'm from the Office of Gene Therapy. So why do we do genome editing? That is because this has some relevance to our product.

So there's a wide variety of a product that come into our office. To your left, you have the ex in vivo modified cells. Those are like genetically modified stem cells and T-cells such

as CAR-T cells.

And then to the right, you also have the in vivo delivered, direct administered lectures that get carried into transgenes.

So in the last couple days, you have heard some excellent talks on AV vectors and lipid nanoparticles.

So what's common about these products, so all these products can potentially incorporate genome editing. So either having the genome editing as one of the main modes of action for the drugs or if nothing else, the genome editing can be used in the development of the drug product.

So by now, we are all familiar with genome editing tools in general. So these are the tools for making precise additions, divisions, and alterations to the genome in living cells.

But there's more than just CRSPR CAS 9. And there have been other designer nucleuses like CFNs or Talens before CRSPR come on stage, right?

And right now, there's now all type of editors such as base editors and prime editors, as

well as other tools that are actually not showing on this slide.

So this is rapid expansion of technology tools. It's very important for all the stakeholders to actually understand the specificity and the efficiency of the genome editing tools.

For the gene therapy part, actually, it's important to understand, there are potential to achieve the therapeutic effect, and what's the risk of intended genome modifications as well as the long-term effects of the both on-target and off-target genome editing.

So there are many technologies methods can be used to evaluate genome editing tools. And today, I am going to focus on some of our past research that used high group sequencing, or next gen sequencing, NGS, in the study.

And I'll share a few things we learned along the way. So we're going to talk about some differential activities between different types of endo-nucleus.

The example I will give you here is the Talens versus CRSPR CAS 9 as well as different versions of certain types of editors such as base editor.

We'll also discuss what the cell type influence editing outcomes and impact of the other components, the non-nucleus editor components, on editing specificity.

Because right now, the editors look at it more complexing post-structure and the functionality.

Okay, so before we start over the first example, let's go over quickly how the so-called conventional genome editors work, how they handle genome editing efficiency.

So, this combination of nucleus, what they do is they create a cut, generate a double strand break at the genomic sequence.

And what happens, and to follow this, it depends on the cellular machinery, heavily depends on the machinery, such as what kinds of components of the machinery present in the cells at the time

of this break was created.

So one way for the cells to fix it is through this new homologous enjoining, and the results are quite open for the end source conditions, or the endose (phonetic).

These can be relatively random. And the other way the cells repair it is through the homology needing the repair or the HDR if there is a donor template that has the homology sequence, the DNA sequence, near the double strand break.

So these HDR needing the repair usually are more precise so you can put exactly what the sequence you want because the end of endose right can be random.

Now just because it's random doesn't mean it has no therapeutic value. As many of you know, the first and current VD, the only approved genome editing product, Casgevy, actually relies on the ability of CAS 9 to create an endo at the cat one binding site in the PCL 11 aging.

And by doing this, this abolishes the finding of the cat one to PCL 11 A and increase

the (inaudible) production.

So the HDR also have wide applications. One example here is that you can use HDR to first knock out a -- I'm sorry, let's go back -- to knock out the TCR gene, and in the meantime, you insert your transgene, in this case a car transgene into the TCR locus.

And this is commonly used in the heterogeneric CAR-T production. So the first story I'm going to tell you is how we studied the differential activities that were used by Talens and CRSPR CAS 9. So this is a study we did quite a few years ago.

And so here is the study design. We targeted three genomic load sites, right? So the first one is the JAK 2 gene at where the JAK 2 V67F occurs. This is one now of the most common mutations in hematological malignancy.

And the second one is the SERPINA 1, also called alpha antitrypsin, at the site of the AT, so called the PIZ mutation, which caused the alpha antitrypsin deficiency.

And for this reason, because we're targeting the disease, really the mutation, we actually used patient-derived IPSCs which carry this specific mutation.

And the third site we target is the AVS1 site. As many of you know, this is one of the commonly used safe harbor sites for integrating transgenes in the cell.

So, in parallel to delivering this genome editing endonucleus into the cell, here we also have a control arm and deliver the homologous donor.

So one example for the AAVS 1 was showing here, you have the left arm, right arm. There's a homology to the sequence around the cutting site.

So we then use the amplicon sequencing, the deep sequencing, to analyze what happened right around the cutting site to determine what kind of endos and what endos occurred at this cutting site.

And also the frequency in the case of

Talen or CRSPR CAS 9 mediated cutting. So you can see here, actually, we were kind of surprised. You can see a big difference that the CAS 9 mediate induced a large amount of a much higher level of endo at this load site as compared to Talen.

So at least tenfold highs or even much higher. But in the meantime, if you look at the HDR efficiency, they are not so different. In fact, they are quite comparable to each other.

So this tells you that when choosing the editor, you need to really consider what's the intended outcome. So the editor really matters.

So in this case, the CAS 9 used to be at a far greater endo induction than the -- or the gene disruption in the case of such as the Casgevy, right, than Talens.

However, in this case, the efficiency of immediate in the HDR seems to be quite equivalent.

So we then also look into how the cell type affects, in this case, the endo frequency. So we compared elementary T versus the IPSCs. And

again, in addition to the target site, we also -- I'm sorry, target site, we also analyzed, used the deep sequencing to analyze those incomatic (phonetic) predicted most likely off-target site, 14-15 of them.

So each has their risk. This indicates that there is a statistically significant increase in those, in the experimental group, as compared to the mock transfection.

So you can see there is a lot of at risk here in the 293T cells compared to the IPSC. So, that tells you the cell type difference, it may influence the outcome.

And this also correlates to the on-target efficiency as you may notice here. The on-target efficiency in the 293T is also in this case about four-fold higher.

If you look at the AVS 1 site, the difference is even much striking. So the 293T, you have over 40-fold higher of endogeneration than the IPSCs.

And also, again, just like the JAK 2

site, you'll see a correlation of increased off-target editing when the on-target editing was also higher. You'll see this correlation.

Okay, so, now I'm going to switch gears. In addition to the targeting the deep sequencing, here's a story how we used the whole genome sequencing in odyssey.

So the so-called unbiased sequencing to study editor specificity. The example I'll give you here is the cytosine base editor. So as you know, the base editor is a fusion protein, right, of a catalytically impaired CAS 9 that fused to a cytosine deaminase.

In this case, there's a before, CBE base editor is -- this deaminase domain is APOBEC1. So when this enzyme encounters a single strand DNA which created by this CAS 9, the cytosine that we think is rich, the window can be converted to a uracil, right?

This uracil during the DNA replication will be recognized as a T. That's how the C2T mutation happens.

So the reason that we jumped into this study was there was concerns. Around 2013, there was a group of studies that found this APOBEC morphogenesis pattern in human cancers. And they also identified it as so-called APOBEC mutation pattern.

This TCT or TCA motif. So when this APOBEC domain that deaminase was effused to a dCAS where it's always pressed that also caused this unwanted mutation.

So to answer that question, because from those past study, this APOBEC mutation is kind of random. There's no hot spot. So how do we locate them?

We can now use targeted deep sequencing. So the only way to look at it is how genome sequencing is unbiased way. However, whole genome sequencing also has limitations such as sensitivity, right?

So I think this was discussed in yesterday's metagenomic session. So one way to overcome this is we use the approach that whole

genome sequencing of the single IPSC clone, aggregated clone.

So here is the overall approach. We added in the cells and the clones, identify those ones that have been successfully edited at the on-target site.

So this is where we published the study. So I will go quickly. And we have found that some of the clones have significantly increased numbers of C2T mutations.

So what they show you here, each one, each bar is a clone, and each color represents one type of mutation. The red is the C2T notation. So you can see some of the clones are almost dominated by the C2T. The absolute number is also much higher than the control clones, which I forgot to mention first, these are the control, what you expect to see, a non-edited IPSC clone.

And also, if you analyze those mutations, that we also identify these local APOBEC with the genesis signature.

So, and these mutations, when you map

them onto the genome, they are evenly distributed among the chromosomes. So here is the chromosome size. You can see they pretty much just randomly distributed around the chromosome.

So, which means that to identify them, an unbiased matter has to be used.

Okay, so what about the other version of cytosine editor? So, here's another version or variant of the CBE, the so called CBE 4. It's created again by Dr. Debbie Lu's (phonetic) lab.

So this one only has two mutations, right? The mutations on the APOBEC domain of this editor, these two mutations.

And in their study, they also, when they look at 293T cells, they see a reduction of the C2T mutation. We see the CAS 9 binding independent of the mutation.

So what about in the IPSC? So we used the same approach. And here's the results. Again, each bar represents one IPSC clone. In this case, we also included an analysis of the adenine base editor.

So this one has been from our study as well as many people's reports as a much higher fatality. So they have a less unwanted off-target effect.

So, as you can see, when it's compared, the YU1 version compared to AV, they are pretty comparable. It's also where the level is similar to the control clones. However, in the C2T mutation, in this group, you do see an increase in the C2T mutation, in the YU1 edited clones.

Now, here is a summary of the mutation type, right? Again, the red one is the C2T mutation, but you see a slight increase but it's not to the degree of the pre-edited version of four, which some clones pretty dominated the mutation landscape.

And also, when you compare the absolute numbers, so here's the previous three or four, you can see the clone. Some clones, the mutation total numbers are pushing from 1,000 to over 2,000, but in comparison, the other version of the CB, you have about tenfold lower.

So this also tells us the different version of the same type of editor can give you a quite different off-target profile.

And so that's basically the summary. So from using those high group sequencing, we have found that different genome editors may trigger different cellular response and that the editing efficiency specificity can often be cell type dependent.

And also, here is just an example of how the NGS can be important for evaluating genome editing. So such as the target of the sequencing to understand the off-target effect, and the genome unbiased may be needed for certain editors where you don't know where to look, such as in the cytosine base editor.

So, here's all the people from our lab and our collaborators who have contributed to these studies. I'll be happy to answer questions at the end of the session.

DR. LAGASSE: Now, please welcome Dr. Daniels to the stage.

DR. DANIELS: Okay, before I start, I'd like to thank the organizers for giving us the opportunity or me the opportunity to present some of the work from our lab on an assay we developed for assessing influenza neurominidase.

So as many of you know, there's been a big push in recent years to make more efficacious influenza vaccines.

And one of those strategies is to incorporate the neurominidase antigen, or higher amounts of neurominidase antigen into the vaccines, but currently we don't know which NA should go in the vaccine or which strategy is most optimal. And we hope this assay can start to help assess these two questions or address them.

So what I want to do really briefly in this talk is introduce currently influenza vaccines, the benefits of incorporating neurominidase antigens, and then the main barriers that currently exist for adding NA antigens to these vaccines, and then just give a brief snapshot of our current work on the assay

development that we've been doing for selecting vaccines transfer to neurominidase, which ones should we actually use, and then how to profile those responses in say a vaccine trial in order to try to identify a correlate of protection.

So if we look at influenza vaccines, there's tons that have been licensed. If you're not aware, you can actually request specific ones. I like to tell my parents this.

They're all produced via three different platforms. One is viral based, and what I've shown here is just four different viruses of the previous quadrivalent vaccine.

And these would be made attenuated and this would be something you would find in flu mist. And then the more predominant vaccines that are on the market are inactivated or split versions of these viruses, where all we've done is treat them with detergent.

And the more recently, there's been a recombinant protein based vaccine that just contains HA.

One thing to keep in mind is the viral based vaccine through either propagated in eggs or more recently in cells, and the recombinant proteins are made in insect cells.

And the product qualities are controlled by these systems. So in case of viral based vaccines, the amount of NA that you would have in the vaccine would be dependent on the viral -- the property of that particular virus, whereas in a recombinant approach, the product is controlled by the cells or the design of the protein.

So for instance, there's no NA in the recombinant HA vaccine. So as you're well aware, seasonal influenza vaccines show variable efficacy that ranges borderlines around 50 percent, but varies by season.

And as I alluded to in the intro, one strategy to improve them is to try to actually incorporate the NA antigen which can have these types of benefits.

So current vaccines focus on hemagglutinin or HA that's on the cell surface.

The function of HA is to bind a sialic acid, mediate viral entry, and ultimately fusion and delivery of the viral component genome to the host cell. And that's the main target of current vaccines.

NA has an opposing function, and its role is to cleave sialic acid to sort of promote the mobility or release, people like to refer to it as the gene that promotes release from the infected cell, by eliminating the ability of HA to persistently bind.

So what we know from literature back in the '70s is that existing NA antibodies in a human clinical study could reduce the infection severity. And we also known from a follow up study that an NA-based vaccine could reduce illness from a challenge as well.

The other benefit of having NA in a vaccine is cross protection. So what I'm showing on the left is the nine subtypes of NA versus the subtypes of HA. That gives you 144 combinations. And the current vaccines that focus on HA give us

about -- cover about 18.

If you just include NA in this IV-based vaccine, you would have 46 of the 144 combinations. So you'd approach a third of the entire space.

The other example is in antigenic drift, which is where this assay we've developed comes in handy. Antigen drift just means these proteins evolve over time.

So for instance, in this example, each bar is showing when the antigenicity of the particular antigen changed.

And as you're aware of, vaccine strain selection, that occurs say six to nine months before the actual season. So on the left, we have identified the selected strain, and by the time we administer it nine months, six months later, you can see the HA is now antigenically distinct but the NA is still the same. So you can have a benefit from that.

So the main barriers for NA vaccine is really simple. One, what's the optimal way to put

NA in a vaccine? It's not to make a viral based vaccine.

Viruses by definition usually have at least 10 to 1 HA to NA content. We have looked at that. So what we've shown in one publication is you actually can modify the NA content of vaccines just by changing the viral premise.

I think we increased four-fold, but it's still not probably sufficient for a vaccine. The more common strategy is to supplement those vaccines with say recombinant NA, and that was introduced by Ed Kilborne's group back in the '80s. And we've done some work with that as well and then more recently in MRNALNPs.

And then lastly, we need an antigenic assay for selecting or identifying a suitable NA for that vaccine. And so when you just think of circulating virus diversity, which NA should actually be in that vaccine?

Currently, people use the NA that's in a strain that's selected based on HA antigenicity, so it doesn't make any sense because they can be

different.

So here's a little bit of our work on this NA active site proximity assay for measuring NAI titers.

So if we look at NAI or neurominidase inhibiting antibodies, they basically prevent cleavage of sialic acid.

So NA is an enzyme that cuts sialic acid. What I'm showing here is a small reporter substrate. It has sialic acid in purple. It has a fluorescent molecule blepharon on the right.

And so when that molecule is processed by NA, you get fluorescent signals. The issue with this small substrate is if you put anti-sera mouse, anti-sera on these viruses and measure NA activity, I'm showing you on the graph, you can see you don't get much inhibition.

So what people have known for a long time is that you need large substrates, so they use glycoproteins for measuring NAI antibodies. So glycoproteins have branch structures of adenine glycans, or have sialic acid on the end.

And so theoretically, if you have an antibody that binds around the active site, it would sterically block the ability for the enzyme to bind that sugar. And that's what you see over on the right. All of the sudden, the same sera will give you good inhibition of the enzyme.

One of the main issues with this assay, and I think people overlook it quite a bit, is that you're using a multivalence substrate. You're also using a virus.

HA targets the virus to the multivalence substrate, which gives you an increase in the apparently NA activity. So any HA antibody that blocks receptor binding is going to give you a wonderful NAI titer, and that's what I'm showing you here with three different monoclonals. You can see the titer is 40,000.

So what we did is try to address this. We sort of took this principles all into account and we created a simple assay that I'm just going to refer to in terms of how we do it.

We take a virus. We add one reagent.

We had sera. We incubate. We add another reagent. We incubate. We add another reagent. We measure. We're done.

Here's the results from the assay. So on the left, we're showing ferret anti-sera again, the same exact ferret sera I showed you before, and one additional sera.

And you can see that we get a signal increase with the black and the red sera that are against DNA. And we see no signal increase with the negative sera.

If we reuse the same monoclonal antibodies that gave you a 40,000 titer, in Ella, it gives you zero in this assay. So it removes the HA-dependent effect.

To prove that even further, we took a bunch of reassortant (phonetic) viruses that have H6 in the particular vaccine strain NA, and then we mixed them with the exact same ferret anti-sera, all generated, all have H6 antibodies.

And you can see that we get signal only for the ferret sera that matches the NA in all of

these examples.

We went a step further to say, hey, how did this coordinate with Ella? So we took a panel of 27 monoclonal antibodies. We ran Ella, we ran our assay. You can see the titer difference on the table. I think we had three deviations, and they were generally where we saw Ella titers around 30 or 40.

But you can see the correlation plot is close to a line. It gives a Pearson correlation coefficient, or an r squared, of.81. And that's for N1, N2, and type B, which are in a vaccine.

Then we ask, can this provide antigenic data? So, to try to simplify this, we just have five viruses. They're from 2007, shown as like Brisban 07, California 09, Michigan 15, Brisban 2018, Victoria 2019.

And then we have ferret anti-sera against all these NAs. And then we run the assay. And essentially, if you get the same curve, you're antigenically similar. If you get a distinct curve, like for Brisban 07, you know you're

antigenically distinct from the other anti-sera.

So in the top left, you can see Brisban as antigenically distinct from the earlier --

From the more recent NAs. You can see California as antigenically distinct from the previous Brisban 07. And then you can see that Michigan 15, Brisban 18, Victoria 19 are all antigenically similar.

The reason we did this is because we previously looked at these exact same NAs with Ella and you can see the correlation or the pattern of the results is almost identical. And this is done in an afternoon and not in four days.

Then we took human sera and we did a blind analysis. So we got these from our collaborator at NIH. They sent over sera that was from a clinical challenge study.

And we measured. We used our assay to measure steric inhibitory antibodies in black as well as active site inhibitory antibodies, or enzymatic ones in blue.

And then once we were done, we got this

Ella date from them. You can see when you combine the two different phenotypes there's a clear pattern match. The interesting thing to us was the high prevalence of active site antibody inhibitors in humans.

You can see black bars are steric. You can see people that only have steric, like patient three. You can see people that only have enzymatic, like patient five. And then you can see people that have both, like patients eight, nine, and ten.

And so now you can start to use this mechanistic insight to ask do one of these or both of these correlate better with protection in humans or when you're doing a vaccine design, which type of response are you actually aiming for?

This is not what you would see with naïve animals. You would basically see black bars everywhere. So you have to also ask how much is this naïve animal informing us on what's going on in humans, as many people have alluded to.

So the conclusions, I think this assay, we clearly demonstrate that it can provide NA inhibitory antibody measurements. It can be used to assess NA antigenic changes. Think of this as it's automatable.

You can use the exact same reagents that the WHO collaborating centers use for HA antigenic drift. They have anti-sera. They have the strains. You just run our assay instead of theirs.

You're compatible with the same reagents. That's what I just alluded to. It distinguishes between steric and enzymatic NAI antibodies, something Ella can't do. It just goes down.

And then we believe that you can implement this to help identify suitable NAs for a vaccine and also profile those responses.

And finally, I would just like to thank all the folks in the lab, Jin Soma, Galina Mira, Goa Tahir and Sylvie have contributed to this presentation, or all the work here, because I certainly didn't pipette. And then a whole bunch

of collaborators. And thank you for your attention.

DR. LAGASSE: At this point, we'd like to have both speakers come to the table and answer questions. Are there any questions online?

DR. ELKINS: While folks in the room think about it, I have the online questions. And also, people who are doing flash talks while we work through questions, if you could cluster and differentiate in the upper righthand corner of the room and get ready for the flash talks, that would be helpful.

All right, online. For Zhaohui first, how is the transfection efficiency normalized for the comparison of CAS 9 efficiency and HEK293s versus IPSCs?

DR. YE: Right. Great question. So in this case, it was not normalized. But that's a valid point, especially when you're comparing the on-target antigen efficiency. That's a very valid point.

But the one thing we're trying to point

out is that the correlation between the on-target and the off-target effect, but that's a good question. In that particular experiment, it was not normalized.

DR. ELKINS: All right, and then I'll cluster the ones that are related to CAS 9. Do your results for Talens versus CAS 9 suggest that Talens are actually a better approach for gene repair or gene insertion, given the strong selectivity for Talens to induce HDR over INDL formation.

DR. YE: Is that a question or comment? So our data doesn't really suggest Talen is better, right? So even in terms of HDR efficiency, CAS 9 is as good or you'll actually see the AVS 1 side, you have about two-fold higher efficiency than Talen.

Then again, one point that we're trying to make is it really depends on what your intended purpose is for your therapeutic drop keep element, right?

So in a Casgevy case, then you actually

want to add, to be able to abolish the gotten sequence. So in that case, at that particular site, the CAS 9 might be a better editor.

DR. ELKINS: Okay, and then a couple of versions of the same general questions. Why does CAS 9 demonstrate different efficiencies in different cell types?

DR. YE: So one thing is that I think the first -- so similar data we presented as the first question alluded to that there is main release to the transaction efficiency.

And also, when you think of this, editing may also relevant to epigenetic states, right? How it is accessible to the editors as well as the overall population, where the cells are, what percentage the cells are in which cell cycle.

So that all contributes to the difference. I forgot the -- the question is about cell type, right? Yes.

DR. ELKINS: Right, different efficiencies in different cell types.

DR. YE: Yes.

DR. ELKINS: All right. Thank you. Switching to Rob. Since influenza infection itself is supposed to induce NAI antibodies, in an adult challenge study, why do only less than 50 percent of adults have NAI titers as measured by the NASF assay? And is the NASF assay, does that assay have a lower sensitivity than Ella? Perhaps those two go together.

DR. DANIELS: Sure. I think one thing to look at is the quantity of the enzyme that's in the assay is going to dictate your sensitivity.

Ella is done for 18 hours at 37 degrees. Our assay is done at 10 minutes. We can go to 2 hours to increase it by tenfold. That'll match it to Ella, and we can go overnight and decrease it by 100 fold.

So I'm pretty sure if we just do an overnight incubation, we would get higher titers than we've shown there. And the consistency of the titers across the NA amount, if you go to longer incubations are basically, it's completely

consistent where Ella's changing titer based on the NA amount that's unavoidable.

DR. ELKINS: Yes, and there's another question about the relative sensitivity of the two assays. So is there anything more you want to say about that?

DR. DANIELS: Yes, so that was the sensitivity. So I think you can match sensitivity easy by just prolonging the incubation.

We spent a lot of time developing it just to show it works. The last one was in a human challenge study, what do you see?

Well, interesting. So if we do Ella, we have no idea what we see. We see something go down. So when you use an assay that differentiates between enzymatic inhibition versus steric inhibition, you actually learn something.

You learn that you have two different types of antibodies. We hypothesize that you would have steric based antibodies in pediatric populations, and over time, you would start to develop these NA inhibitory antibodies.

The most interesting thing about this is there's several recent papers that seem to allude to the fact that it's difficult to find NA inhibitory antibodies or ones that bind the active site.

And we actually have no problem finding these, either in humans or in monoclonal antibody screening. It's just doing a clever way of looking at the assay.

DR. LAGASSE: Are there any questions from the room? I have a question for you, Rob. What do you think are the limitations of your NASF assay as far as what would you do as far as potentially making it better with a second generation?

DR. DANIELS: I didn't show this. So the first one that we did, we used a particular binder which had varying affinities across NAs.

So it had a one step ahead where you have to identify the binding affinity. We've actually done in collaboration with a chemist, put together a ligand that has pretty much the exact

same affinity for all NAs. So you can use just a fixed amount for everything. And then you can modify the, let's just say the footprint, so that you can get information on how far away is that antibody from the active site. So that's the second generation that's in the pipeline.

DR. LAGASSE: Yes, thanks. Well, if there's no more questions in the room or online, I would like to thank everyone. And we're going to move on to the next section of our session.

We'd like to thank the speakers, of course. So the next section of our session is the flash talks, where we'll have a number of speakers come up and they will each have about two or three minutes to -- two minutes to go through their talk.

So I'd like to welcome the first -- well, all the speakers, they'll just come up right after the next speaker. So the first speaker is Dr. Jankowska.

DR. JANKOWSKA: Good afternoon. My name is Katarzyna Jankowska. I'm working in Dr.

(inaudible) lab. And here is the title of my talk and poster number is four.

The central premise for incorporating synonymous variants into the genetic sequences of optimizing biopharmaceuticals and gene therapies is that this changes in other protein primary structure.

Nevertheless, the current studies show that even synonymous variation may impact MRNA expression and protein confirmation, which may lead to protein deficiency and disease manifestations.

We reported comprehensive in silicon (inaudible) assessing the impact of single synonymous variants on ADAMTS 13, and highlighted numerous variants that can affect protein functions.

ADAMTS 13 is the protein which is essential in (inaudible) studies that this deficiency may lead to life-threatening disease called thrombotic thrombocytopenia purpura.

Our recent studies predicted that nine

of ADAMTS 13 single synonymous variants can affect the binding size of (inaudible) and that is including the variants 972C2T that was predicted to gain the binding site to microRNA 221.

To evaluate the effect of synonymous variation under all of microRNA in ADAMTS 13, functions the binding of microRNA 221 to ADAMTS 13 coding sequence were validated by luciferase supported assay and in cellular systems.

Our studies demonstrated that microRNA 221 can modulate ADAMTS13 genome protein expression, which can be further disturbed by a single synonymous variation or code optimizations.

This study suggests that any genetic variation may also affect microRNA binding to ADAMTS 13 and demonstrate the potential impact of single synonymous variants in modifying important characteristics and disease severity.

Thank you so much for your time and attention. I will be happy to take questions during the course.

DR. LAGASSE: Please welcome Dr. Oakley.

DR. OAKLEY: Okay. Hi, my name is Miranda Oakley. I work in the Laboratory of Emerging Pathogens. I conduct research on malaria and Babesia in Dr. Sanjay Kumhar's lab.

So cerebral malaria is a major cause of malaria mortality and occurs mostly in young African children. There's an urgent need to develop adjunctive therapies that can reduce the high mortality rate from cerebral malaria.

There's a very good mouse model of cerebral malaria. It's the plasmodium Berghei ANKA model. And it's used to study experimental cerebral malaria in mice.

The objective of our project on my poster is to preform single cell sequencing of pathogen brain sequestered CD8 T cells during experimental cerebral malaria and P Berghei ANKA infected mice.

Our experimental design is that we infected susceptible C57 BL6 mice with a million parasites and then brain sequestered leukocytes were prepared from profused brain tissue so that

we look at the CD8 cells that are binding to the endothelium and are pathogenic.

And three groups of mice are uninfected controls are mice that are infected but non-

(inaudible) and then our infected mice that actually exhibit symptoms of cerebral malaria.

After we prepare these brain sequestered leukocytes, we performed single cell sequencing on 40,000 cells from five mice using the BD rhapsody single cell analysis system.

I have a lot of results on my poster. Poster 8, I will highlight three of these results. We found that brain sequestered CD8T cells are heterogeneous rather than homogeneous population.

We have more than nine different clusters that sequester in the brain. Second, we looked at differentially expressed genes comparing our mice with cerebral malaria to our infected mice without cerebral malaria, and comparing our infected mice without cerebral malaria to uninfected controls.

And then lastly, we've created this transcriptional atlas of the pathogenic CD8T cell. We've looked at various types of classes of cells. We've looked at transcription factors, cytokines, checkpoint inhibitors, and T and F receptors, super families, as well as other families such as like signal transection molecules.

And we can look at all the genes in each of these categories and see which ones are differentially expressed in the CD8T cell.

So again, my poster is Poster 8. Thank you.

DR. LAGASSE: Please welcome Dr. Fatima.

DR. FATIMA: Hi, everybody. My name is Tahira Fatima. I am a star fellow in Dr. Day's lab in TBBB. And our lab's mission is to improve safety of cell and tissues, which are used for therapy.

For my stated objectives, first, I want to compare metabolic to (inaudible) based detection acid and immunogenic acid, and also to develop metabolic markers for detection of

flaviviruses in human IPSCs.

According to my study design, first I infected cells with Zika MR766 strains and then we treat at MOI1, and then collected mock and infected cells between 0-96 hours post-infection.

And then I tested these cells for viral detection using restrum (phonetic) block and QRDPCR. Simultaneously, I prepared cells on both sections for LTMS analysis to identify any shift in metabolite.

And also I highlighted the results. In A, I'm showing Zika detection by restrum block at 48 hours post-infection. And in 2PCR, we detected Zika at eight hours.

In case of Dengue retreat, we did not detect DENV retreat in restrum block. However, QPCR data showed DENV positive result at 48 hours post-infection.

So in contrast, LCMS successfully differentiated between infected and mock cells at all time points, as I'm showing in B, the one camouflaged. And we identified six metabolite

markers so far. This is a potential panel in C.

So for my future experiment, I plan to validate candid biomarkers in patient samples and also to extend this technology to other viruses such as herpes viruses.

Thank you so much. And if you have any questions, please visit me at Poster Number 38.

DR. LAGASSE: Please welcome Dr. Klenow to the stage.

DR. KLENOW: Hello, everyone. My name is Laura Klenow and I am from the Office of Blood Research and Review. And I just want to direct your attention briefly to the Leishmanin skin test.

So there are locally acquired infections as well as travel associated cases that have started to be popping up in the United States recently.

And in the past year, there have been several papers that have highlighted these cases, as close as Washington, D.C., which is right next door.

So one of the things that our lab has been focusing on is that we're very aware that this is an issue in the U.S. because we already have the awareness of the potential sandfly vectors that are highlighted in these light blue states.

So one of the things that we want to look at, particularly because we have an emerging epidemic of Leishmaniasis in the lower United States, particularly in Texas and Oklahoma.

And so we really need to focus on having an increased diagnostic for not only blood safety but also for potentially assessing the future immunogenicity of any vaccine candidates.

So in looking to assess these unmet needs, one thing that our lab has been focusing on is the development of a diagnostic that is reliable and scalable so that we can meet these needs for not only surveillance, but also for vaccine efficacy.

And one of the things that we have done is we have very surly characterized a strain of

visceral Leishmaniasis, L. donovani, and we have focused on creating a reliable and reproducible layoff flies antigen, which is a GMP grade Leishmanin antigen.

We have tested this potency of our antigen in both vaccination and in latent infection models. And what we have seen is that with our antigen, that we are able to reproducibly induce a delayed type hypersensitivity immune response in both of these models.

And so what that's telling us when we look at this through high dimensional flow analysis is that it's also mediated by CD4 T-cells. And so we're able to combine all these results in not only a very nice diagnostic for surveillance and emerging epidemics, but also as a really nice test for future vaccine trials for the immunogenicity and efficacy of upcoming vaccines.

And I'm Poster Number 40 if you would like to hear any more. Thank you.

DR. LAGASSE: Please welcome Dr. Konduru to the stage.

DR. KONDURU: Good afternoon. I'm Krishnamurthy Konduru, Staff Scientist in the Office of Blood, LLV.

So Dengue and Zika widest outbreaks have increased raising CD's public health concern. Most of these wider post-infection are 80 percent infections are asymptomatic. The remaining 20 percent are flu-like. Some develop serious disease. The outcome is entirely different.

Dengue can cause severe form of dengue disease. Zika can cause fatal death. So differential diagnosis very important to provide support to specific care. (Inaudible) have extensive (inaudible) activity between these wider cases because of high homology sequence between these two.

Nucleic acid test is affected by shock (inaudible) only presents for 12 days, approximately, 12 days with low viral loads.

So we want to explore microRNA in plasma as non-human biomarkers for differential diagnosis between Dengue and Zika viruses.

So this is our workflow. We used 10 samples from each pathogen, Zika, Dengue, and controls, profound by NGS. NGS identified hits, 1,945 microRNAs. Then we narrowed down.

Here, for Zika, just these volcano plots, we get to show that red dots are upregulated microRNAs, green are downregulated. Similarly, with Dengue as well.

From them, we identified 80 candidates, Zika, 35 Dengue. We did validation by QRTPCR with the lots sample sets. From them, we identified 22 microRNAs as potential candidates. From them, nine shows highly significant.

Among nine here, we have showing a representative three candidates as three categories. The first on, one left, microRNA 3195, able to discriminate all three Dengue versus Zika versus controlled (inaudible).

And another category, at least five microRNA identified. For example, microRNA 328, able to discriminate Dengue from Zika or control.

So the left one, the third category, at

least two candidates, we identified here, microRNA 369, able to discriminate Zika from control or Dengue.

So, we got autocycles for each microRNA here. I'm showing just two assay examples, microRNA 3195 to calculate under the area curve, for this.79.

So other microRNA, 4485, that you use this.76. Now, we are assembling at the panel to give a powerful diagnosis.

So we are using both mission learning algorithm as well as manual assembly. Here, two microRNAs increase the powerful diagnosis.

For example, the blue line increased from.79 to.762, almost.95. So currently, we are using these, testing these panels with large samples.

Thanks. I have Poster Number 42. Thanks.

DR. LAGASSE: Please welcome our next speaker, Catherine Jean, to the stage.

DR. JEAN: Hi, everyone, my name is

Catherine, and I'm in the lab of Dr. Zuben Sauna. Today, I'll be presenting our research on high-throughput APTT and one-stage APTT-based Factor VIII potency assays, namely in low volumes of mouse plasma.

So what is APTT, right, and why do we need to optimize it? Essentially, APTT is a conventional coagulation test often used in clinical laboratories for the function of diagnosing functionality and intrinsic coagulation pathways.

Here, we'll be looking at Factor VIII-based ATTP assays. Factor VIII is a blood clot formation protein that we find deficient in people with hemophilia A.

This needs optimization because in pre-clinical studies, as well as in research labs, the amount of plasma available to conduct this research is often minimal, and even if it's enough for one test, often it's very few for a second test.

So our aim is to develop a high-

throughput APTT assay to test small volumes of mass plasma. And we did it.

So we were able to show 80, 77, and 90 percent recovery in our in-house developed microplate assay. We were also able to show similar levels of success in a comparison analysis using common hemostasis drugs, such as Afstyla and Altuvio.

We did a comparison of the ACL TOP, which is a machine often used in clinical laboratories for assessing APTT.

We did that compared to our in-house microplate assay. And our results were promising. In short, our sensitivity was as low as 0.00015, and our assay took less than 10 minutes.

Check out my poster at number 44 for more questions. Thank you for your time.

DR. LAGASSE: Please welcome Dr. Pacheco- Fernandez to the stage.

DR. PACHECO-FERNANDEZ: Hi, everyone. My name is Thalia Pacheco, and I'm going to be talking about how we have been identifying

Leishmania parasites in the bone marrow at the Nacasi (phonetic) lab.

So, Leishmania is a blood-borne pathogen that is very recently became endemic to the U.S. So that particularly, the contagious manifestation of the disease is what is being prevalent in the U.S.

This manifestation causes skin lesions and ulcers. So, this obviously increases the risk of transfusion, of transmission via transfusion, via vector transmission and also via organ transplantation.

So there's a need for new methods of detection. The thing is that current methods of detection have very low sensitivity for latent infections.

So what we did was to use a combination of computational and molecular biology methods to detect these parasites in mouse model of cutaneal Leishmaniasis.

So we found two main findings in our studies. The first one is that the cutaneous

parasites are capable to reach all the way to the bone marrow in very low numbers, which was previously not identified because of these low numbers.

These parasites particularly reach to the stem cells and the monocytes. Now, also you're seeing single cell RNA sequencing and flow cytometry. We found that the main changes driven by these low numbers of parasites in the bone marrow are modifying the myelopoiesis, which has raised a calibrated differentiation to monocytes and neutrophils, and also (inaudible).

So, we tested these results in two models, one which is the top one. Oh, not that one. Yes, the top one is in a viral infection of Leishmania, and the second one is in a vaccine model using a life attenuated vaccine.

So overall, these results show two things. One is that this method is good to prove the safety of the blood supply, and the second one is that we can also evaluate immunogenicity of possible vaccines.

So if you want to know more, please stop by Poster 18. Thank you.

DR. LAGASSE: Please welcome our next speaker, Dr. Pilewski, to the stage.

DR. PILEWSKI: Okay, hello, my name is Kelsey Pilewski, and I am part of the gastrointestinal viruses unit, also known as the PAR Lab.

Today, I'm going to tell you a little bit about my research, looking at the humoral antibody response to human norovirus.

Norovirus is the leading cause of non-bacterial acute gastroenteritis in all age groups. It's also extraordinarily diverse, with greater than 30 different genotypes that are capable of infecting humans, the frequency of which is shown on the righthand of the screen.

Due to this diversity, along with the fact that we do not have a traditional subculture model or small animal model system, has really led to the delay in the development of effective vaccines against norovirus or specific

therapeutics.

Due to this, understanding how to elicit long-lasting, broadly protective immunity against norovirus really remains critical.

In my study, I am looking at the humoral immune response following controlled human challenge with a G22 norovirus.

I found the following challenge, all individuals elicited a robust antibody response to the challenge agent, with IGG antibody titers peaking at 30.

Interestingly, all individuals also had diverse antibody titers to diversity types such as G24, G26, and G217, shown here.

Interestingly, although all individuals had cross reactives, binding antibodies to the different genotypes, we found that norovirus G22 was the only one or the challenge with G22 norovirus really only induced neutralizing titers to itself, and we really didn't see any increase in functional or neutralization titers to the heterologous genotype.

Finally, using a novel competitive neutralization assay that we recently developed, I looked at the contribution of specific sites to the observed neutralization phenotype over time.

And what I found was that both conserved and highly variable immunodominant sites are both important for these broadly neutralizing activities, and that this activity really changes over time.

And so if you'd like to hear more, I look forward to seeing you at Poster 20.

DR. LAGASSE: Our next speaker is Dr. Rajasagi.

DR. RAJASAGI: Okay, move forward. Okay. Before we start, I would like to thank the audience for giving me this opportunity.

I'm Naveen. I work in the lab with Dr. Meyenmajor in Seaport (phonetic). We work with hepatitis C virus.

Hepatitis C virus is a post standard interloped virus. The low proteins E1 and E2, they are responsible for the impurity virus. It's

officially the hepatocytes, the natural proteins. They help the virus be (inaudible).

The hep C is a blood-borne virus. It's a major problem in people who share needles. It's a major infectious cause of liver cirrhosis and also liver cancers.

There are antiviral treatments that are available, but these don't prevent reinfections. They cannot protect against infections.

Unfortunately, there's no effective vaccine against hepatitis C virus to prevent infections. So our group is interested in developing different vaccine approaches to tackle or come up with a vaccine kinds for HCV.

We mainly, for this study, we mainly focused on developing these vaccine approaches, mainly target humoral immune responses.

This is based on the evidence with support of the important role which neutralizing antibodies play against HCV.

And coming to the extensive design, we used mice (inaudible) non-replicating antivirus

(inaudible) or the common 14, which is formulated with the adjuvant F43 or the E2 formulated with the CPG alum.

So (inaudible), what we chose to do is because it's a main target for the neutralizing antibodies, so after planting the bunch of the mice at day 21, and we sacrifice these mice at different timepoints post-boost.

And we cultured the spleen, lymph nodes, and also we cultured the serum for the analysis. So, examined the effectiveness of the (inaudible) we used. We looked at what the similar response but also the humoral response.

We looked at the CD response we did (inaudible) assay. Here, we can see, on the X axis, these are the vaccine groups and the proportional (inaudible).

What we found was like the group use of it is stronger (inaudible) response. However, the minimal response was observed in the mice which eliminates with these recombinant proteins.

Next, we looked at the (inaudible) in

general, the recent responses. These are collected up in the lymph nodes. So these responses are very important because the injection of the T-cells (inaudible) is important for the formation of high-quality, high-efficacy antibodies, and also for the generation of long-lived antibody producing memory cells.

We found that bodies, all the groups were able to produce (inaudible) recent responses are very (inaudible) reproduced in the vectors and also in the E2 adeno vector groups.

Coming to the last slide, the most important is the humoral responses. We have good titers in all the three groups. They had high levels of antibody titers in the mice (inaudible) with the recombinant proteins.

And the good thing is, the antibody titers did not go down post-boost, which is actually a good point when we are looking at the long-term for potential vaccines.

Coming to the notation capacity, the CM from the mice which had immunity, which was E2,

also had the E2CPG alum, they exhibited a higher neutralizing capacity than the adeno vector mice.

So what I confirmed is although the outside group gives produced a good (inaudible) response, the common 14 in use is better quality antibody response than the adeno vector group.

This I would like to end. Thank you for listening.

DR. ATREYA: This closes the flash talk session, and then we have 12:30 p.m. We have the posters. You guys can have your lunch. And the even number posters are going to be shown today. They are on different topics, advances in computational science, methods in biomarker discovery, imaging and reimaging decisions, and immunoresponse to vaccinations.

Thank you all for attending this morning's session.

DR. ELKINS: And speakers and speaker lunch guests, you know who you are, you have boxed lunches with your name on it at the kiosk in the lobby. And we have a dedicated lunchroom since

outside is not so good, at the end of the hallway behind the kiosk.

We will reconvene promptly at 2:00.

(RECESS)

DR. KURTZ: So, if everybody could take their seats, we're hoping to get started on time here, because one of our speakers needs to catch a flight.

So, Dr. Golding is going to introduce our first two extramural speakers, and we'll hopefully get the session started. Thank you.

DR. GOLDING: Welcome everyone to the last session of the wonderful symposium. And I would like to start by introducing Dr. David Montefiori, who is a professor and Director of the Laboratory for HIV and COVID-19 Vaccines Research and Development in the Department of Surgery, Division of Surgical Sciences at Duke University Medical Center.

His major research interests are viral immunology and HIV and COVID vaccine development with special emphasis on neutralizing antibodies.

In addition, Dr. Montefiori is also direct a large vaccine immune monitoring program supported by the Bill and Melinda Gates Foundation, and has served as national and international resource for standardized assessment of neutralizing antibody responses in pre-clinical and clinical trials of candidate HIV vaccines since 1988. Our hats off to him.

At the onset of the COVID-19 pandemic, he turned his attention to SARS CoV-2, which a special interest in emerging variants and how they might impact transmission, vaccine, and immunotherapeutics.

His rapid response to emerging SARS CoV-2 variants of concern provided some of the earliest evidence of the potential risks variants posed to vaccines.

In May 2020, his laboratory was recruited by the U.S. government to lead the national neutralizing antibody laboratory program for COVID-19 vaccines.

Today, he is going to share with us his

experience in developing neutralizing, validated neutralizing assays against both COVID-19 and the HIV.

And he's changed his title, so I am going to let him take the lead.

DR. MONTEFIORI: Thank you, Hana, for that wonderful introduction, and I'd like to thank the organizers for inviting me here today.

All right, so, the title of my talk today is Assessing Vaccine Elicited Neutralizing Antibodies Against SARS CoV-2 and HIV.

And I'd like to start out with a brief introduction into my program. As Hana mentioned, since 1988, I've been an essential laboratory for neutralization assays for the HIV vaccine trials network back when it was called the AIDS Vaccine Evaluation Group, AVEG.

And then for the Dades pre-clinical program, we've been the central laboratory for all of the non-human primate studies and a lot of studies in smaller animals since 1993, and that's still going.

In 2006, we became a central lab for the Gates CAVD and for the Duke CHAVD, and both of those are still going, and then more recently for the Duke CFAR.

And then in 2020, we got recruited, as Hana said, to get involved in the COVID-19. So I've been part of the COVEPN, which is just another name for the HVTN, and then the U.S. Government's COVID-19 vaccine program and the Moderna COVID-19 vaccine program.

So, a lot of what we do focuses on immune monitoring. And we've been a GCLP compliant laboratory for over 20 years.

We also have a basic research program, though, that benefits from all of the samples that we get from the pre-clinical and clinical studies, and we do a lot of research with those samples and other samples that benefits our immune monitoring.

We are very focused on how to improve the way we assess neutralizing antibodies. So we look at the antigenic diversity of the viruses. We develop reference reagents. We study

mechanisms of neutralization in the scape. We develop reagents to map neutralizing epitopes.

The data is useful for interpreting structural studies. We've been involved in identifying and characterizing broadly neutralizing monoclonal antibodies for HIV, and we're very interested in immune correlates.

And all of this feeds into being able to design new immunogens, to perform pre-clinical and clinical studies, how to design those pre-clinical and clinical studies, identifying lead products to move through the various phases of clinical testing.

And then our experience with COVID and the Moderna program, actually being involved in regulatory approvals and my first direct interactions with the FDA.

So again, my lab was recruited in May of 2020 when Operation Warp Speed was formed. That was later, became the Countermeasures Acceleration Group under the Biden Administration.

And the government was investing

billions of dollars in a number of companies to develop their vaccines and take some of the financial risk out of it.

And in return, they wanted the assays to be done, the binding and neutralizing antibody assays were the highest priority, in laboratories that they designated.

And so they started a program to have the laboratory program, and eventually it became a lot of CROs doing the assays. But I was asked to help with the neutralizing antibody program, and to develop a pseudovirus neutralization assay, since I had a lot of experience with that for a number of years for HIV.

So one of the first things I did, a lot of people had neutralizing antibody assays very early in 2020. A lot of people had neutralizing antibody assays for SARS CoV-2, live virus assays, pseudovirus assays.

And I wanted to understand how they compared to one another. And so we initiated this program with Tom Denney and we called it SNACS,

the SARS CoV-2 Neutralizing Antibody Concordance Survey.

Our primary objective was to gain an understanding of the variability of the results in the different assays, which in turn provides insights into the comparability of published data sets that were starting to come out at the time, and the design of future standardization efforts, which I was very much involved in.

And also, because these assays were in the early stages of development for the most part, and the limited number of samples, I urged caution when drawing general conclusions about the potential superiority of one assay over another.

And we had a total of 54 assay results submitted from 46 sites. There were 20 live virus assays and 34 pseudo virus assays, 23 of which were lentivirus based and 11 were VSV based. Those assays were quite diverse.

We did take a survey of the experience that the laboratories had, whether or not they were GCLP or GLP compliant. A number of spike

proteins, they had a lot of different names, but they all boiled down to either the ancestral or the D614G variant.

A number of different cell lines are being used. Spike characteristics varied. Some people were deleting the cytoplasmic tail for increased expression. Other people weren't. Some people were codon optimizing. Others weren't. So we had a mixed bag there.

And then a lot of different assay readouts. So considerable diversity in all of the assays and how they were being performed.

The samples that we had available to share with all of these laboratories, there were samples comprised of high, medium, and low titers.

Some were in triplicate to look at repeatability. We had four negative samples to look at specificity. So 21 samples total that all of these laboratories ran in their assay.

And I'm only going to show this one data set because I think it was the most revealing data set. So this is a range of titers that all of the

laboratories obtained in their assays.

The live virus assays are in red. The lentivirus based pseudovirus assays are in green. And the VSV based pseudovirus assays are in blue.

And the numbers along the X axis are all of the different laboratories blinded. And you can see that it's a mixed bag all throughout. You don't have a clustering really of one type of assay at one end versus the other.

There is a little bit of a clustering of the lentivirus based pseudovirus assays in green on the far right, but you also see them spread all the way out and one of them on the far left.

The live virus assays in red, you see one of them was the least sensitive on the far left and another one was the most sensitive on the far right. So there was really no clear association between the assay type and the sensitivity that they have.

And that was a big question at the time. There was a feeling that live virus assays, which were also called authentic virus assays, and

whether or not they were going to be a more reliable readout than pseudovirus assays.

And so that's some general conclusions. We saw about a two log difference in ID50 titers and a one log difference in ID80 titers across all of these assays.

There was greater precision for ID80 than ID50. We see the same thing for HIV. And there was greater concordance among pseudovirus assays than among live virus assays.

So we went ahead and adopted an assay that was actually developed at the VRC by Barney Graham and Kizzi Corbett. We used these targets, 293HT cells that we got from Mike Farzam and Hui Hui Mu. Very generous, both of these groups in sharing the reagents and allowing us to work with them.

I'm showing a schematic here of this particular assay. And we optimized, qualified, and validated this, and all of this was vetted by the FDA. So it's a 96 well-played assay. We run four samples on a plate, eight delusions in

duplicate, and we have a positive plate control for every plate.

It's luminescence based. We generate those response curves and then the results are QC'ed. We have acceptance criteria for the assay. We have, in addition to the plate control, we have high, medium, and low run controls and a negative control, and a number of other parameters that we look at in terms of the QC'ing all of the data and the acceptability of it.

This is just the workflow for generating all of the documents that we sent to the FDA. It was quite an extensive process and we have over 50 documents in our master file now.

And this is just quickly an outline of the GCLP compliance sample and data flow for all of the work that we've done and continue to do for the Moderna program.

A lot of work went into this. 2020 was a very busy year for us, and part of 2021. We have validated and revalidated this assay for eight variants, most recently JN1 and KP2.

And the validation for the D614G variant, which was the variant circulating during the time that the Moderna and Pfizer phase three trials were being conducted, there was a real rush to get the assay validated in time to do a correlates analysis in these efficacy trials. And I was involved in the Moderna efficacy trial and doing the neutralization assays for the correlate study.

And we got those assays done in early 2021, and I was very relieved to find that our assay did correlate with efficacy and it's shown down below there in the red box. Pretty significant correlation for both ID50 and ID80. A little better correlation even then for the spike in RBD specific binding antibodies.

So this is really nice. Very comforting. And of course, these findings strengthen immunobridging for regulatory approvals.

For example, I'm showing non-inferiority of the neutralizing antibody responses in children

less than 18 years of age.

This is a list of all of the SARS CoV-2 variants that we have in the lab. In the red box below there, that's JN1 and all of the subsequent ones showing the variability in spike, and we focus a lot on the variability in the RBD.

This is a slide that I look at a lot, stare at a lot, and think about a lot, and keep adding the next variants to. And we continue to do this.

I'll show you an example now of some of the neutralizing antibody data that we've generated to continually look at new variants and the extent to which they are evading neutralizing antibodies.

Right now, the sample set, the serum sample set that we've mostly focused on are from people who have been fully immunized with the Moderna vaccine. So they got the first two doses of the ancestral spike at 100 microgram dose, and then they got the third dose at 50 microgram, and then they were boosted with the bivalent Wuhan

BA5, and then about eight months later, they were boosted with XBB1.5. And we're using serum samples from the day of boost and then four weeks after the boost to look at neutralization of sequential variants.

And so these are the results on the top for people that, as far as we know, were not infected previously, in the middle for people we know were previously infected, and then the combined group on the bottom.

On the left is before the boost, so this is basically eight month durability data, and then on the right is four weeks after the boost. And it's again, you can see over time, so as you move from left to right, the variants are becoming less and less susceptible to neutralization.

After XBB1.5 boosting, the titers against XBB1.5 range from 1500 in the non-infected group to 3600 in the previously infected people, 2755 average.

To me, that's a pretty good titer. In the initial phase three efficacy study, after two

doses the geometric mean titer to D614G was about 2,000. There was over 90 percent efficacy there. So I'm always looking, personally, at least, for titers that -- a geometric mean titer of around 2,000. We have a ways to go there now for JN1 and its offspring KP2 and KP3 and KP3.1.1, which is a predominant variant at this time.

We also got involved in calibrating. I know calibrating is important for the FDA. And we embarked on this because we were doing neutralization assays for the Moderna program, and Monogram Biosciences had the honor of doing the neutralization assays for all of the other programs.

But we were getting different titers in our assay. Both of these assays were validated and vetted by the FDA. And so, our objectives here for calibration were immune correlates, understanding that licensure for ages less than 18 years and immunobridging regulatory approvals, and then making decisions about when to boost and what to boost with moving forward.

And we were concerned about the four-fold difference in titers that we were getting. And so we did calibration, and with the help of Yunda Wong and Peter Gilbert and others as statisticians, we decided we needed 248 convalescent serum samples.

These were all from early in the pandemic when D614G was dominant. And another set of samples were similar to the ones I described from Moderna, but these people only received the initial Wuhan spike vaccine.

And then we looked at the WHO international standard that was available at the time. And there were three calibration approaches.

And so these are the results on the left. The titers with the 248 convalescent serum samples, you can see Monogram's titers in red are about four times higher than ours. And that was true for the vaccine sera and the WHO international standard.

And so the statisticians used three

different approaches to calibrate. One was based on the international standard. Another one was -- the other two were based on the convalescent sera using a bivariant normal distribution model or a linear regression model.

All of them performed pretty well. But approach number two with the concordance correlation coefficient of.87 was the best. But it showed that you can calibrate these two assays.

And more recently, we did calibration again with Monogram for several additional variants. And you can see non-calibrated titers on the top. Their titers were again higher than ours for pre-Omicron but not post-Omicron variants. And with calibration, everything comes in line very nicely.

So now, I'll switch to HIV. Very different situation with HIV. It has a somewhat smaller spike. It has at least a half a dozen broadly neutralizing epitopes that are susceptible and are major targets for vaccines.

We know a lot about those epitopes and

we know a lot about the antibody lineages that give rise to broadly neutralizing antibodies.

The early days of HIV neutralizing antibody assays, there was a lot of inconsistency and confusion. And I was there right from the very beginning of this. And I remember Marjory Robert Guroff's first demonstration of the antibody mediated neutralization she published in Nature in 1985.

And then after that, multiple cell types were used to propagate the virus and use these targets for neutralization. Numerous human lymphoblastoid cell lines and peripheral blood mononuclear cells, a lot of ways of reading out neutralization.

But then the pseudovirus technology came along in about 2003. John Cappas and George Shaw first applied this to neutralization and Monogram had a similar pseudovirus assay at about the same time.

And this really changed things. It was an assay that was reproducible. It was

validatable. And the use of pseudoviruses really strengthened our ability to understand what we were measuring, even at the genetic level.

We optimized, qualified, and validated the assay that John and George developed. We successfully transferred this assay to at least 54 laboratories around the world. It's been the gold standard in the field since 2005.

But our validation, qualification and validation of this assay has not yet been vetted by the FDA, and I suspect that we have some work to do to meet their standards.

We did develop an international proficiency testing program that was implemented in 2009. We went through several rounds of optimization. And then based on the results from the third round, and this is laboratories from the U.S. as well as international laboratories were involved in all of those rounds, after the third round, we developed a statistical qualification rule. We manufactured kits. And then those kits went out to a number of reference laboratories

that were highly experienced to get our acceptable ranges.

And this, again, has been used by multiple laboratories for quite some time now to facilitate their GCLP compliance. And all of our methods, all of our detailed protocols for all of this, have been openly available on the websites since the very early days, so that anyone can see how we're doing things and hopefully do things the same way to improve standardization.

So we have this TZMBL assay for HIV that's been the gold standard for many years, but what I was always concerned about is, is it the right assay?

So would the results in this assay correlate with protection against HIV acquisition in humans? And we finally got to test that with the antibody mediate prevention trial.

This was an efficacy trial that tested a monoclonal antibody to the CD4 binding site of JP1 -- of the HIV spike. The monoclonal antibody is called VRC01. There were two trials. They were

conducted the same but in different cohorts, one in North and South America and the other one in Southern Africa.

These people received the IV infusion, 10 IV infusions of VRC01. Every two months, they would receive an infusion for a total of 10 infusions.

And what we were most interested in in this trial was to look at whether or not there would be a neutralization sieve effect. With viruses that infected people who got the treatment be more resistant to VRC01 than people who got infected in the placebo group?

And so there were two laboratories that sequenced the transmitted founder lineage envelopes in breakthrough cases.

Then we had pseudo viruses made from those, and we would test those pseudo viruses for neutralization by VRC01.

This is the program that we put together. So again, the two trials, one in sub-Saharan Africa, one in the Americas. For

sub-Saharan Africa, Carolyn Williamson's lab did the envelope sequencing and the founder variants selection for the HV10704. Jim Mullins's lab did the sequencing and the founder selection. Those sequences went to Gene Whiz, the contractor. They synthesized the genes and put it into our expression plasmid.

And then for 703, those plasmids were sent to Lynn. For 704, they were sent to my lab. We resequenced the envelope chains in those plasmids and made sure that they matched the sequence that Carolyn and Jim got. And then we produced the pseudo viruses, titrate the pseudo viruses, and did neutralization assays with VRC01 in autologous serum samples. And all of those data went to the statisticians at Sharp for analysis.

While we were in the process of doing this, Lynn and I exchanged plasmids, regrew the pseudo viruses in our lab, and did the assays with VRC01 to test equivalency across the two labs.

We were highly concordant equivalent,

really, in those tests, and that effort was overseen by the director of our quality assurance unit, Marciello Sarzetti Kelso. And John Herall was the overall program manager for this.

This is a really well thought out, very robust program to generate reliable results. And in fact, even though there was no overall efficacy in the trial, when you looked at efficacy just in the group of people who got infected with viruses that were the most sensitive to VRC01 that had an IC80 of less than 1 microgram per mil, this is about 30 percent of the breakthrough cases, there was about 75 percent efficacy against those viruses.

So now we finally had the first evidence in people that the TZMBL assay was a correlate of protection against the acquisition of HIV infection, mediated by a neutralizing antibody.

And those results were further extended and analyzed by the statisticians to come up with what they call a PT 80, predicted titer 80 of 200. This is a titer of neutralization that is required

to prevent transmission of the virus, the acquisition. If you have a titer of 200 against a virus you're exposed to, this predicts that that titer would protect you. It's a very high titer to achieve. It's an ID 50 titer of about 800-1000. Somewhat daunting.

But there are some other monoclonal antibody combinations, a triple combination, in fact, that is expected to do much better than VRC01 that's shown on the right there in the purple curves on the top compared to the efficacy of VRC01.

Those are moving forward into efficacy trials that may be beginning in the next couple of years.

I'm showing the three monoclonal antibodies there at the top, the CD4 binding site of V3 glycan and V2 apex antibody.

And these results will, if we get the efficacy that we're predicting based on PT 80 that is guiding the design of this trial in terms of the antibodies being used and the dose and

schedule, that would validate the VRC1 amp results and the PT 80 threshold and applicability of that to being against other epitopes.

We're hoping that the efficacy will be good enough to lead to product approval for another long acting prep option.

And of course, we'll be generating comparative data sets for next generation products, and for immunobridging to permit expanded access. And there will be next generation BNABs that will be tested in clinical studies for safety and tolerability, pharmacokinetics, ADA induction.

But here, the thought is immunobridging will replace efficacy studies because it will probably no longer be possible to conduct efficacy studies. And both of these, as you noticed, being from the FDA, are likely to require a high regulatory bar.

Now, HIV, as most of you know, is highly genetically and antigenically diverse virus. When you look at it genetically at the nucleotide

level, it can be broken down into multiple genetic subtypes and circulating recombinant forms.

And they have different geographic distributions. So in North and South America and Europe, it's mostly claim B. Southern Africa and in India, it's primarily claim C.

Southern Africa carries the heaviest burden of HIV infections in the world, so claim C is of major importance for vaccines.

And it's important to have reference strains that represent all of this diversity to understand where they are claim specific neutralization phenotypes and for the evaluation of candidate vaccines and candidate broadly neutralizing monoclonal antibodies in combinations for prevention.

This is now looking at the diversity of SARS CoV-2. Right now, shown in the box at the bottom, are the most recent JN1 lineages. And looking at the amino acid diversity in spike, the total diversity that we've seen in SARS CoV-2 in the beginning is about.6 percent of the amino acid

sequences.

This is what HIV looks like now. This isn't the total evolution from the beginning. This is what it looks like now. And it continues to get worse.

So if you compare any two variants of HIV within the same subtype, they could be up to 20 percent different in their amino acid sequence between claims or subtypes. They can be up to 35 percent difference. The total opposite end of the spectrum.

We, a number of years ago, tried to understand some of this diversity. And there is also a need for standardized reference strains in the field. And so, through our Gates consortium in 2008 and 2013, we started what we called our CAVD Standard Virus Panel Consortium and a neutralization sera type discovery program.

Again, this is a five year program, about $10 million. We had a number of specimen acquisition laboratories around the world. We had half a dozen cloning and sequencing labs to get

the envelopes cloned from plasma samples that we were acquiring.

We had three laboratories doing neutralization assays in two very good groups doing computational analyses of the data. We created more than 500 non-pseudo type viruses of all major genetic subtypes and geographic diversity.

We used the subset of those for all of the neutralization assays and came up with reference strains that are still in use today. There are large panels and then there are subset panels.

So this is just a heat map of the neutralization data from over 200 serum samples assayed against over 200 isolates, 45,000 assays. And I asked Allen de Camp, a computational biologist and statistician, to tell me how many and which of these strains would we need to use as reference strains where the results represent the overall diversity in this heat map.

And he used the method called LASSO, and

that showed that you only needed nine viruses to do that. The right viruses. You see in gray up there in the top right the spectrum of representation, and the solid line is the best selection of strains, and nine of them got us an R squared value of.952. We really didn't need any more than that.

So those are reference strains that have been used for quite a few years now in the field and have been very useful. And we did the same thing for claim C since that is such a major subtype of major importance. It was a little smaller in terms of the number of serum samples that we assayed, but we did have 200 Southern African claim C viruses.

These were all transmitted founder viruses. And the same type of analyses, and came up with a panel of 12 viruses just for subtype C.

And we share these with everybody in the field. They're available through reagent repositories. And the whole purpose of this is for standardization.

Now, we also have the breakthrough viruses from the amp trials, and we've been working with those. A number of people have. We've shared these with other people who are interested in them. A lot of work is being done with them.

And we're learning that these viruses are more recent in the pandemic compared to our older referenced viruses, and we're seeing evidence of drift.

So for example, the subtype C viruses have greater resistance to VRC07523 and there's greater resistance to PG2121 and PGDM1400 in subtype B. So this is telling us that there's some need for us to update our reference panels as well.

This drift isn't happening fast, it's happening slowly over decades, but it is occurring. And now these viruses from the amp placebo group are new reference strains that are available for people to use. But we are still in need of many other subtypes and for viruses from

breastmilk transmission.

Ultimately, when an HIV vaccine starts generating the types of neutralizing antibodies we want to see that cross neutralize primary isolates, our goal is to be able to measure the magnitude and breadth of neutralization and be able to compare immunogens and see which ones are doing better to continue to advance.

We don't have a vaccine right now that generates antibodies that neutralize heterologous, what we call heterologous tier two viruses to any great extent, but we do have statistical methods to actually analyze those types of data.

So shown on the left and the right, really, the X axis is the titer of neutralization and the Y axis is the number of viruses that are neutralized at that titer.

Again, this is a type of magnitude breadth plot. It's something that the statisticians can analyze to look for statistical differences.

On the right is where we applied this to

the very early efficacy trial of the GP120 immunogen where there was no efficacy.

But we did detect some weak heterologous tier two neutralizing activity that was statistically significant compared to the low-level non-specific activity in the placebo group. But again, this was too weak of a response to amount of any efficacy. So we're looking for much greater differences with future vaccines.

These magnitude breadth plots have also been used to identify best in class broadly neutralizing antibodies for passive administration as prepped and to identify the best combinations of those best in class.

And what I'm showing here in red is the magnitude breadth plot of VRC01 and then in purple and green and maroon color are the three monoclonal antibodies I mentioned earlier that are going in, are expected to be going into an efficacy study.

Their magnitude and breadth individually, and those ones in purple and green,

they look similar in terms of their curves, but they're complimentary to one another.

What the green antibody neutralizes, or doesn't neutralize oftentimes, the purple one does, and vice versa. And so when you put those three antibodies together you get that black curve. And you can see that that magnitude and breadth is about 50 times better than VRC01, and we're really counting on that translating into being that much better in the field and in people as well.

Another very important property of HIV, you've heard me talking about tier two viruses, heterologous tier two viruses. What is that?

When you phenotype a variant of HIV with serum samples from chronically infected people and look at how sensitive the variants are, you can actually break it down into four different tiers of sensitivity.

You have viruses on the far right there that are really sensitive to neutralization. We call that Tier 1 A. And then you have the green

in the middle there, the majority of viruses. We call that Tier 2. And then between Tier 1 A and Tier 2 is this intermediate phenotype we call Tier 2 B. And then on the left are the least sensitive viruses that we call Tier 4.

And so most of our focus is on those viruses in the middle that are Tier 2 phenotype. We stay away from Tier 1 viruses because they tend to have an open confirmation where epitopes are exposed that are not exposed on Tier 2 viruses, which are the majority of circulating strength.

And Monomer TP120 immunogens will generate antibodies to these epitopes, these cryptic epitopes, that aren't exposed on primary isolates. And those aren't good antibodies. We need antibodies that will neutralize Tier 2 viruses.

And we know that the trimer, the HIV trimer, breathes. It's constantly opening and closing. And on Tier 2 viruses, it's in a closed confirmation more of the time, whereas in Tier 1 A viruses, it's in an open confirmation more of the

time. And in the Tier 1 B virus, it's kind of somewhere in the middle.

And this helps to explain these different neutralization tier phenotypes that we see in HIV when assayed with serum samples from chronically people living with HIV.

So just to summarize, SARS CoV-2 is a new human pathogen for which much is unknown about its evolutionary trajectory as it relates to neutralizing antibodies.

I mentioned that we were required to revalidate the assay for eight variants, and we anticipate having to continue to do that for important variants in the future.

And this extensive assay method revalidation provides assurances of quality results. But HIV is going to require a different path to method validation.

We have done a lot of validation of the TZMBL neutralization assay. But because HIV is so much more complex, it's going to require a different path.

But much more is known about HIV variability and antibody mediated neutralization. And this information may provide a framework for decision making.

I'll stop there, and there are a lot of people -- this is a short list of all the people who are involved in this work and then the people in my lab. Thank you.

DR. KURTZ: Thank you very much. Are there any questions, please?

MR. WANG: Beautiful work. This is Tony Wang from the Office of (inaudible). I am wondering whether you have any comments regarding what kind of improvement you would like to see with the SARS 2 neutralization assay in general.

DR. MONTEFIORI: What type of improvement --

MR. WANG: What type of improvement --

DR. MONTEFIORI: -- in the assay?

MR. WANG: Right.

DR. MONTEFIORI: Oh, it's a wonderful assay. And coming from someone with a long

history of very cumbersome, extensive, time consuming assays for HIV over the years, the pseudo virus assay technology is wonderful, and it's just really nice to see that it's working out so well in terms of being a correlate.

It was a correlate for SARS CoV-2. The pseudo virus technology is now correlate for HIV as well.

Yes, I really -- yes, there's always room for improvement, but again, from where we came from, it's a really nice assay.

MS. KATAGIRI: Effie Strugel, Office of Plasma Protein Therapeutics. So, you talked a little bit about monoclonal antibodies and using combination of those in clinical trials.

Now, we know that a lot of newer monoclonals have modification in VFC to increase their half-life, and as you know, that also affects the bad distribution and how it goes and deposit.

So do you have any advice on how we can improve our neutralizing assays to take into

account this property of these antibodies?

DR. MONTEFIORI: Yes, I mean, that's very -- those are very good points. And a good question. The LS modification for improved recycling of the antibody, prolonged half-life, doesn't really have an impact on the neutralizing activity in the assay in terms of entering inhibition, which is how these antibodies work.

But that said, the VRC01 amp trial, that VRC01 antibody, and those data that were used to derive the PT80 that's being used to design the next efficacy trial, the VRC01 was a non-LS antibody. It was not the LS version.

The three antibodies going into the combo amp trial are LS versions. And those antibodies are likely to localize at the mucosa rather than VRC01 did.

So there's a possibility that they'll be even more protective than VRC01 being at the site of exposure. And that's not something we can measure in our assay.

But we will from that trial get a

revised PT80 because if there is a positive impact of that localization of the antibodies at the mucosa, the protective titer is likely to be different than what we found in VRC01, and that titer will be more useful moving forward.

MS. KATAGIRI: Thank you. I hope you have time for another question. So as you know very well, we are now moving to new platforms of vaccine development, especially messenger and lay vaccines, not just for Coronavirus and RSV but also for HIV.

And there's always the question, A, what is the nature of the immunogen? What is the nature of the immune response? And whether the current neutralization assays are going to capture all the antibodies that are generated by the new platform.

I assume this is some -- there are some efforts to address this?

DR. MONTEFIORI: Yes, so, very good question. A lot of the vaccine approaches for HIV right now are focused on inducing broadly

neutralizing antibodies.

The pendulum has swung toward BNABs. And we now know that one of the problems of not being able to induce BNABs in the past is that we didn't have immunogens that would initiate the correct lineages of antibodies that give rise to BNABs.

And now there are immunogens with information that we've gained over the past ten years or so, it's been possible to design immunogens that will engage the germ line reverted form of these BNABs.

So it triggers the naïve B cell receptor on the right lineage of B cells, activate those cells, to begin making early pre-cursors of these BNABs.

And then the goal is to design boosting immunogens that will continue to mature that response, to drive the somatic hypermutation and affinity maturation that's needed to become a fully mature BNAB.

But where the field is right now, is

they're initiating early pre-cursors. They haven't figured out how to mature them yet.

And I wanted to be able to use the neutralization assay to monitor progress eliciting early precursors of these antibodies. But the early precursors don't neutralize wild type HIV.

And what we had to do was figure out ways to engineer our pseudo viruses so that they would be susceptible to neutralization by these early precursors.

And we basically use the same strategies, design strategies, that people use to engineer immunogens to have high affinity for the germ line reverted antibodies.

We engineered those features into pseudo viruses and made the pseudo viruses sensitive to those germ line reverted antibodies.

And so we are capable of monitoring for certain BNAB lineages, particularly the CD4 binding site lineage BNABs. We're able to detect those early precursors very early on. And with intermediates of these engineering features that

we put into the pseudo viruses, we can also monitor progress in terms of maturing the response before the antibody is able to neutralize wild type viruses.

So, and that's something I didn't have time to describe today, but it's been very useful in the field and primarily identifying interesting people in clinical trials for other laboratories to do their deeper interrogation for the B cells, look at the molecular level, the sequence level, of what those antibodies are, getting monoclonal antibodies out to confirm things.

Our data have been fairly reliable so far, pointing to the right candidates and correlating with what they're finding. So that's -- and we can generate these data quickly.

The B cell interrogations are more costly and take a lot longer, and you can't do it on everybody like we can.

DR. GOLDING: There are no questions online? Well, in this case, I want to thank you very much for very thoughtful and thorough talk,

and good luck catching your plane.

DR. MONTEFIORI: Thank you. Yes, unfortunately, I have to run.

DR. GOLDING: Now we have the pleasure to have Dr. Sette who is going to shift field a little bit. Dr. Alessandro Sette has devoted more than 35 years in biotech and academia to understand and measuring mainly T-cell immune responses and developing disease intervention strategies against cancer, autoimmunity elegy, and infectious diseases.

Dr. Sette has overseen the design and curation efforts of National Immune Epitope Database, IEDB, a freely available, widely used bioinformatic resource that I think a lot of scientists really are appreciating.

Dr. Sette's lab uses knowledge of epitope to define the hallmark of the beneficial immune response associated with effective vaccine as opposed to immune responses that are ineffective or that can actually cause harm.

Importantly, he founded Epimmune in

1997, where he serves both as the Vice President of Research and Chief Scientific Counsel until 2002 when he joined the La Jolla institute for immunology, Head of the Division of Vaccine Discovery and is also the Head of Center for Infection Disease at De La Jolla University.

And at this point, I would like to invite Dr. Sette. The title of his talk is the Study of Adaptive Immunity to Viruses of Pandemic Concern.

DR. SETTE: Okay, so first, yes, okay, so, the current scenario with SARS CoV-2 as we all know, has been a lot of people that have been vaccinated and received multiple vaccinations and a lot of people have also experienced breakthrough infections.

And so we've been curious about defining the interplay between vaccinations and infection in adaptive responses to SARS CoV-2.

And going back to early years in 2020, there was a lot of confusion and concern about adaptive immune responses in SARS CoV-2.

There was polls that said that this was a Coronavirus, and the biweekly immunogenic would be difficult to induce a good immune response against SARS CoV-2.

The other end of the spectrum, people were saying that the overreactive immune response was killing people in the acute phase of disease and infection.

And so we set out to measure immune responses to SARS CoV-2, and we specifically focused on initial status to people who have experienced a mild form of infection.

So we wanted to have a picture of what was a success story, so to speak.

And also, in other studies, we focused on what were predictors of such a mild infection versus a more severe infection. And there's a lot of data that accumulated over early on over the years, pointing out to an important role of T cells in SARS CoV-2 immunity.

There's data we generated early in 2020, but we demonstrated that the best predictor of

having a mild disease course was having early CD4 T-cell responses, by far. That is and has remained the best predictor of mild disease.

Also, early CD8 response correlate. Antibodies do not correlate in terms of early predictors of mild outcomes.

And also, onset of protection in the Moderna and Pfizer actually preceded the appearance of neutralizing antibodies.

There was data from Bertoletti's group in Singapore that showed that the early CD4 T-cell response was actually a predictor of lower viral titers later on.

And finally, we showed, I don't know how you -- does that work to point? No. Can they see it?

DR. KURTZ: No, sorry, only in person, not online.

DR. SETTE: Aha. Okay, so, forget it. Anyway, what you see in the righthand side is another study that was eventually published later in the pandemic, but was done with the early

samples from Italy.

And as you recall, Italy was hit very hard early in the pandemic. And what we saw in a number of collaborations with Italian research centers is that a predictor of a mild disease was having a broad response, not only directly against spike, but also T-cell response against other antigens.

Based on this data, Shankrote and our collaborators at the La Jolla Institute came out with a model in which we posed the notion that protection against infection of course is driven mostly by neutralizing antibodies, as David has shown us a minute ago.

This is what you want in a vaccine preventative setting, to prevent infection. You want neutralizing antibodies.

But in the context of protection against severe disease, really that is driven still by antibodies, but to a large extent by cellular immunity, both memory B cells but also importantly by CD4 and CD8 T-cells.

Now, as I was mentioning in my opening remarks, we are in a situation where there are two important contributing factors to our immunity wall.

By now, everybody has been vaccinated, or most people have been vaccinated multiple times, and most people have also been infected and reinfected multiple times.

And most of these factors contribute to develop and maintain an immunity wall against severe disease.

So we were interested in, as I was mentioning, to understand this a little bit further, and there's been conflicting reports about the effect of breakthrough infections on T-cell responses.

We have seen in collaboration, for example, with a South African group and in other studies in collaboration with John Wang's group, an increase of T-cell responses associated with breakthrough infections.

Other studies have shown very mild

increase. And other studies have shown actually a decrease, which was potentially very worrisome. And unfortunately, I think some of his studies got a lot of press in the catastrophic thing of we're all going to die because the more you get breakthrough infection, the system gets exhausted and apocalypse.

In our mind, it was important to see that these studies are pointing to a decrease where actually only looking at the RBD, which is a small fraction of one of the proteins of SARS CoV-2.

So we thought it was important to really get a global knowledge and analysis of what's really going on at the level of T-cell responses in the context of breakthrough infection.

So we developed this assay early on to dissect the impact of infection and vaccination. And this is conceptually a very simple assay, which is based on measuring T-cell activity and plotting this T-cell activity in a two-dimensional space.

So on the Y axis, you have activity spike. And on the X axis, you have activity against everything but spike, the rest of the SARS CoV-2 proto.

And we validated this early on in the pandemic when actually you could still readily find people that are only vaccinated or infected as opposed to now.

And so you see here, the blue people are people who are vaccinated only. The yellow people are people that are experienced both vaccination and infection. The red people are people that have only been infected. And the gray are people that were neither infected nor vaccinated.

And so you see that the blue people live on the Y axis. So they only see spike because they've been vaccinated and they've never seen the rest of a genome, while the red people live on the diagonal because they've seen both spike and the rest of the genome. And the yellow people have seen spike twice, once when they were vaccinated and the other time when they were infected. So

they also live on the diagonal. But they have a higher content of spike, if you wish, in their immune responses.

We more recently started a study where we looked at people that had experienced breakthrough infections, symptomatic breakthrough infections, and were part of several hundred people, longitudinal cohorts that we follow at La Jolla Institute over time get blood every once in a while.

So we had the pre- and post-symptomatic breakthrough infection samples. And so, when we looked at the blood, in fact we saw that about 30 percent of the people that, according to them, never had a positive SARS CoV-2 test, nor ever experienced any symptoms, nevertheless they had reactivity against the SARS CoV-2 non-spike rest of the genome.

So presumably, they have had an asymptomatic infection. We show that actually having these pre-symptomatic infections mattered in the sense that people that had this

asymptomatic infection actually responded better. So much for exhaustion -- responded better in the context of the breakthrough infection, both in terms of CD4 or antibody responses.

As I was saying, there was no sign that we could see of this being associated with the breakthrough infection being associated with T-cell exhaustion.

In fact, the T-cell after breakthrough infection seemed to have increased all the functionality as defined by the capacity to secrete more than one cytokine.

And this to me was the most cool piece of data out of the study in the sense that when we look at the comparing the reactivity, the T-cell reactivity, before and after the breakthrough infection, as we and many others have shown, in reality, most of the T-cell response is not impacted by limitations in the variants.

But we also saw both in the case of Delta or Armitron, there were new epitopes appearing.

So these T-cells actually recognized the sequence of the variant, the half cause of the breakthrough infection. There was no T-cell activity against that peptide, that mutated peptide, before a breakthrough infection, but there was reactivity after infection.

So that means that your immune system keeps up with viral evolution. So as SARS CoV-2 variants pick up more variants, your immune system actually takes note and develops a matching piece of activity, that matches this appearance of new mutated sequences, which I think is pretty cool.

So, and this data was published earlier in the year. Major points, boost in magnitude. I did not show you this, but obviously, it goes -- it's not unexpected that people that were vaccinated and then had a breakthrough infection had another important maturation, if you wish, of their immune response, because they started to now recognize also non-spike antigens.

So they had a broader spectrum of response, which is as you might remember from one

of my earliest slides, we showed with Italian samples that having a broad response is actually associated with milder disease after, in return and published.

So, going back to an issue of vaccination, there is a combination of keep on getting boosted with SARS CoV-2, particularly with now the updated vaccine, which is a good thing.

We were curious about some fairly basic questions of what happens when you boost people multiple times. And again, is that a good thing? A bad thing? And what happens to, for example, your magnitude, as you keep boosting? You get every time same increase or eventually you get to a point where you plateau out. What happens to the durability of both antibody and T-cell responses?

Do you increase the magnitude but then your DK has a similar slope? Or does the slop change as a functional repeated vaccination?

And while conceptually simple, those questions are difficult to answer in the real

world because for example, it's very difficult to find someone that was vaccinated only once, never boosted, and never experienced a breakthrough infection.

We have a few of those that we affectionately refer to as the unicorns in our cohort. But it's becoming increasingly difficult to get any statistical power where you can compare apples to apples, this kind of thing.

And this was actually also made more difficult by real life. So this is the study design of this cohort. Several hundred people, as I was saying, that had the first two vaccinations and the third and the fourth.

And obviously, this was being done at the same time that Omicron and Delta variants were ravaging through.

And so, we selected about, I think it was people that had received different number of boosters, and again, never tested positive nor experienced any symptoms.

And as you can see here, if we run these

people that were never affected, according to them, through the two-dimensional assay, we can clearly see that about 30 percent or more, it depends where you draw the line, had experienced -- had been infected. They recognized non-spike antigens.

And so, these are people that presumably have experienced an asymptomatic infection, because these are all people that when asked, through the main blog, they were asked whether they had any respiratory episodes.

So how does it look in terms of the response? This is the antibody response. It's actually, I think it's a cool graph.

You can see, I can't point, but you can see, again, vaccinated, it goes up and then goes down. Then the booster goes up and goes down. Third injection, up and down.

But you can see where the slope inches up. So it basically, as you keep boosting, you inference not so much the magnitude but also the durability response.

And the other point here, I'm not going to go into detail in the interest of time, but it does matter if people have had an asymptomatic infection, that kind of counts like an additional booster.

So people that had experienced asymptomatic infection have in general more stable antibody response as if they had had multiple boosters.

We and others have reported previously, and this confirms, that the T-cell response is actually relatively stable, if you look in a six- to eight-month period.

So, I'm surprised that there's not much of an action going on here.

So we were curious at this point, okay, apart from the effect of booster vaccination of magnitude on antibody and T-cell responses in DK antibody and T-cell responses, is there a qualitative difference in memory phenotypes of the antigen-specific T-cells.

And so we approached this by single cell

sequencing. So we sorted the antigen-specific T-cells identified by the aim assay and then we did single cell sequencing.

And you see here, we actually sequenced a million, more than a million antigen specific T-cells. To the best of my knowledge, this is the biggest study in terms of number of cells that have been sequenced that are antigen specific for acute respiratory infection.

So, what do we see? These are the CD4 T- cells, and you see here, you see what you expect to see. You see center of memory, TH 1, TH 2, TH 17, like, and you see also this TFR, very prominent T-cell subset.

It's resemblant of T or regulatory T-cells. And I'll get back to that in a minute. At the level of CD 8, again, you see what you expect to see, a large group of granzyme high CD8 particles, are your legit killers, and then other effector population and so forth.

So, first things, what we were interested in is there T-cell exhaustion as a

function of repeated vaccination and as a function of asymptomatic infection? And there is not.

If you look at the CD 4 T cells, whether they experienced asymptomatic infection or not, they keep secreting their cytokines, CDA T cells do not change in their subset of exhausted T cells. Again, regardless of whether these people have experienced an asymptomatic infection or not.

And to reiterate this point, in the context of the people in red here that have had asymptomatic infection, if anything, they have higher effector function, defined as TH 17 like, population or for the CDA T cells, the granzyme positive T cells.

So, there is no exhaustion that we can see at all as a function of multiple vaccination, and having had asymptomatic breakthrough infection, doesn't give you exhaustion, either. Again, in terms of perhaps countering the catastrophic point of view of the badness of repeated exposure.

But what we do see instead, which is

very interesting to me, is this P follicular regulatory T cell.

And what we see is that this population increases over time, but only in the people that have had this asymptomatic infection.

So we have a situation here where the repeated vaccination and the breakthrough infection preserves and increases the adaptive immune response, both in the antibody and at the T cell level.

But at the same time, there is a regulatory T cell population that develops. And actually Dunafarber (phonetic) has very similar data in their data looking at tissue resident memory T-cells.

So our hypothesis is that this is actually a good population in the sense that this is a population that may be linked with limiting or preventing tissue damage in the context of a breakthrough infection.

Remember, these are all asymptomatic infections. This is, at this point, a

speculation. And the way to address this properly, and the reason for doing both experiments, is to show -- to look at people that have had symptomatic infections.

So we would predict the people that have had symptomatic infections maybe have less of this regulatory population that prevents may be involved in preventing the immunopathology of a stated liver infection.

And I think we went through all these conclusions. In the last -- how much time do I have? Okay, so in the last few slides, I wanted to talk about avian flu, highly pathogenic avian flu, which is of course an issue that is of concern right now as a potential pandemic or outbreak, so forth.

So I just want to bring you back to some basic observations made during the pandemic, and the observation was that again, the T cell was largely -- T cell response was largely preserved at the level of different variants because the variant mutation were concentrated and rapidly few

compared to the very large breadth of epitopes recognized by the human T-cell response.

And also, there's data that showed that people that had pre-existing immunity, presumably because of recent exposure to common cold Coronaviruses, we're talking about at the start of the pandemic, actually did better in terms of disease severity compared to people that did not.

And this all went way down to actually different rates of mortality. So the concept that I'm putting forth is that if cross reactive preexisting T-cell immunity may be important and a factor in planting disease severity, not implementing infection, but in influencing the disease severity.

Okay, so highly pathogenic avian influenza. We all know there has been a number of different pandemics, 1918 the most famous killing tens of millions of people, and has been the recent spillover of HPAI into not only poultry but also fields or cattle and wildly detected in wastewater.

So, really of concerns. I want to point out also that the label of highly pathogenic is actually referring to highly pathogenic for birds.

So if you're a bird, the H5N1, highly pathogenic is a thing, how highly pathogenic is, for example, in cows and other mammals, is more debatable. In fact, in most cases has been rapidly mild.

And in humans, the pathogenicity is very all over the place, in some cases in the old studies were up to 50 percent recently, has been no, that I know of, severe case of severe disease associated with human infection with the 2, 3, 4 claim.

And it's also complicated to really know what is the pathogenicity for humans, because A, this can continue to evolve, but also in many cases, probably infections have not been detected in the first place, so you don't know what the denominator is, the real number of infections.

So, nevertheless, it's a very concerning situation that we need to monitor and follow

closely.

Now, is there immunity, pre-existing immunity, to humans, in H5N1? So, we heard earlier on the fact that actually neuraminates is an important antigen.

Actually, antibodies against neuraminates are consequential in terms of potentially having an anti-viral effect.

And H1N1 is one of a currently circulating influenza in humans. And H5N1 has the same N1. Not exactly the same, but basically, there would be some degree of potential cross-activity at the antibody level against neuraminates. In general, VHA does not cross react.

But there's also -- and there is evidence in the old swine origin 1957 pandemic that preexisting immunity really can be influential in the case of influenza.

And what was the observation back then was that old people, for one, as an old person, I can say sometimes things are good for old people,

so the older people in that context fared better in terms of disease severity than younger people.

And that was correlated to a fact that again, H1N1 had circulated before the 1957 in humans. And so people that were old enough to have had exposure to that had some degree of preexisting immunity, planted presumably disease severity from the swine origin H1N1.

So what about T cells? So there's some bioinformatic analysis, and the first thing that we were curious to ask was is there a fundamental difference? Is H5N1 a fundamentally different beast in terms of what antigens are recognized?

And if you do an analysis of what is published overall literature of what humans and animals recognize in H5N1 as opposed to the currently circulating in humans influenza, the hierarchy of what antigens are recognized is essentially the same.

What degree of conservation is there, or is there conservation, between the targets of T cell response? And again, this is a bioinformatic

analysis where we went in and defined what -- not defined. We curated what the scientific literature had defined as dominant T cell epitopes in the currently circulating influenza strains and subtypes, and asked, are these epitopes which make up existing T cell response in humans right now, are those conserved or mutated in H5N1?

And as you can see in this slide, we are going into the den, essentially, the epitopes are conserved to a large extent in the 50-70 percent range and also as the claid of H5N1 has been evolving, there is no evidence that it's picking up more mutations in the dominant vectors.

And finally, we did some experiments. This is with lab. So here, it's a simple mind of experiment in which you basically have the dominant T cell epitope seen in humans from the currently circulating influenza and you ask, if I synthesize the matching peptide from H5N1, is there still cross reactivity?

So the T cell that are elicited by the currently circulating influenza strains, do they

also recognize H5N1? And as you can see in the slide, that is absolutely very strong preservation of the T cell reactivity.

In other words, the reactivity that you get from normal donors in the San Diego region that we had for this purpose, are capable to recognize also the H5N1 matching thing.

So whether this is going to be actually consequential is a matter of debate in the sense that the degree of conversation is less than there was between -- at the time of the swine origin pandemic, but it's more than it was between common cold and SARS CoV2. So by that criteria, it's somewhere in the middle.

And so at this point, it's a very soft conclusion. But we would expect that where to be a widespread human transmission of H5N1, the existing piece of response may be able to some degree to impact disease severity.

And with that, I'd like to end acknowledging some of the main contributors of this study, all the COVID stuff, the early stuff

in particular, was done in collaboration with Shane Crawdey lab, Al Bagrifoni, and Ricardo Silva have been doing a lot of the work with the breakthrough and setting up the immunophenotyping studies. And Jacinta and Greg and BJ have been involved in the single cell studies to a large extent and collaborations and funding as well. Thank you.

UNKNOWN MALE: I have a question about the TFRs. Were they from paid for blood?

DR. SETTE: Yes, this is all paid for blood.

UNKNOWN MALE: Did you have a chance to look at any tissues or lymph nodes, if they're also increasing?

DR. SETTE: No, we have not. And I was mentioning, there is also study from Donafarber where they have access to organ donors, material from organ donors.

And in the tissues, they see something, not exactly the same but very, very similar, but also they call regulatory population.

UNKNOWN MALE: And I also saw a note. They are not inhibitory? They don't have IL 10, TGF data?

DR. SETTE: They do not. They do not have IL 10. So they are not classic T regs, at least in our hands.

UNKNOWN MALE: The tissue damage control, then what function -- what cytokines would do that tissue damage control?

DR. SETTE: There is a bunch of other markers that are associated with regulatory function. I mean, IL 10 is not the only thing that can now regulate T cell response.

UNKNOWN MALE: IL 10 is the classical TFR.

DR. SETTE: Right, right, right.

UNKNOWN MALE: It's not the same. Is only facts through three posted? Are they?

DR. SETTE: They are.

UNKNOWN MALE: Okay.

UNKNOWN MALE 2: I'm quite intrigued with the slide right before this, where you showed

that, if I remember correctly, like less than 6 percent conserved T cell epitopes, that's sufficient to limit disease severity in the case of SARS CoV-2? Am I right, or no?

DR. SETTE: So there, I was trying to be conservative. In terms, if you look at the degree of how frequently you find an epitope which is conserved between SARS CoV-2 and common cold, that's 6 percent. So that's completely different --

UNKNOWN MALE 2: Oh, okay.

DR. SETTE: -- viruses. And so, that's a low estimate. And nevertheless, the data was very clear that that can have an impact. So I was trying to sandwich between two, a low end and a high end, wherein the swine flu was 70 percent or so conservative.

UNKNOWN MALE 2: The other thing is, I have this question for a long time. When you run cell immunity, like those assays, I know your lab will look at an NP or maybe R3A and those other things.

How do cellular immunity really work in the case that an epitope is derived from a protein that is primarily found intracellularly?

So is it mainly through CTL, meaning killing of the infected cells, or are there any other magnets for cellular immunity to control the disease severity if the epitope itself is derived from a viral protein that is primarily intracellular?

DR. SETTE: So, well, I guess this is a very fundamental basic question.

UNKNOWN MALE 2: I'm trying to improve my immunology.

DR. SETTE: Right. Yes. So, in general, yes, CD8, and I don't want to be too trivial, so CD8 T cells in general recognize things that are inside the cell but there are pathways called cross presentation where CD8 T cells can actually recognize things that are entering through an exogenous pathway.

CD8 T cells can kill directly through a variety of different mechanisms, one of which is

to make a big hole in the plasma membrane of the target cells and there's granzymes and other things, as well as they secrete a bunch of other cytokines, such as (inaudible) TNF but can have also bystander effect.

CD4 T-cells, it's pretty much the same thing except in very much different equilibrium, in the sense that CD4 T-cells tend to see mostly things that come from the outside, but also if you purify class two molecules and allude to the peptides that are bound to class two molecules, a large fraction of them are also derived from inside the cell.

So it's not that a CD4 T-cell can't recognize things from inside the cell, but the cell that they recognize with the finish, they need to express class two, and class two molecules are expressed by lymphoid origin cells while class one is ubiquitously expressed by every single cell of the body apart from red blood cells and neurons and stuff like that.

But, so, in general, CD4 T-cells with

aid in the development of antibody response, but there are also cytotoxic T-cells that kill and secrete bystander cytokines.

DR. GOLDING: I think we need to move on because we have another two speakers. So, Jerry, would you like to --

MR. WARE: Jerry Ware.

DR. GOLDING: You'll be the last one.

MR. WARE: Viral Products. This is a speculative question for you. Based on everything you showed both for SARS CoV-2 and influenza, if one believes that a broad T-cell response actually aids in protection against severe disease or disease, to me, that suggests that one would not limit a successful vaccine design to a single anagen.

Do you have any comment you want to make on that or am I reading too much into your data?

DR. SETTE: Yes, and I believe actually having multiple vaccines, multiple antigens in a vaccine is potentially of interest.

And certainly, one could answer that

live influenza vaccines have multiple antigens or pertussis vaccine has for different antigens.

I think one has to balance the fact that one maybe beneficial developing something that has multiple antigens is by definition more complex for a variety of reasons.

You have to purify, say four different things, may interfere with each other and so forth. So I think it's a balance between different components.

And also different modalities to delivery may induce prevalently only CD 4 T-cells or both CD 4 and CD 8 and so forth.

DR. GOLDING: Thank you very much. I think we have to move on.

DR. KURTZ: Thank you, everybody, for joining us. And so, we are in the final stretch of our CBER symposium. And I have the privilege to introduce our last two speakers for the day who are colleagues of mine from the Office of Vaccines here at the FDA.

So I'm going to introduce both of them

together and they will take joint questions at the end of their two talks to end us out.

So today, we have talks from Dr. Akkoyunlu, who is in the Division of Bacterial Parasitic and Allergenic Products.

And his lab mostly focuses on the development of immunity and immune needs to specifically develop B cell immunity to conjugate vaccines.

So Dr. Akkoyunlu has been here at the FDA for over 20 years and he is a principal investigator in DBPAP.

And our last speaker for the day will be Dr. Weiss, who has also been at the FDA for almost 25 years in the Division of Viral Products. So Dr. Weiss, her lab focuses on viral pathogenesis as well as the development of neutralization and understanding neutralizing antibody responses for viruses including HIV, influenza, and most recently, SARS CoV-2.

So with that, Dr. Akkoyunlu would like to come and give his talk, we'd appreciate it.

DR. AKKOYUNLU: Okay, thank you for the opportunity to give this talk about early age immune responses to vaccines.

So I'm part of the Laboratory of Bacterial Polysaccharides, where we regulate bacterial polysaccharide vaccines.

So these vaccines target and capsulate bacteria. Some of them are listed here. Nice, I manage this, streptococcus nomania, group B streptococcus, and they can cause debilitating, very serious diseases such as bacteremia, meningitis.

And another very important common feature of these bacteria are that they mostly target newborns and infants.

So they are encapsulated. The capsule is composed of polysaccharides and the structure of these polysaccharides dictate their serotypes and serogroups.

And the capsule itself has very loose properties. Some of them are listed here. They can inhibit complement, phagocytosis, and it's

always true that the more the capsule, the more invasive the bacteria are.

And then we have also contributed to this while we go showing that some bacterial polysaccharides can inhibit LPS-induced recognition by the host. So it's veering its property to evade the host's immune system.

And it's been a long time since anybody developed against these polysaccharides was recognized in 1930. It was shown in a skin test, hosts have antibodies against polysaccharides.

And then in 1940s, based on these observations, people started to think about immunizing.

And here you see a paper from 1945 about immunizing with pneumococcal polysaccharides. But the problem with these polysaccharides vaccines are they are T cell independent type two antigens, which means that they only induce short lived antibody response, composed of mainly IGM and IGG3.

And they elicit big memory response to

booster immunizations. And more importantly in infants, and especially those who are younger than one year old, they don't induce any antibody response.

As I said earlier, that this age group is the most vulnerable to these pathogens. But nevertheless, these efforts to develop vaccines proceeded, and you can see a list of some of the vaccines that were polysaccharides, pure polysaccharide vaccines approved, and they are all indicated about infancy.

So why are they polymerogenic? So typically, this multivalent structure of the polysaccharides are recognized by the B cells BCRs or B cell receptors, and this recognition is not sufficient to activate these B cells.

And sometimes, it's actually inhibitory. And during an infection, though, there are a lot of bacterial products that can get a ticking signal, then these B cells would respond, for example, they have PS.

But it was for a long time, it was not

known what was the second physiological second signal. And this question was -- so by the way, again, this recognition of BCR, polysaccharides of BCR, doesn't lead to antibody response, but this question of the second signal, physiological signal, was answered in 2001 by several papers, by using a TACI knockout mouse.

This mouse is expressed on B cells, this TACI. They show that these mice didn't respond to polysaccharides at all.

And so TACI's a receptor on most B cells, as I said. It has two ligands, BAFF and APRIL. They are TNF family ligands.

And they can then provide the second signal and then allow these B cells to respond. And we became interested in this system because of the similarity between the TACI knockout mouse and the early age newborns.

So then we focused on the newborn mice and looked at -- measured TACI levels first. And we saw that, as you can see here, they were severely reduced compared to adult B cell TACI

levels.

So as a result, when you expose these details to BAFF and APRIL, the ligands, there's not enough TACI to induce the second signal. So you cannot get antibody secretion from these B cells.

So we believe that at this point that newborns don't respond to polysaccharides because they lack this expression of TACI. And then we further tested this hypothesis by using CPG, which we had earlier shown that CPG can upregulate TACI on adult B cells. You can see here in this full cytometry.

And we did the same thing with neonates. And indeed, in neonates also we were able to induce the upregulation of TACI expression, which then resulted with responsiveness to BAFF and APRIL.

Now these newborn B cells were also able to respond to BAFF and APRIL. Then we moved on to in vitro experiments where we immunized neonatal mice. We can see here that when we add CPG to NP

cycle, the present type T cell in phenotype two antigen, now we can induce antibodies, even in newborns.

And this was because of the TACI upregulation because the TACI knockout newborns were not able to respond, even when we added CPG to NP cycle.

So these collectively, these data then showed that newborn unresponsiveness to polysaccharides, the independent type two antigens, was because they didn't express sufficient amount of TACI, and this observation was subsequently shown that in human newborns also, and infants, their TACI levels are low, so most likely the same scenario applies to humans also.

But the field has moved on, and with the efforts of many labs and especially the late John Robbins, the polysaccharide vaccines were made more immunogenic by conjugating the proteins.

And this conjugation led to more immunogenic polysaccharide vaccines because it

involved T cells. So now they became -- the vaccines became T cell dependent.

And for a long time, how T cells were involved was not known, but Dennis Cappas's lab then showed that these T cells actually recognize the polysaccharide in the MAC2 when they are bound to the peptide.

So there are polysaccharide specific CD 4 T cells which then provide this T cell help, and you get T cell dependent immune response.

And then this led to development of many vaccines including ML4 influenza and pneumococcal conjugate vaccines. Now they are part of the routine immunization schedule.

But the problem is that they need to be administered multiple times, like most other protein vaccines to independent response.

And this in the field is recognized as a window of vulnerability, because during this time until they complete the whole regimen, they are still susceptible to a degree to infection with this bacteria.

So this is a burden. And the need to administer multiple doses also leads to compliance issues.

There was this study in 2016 showed that in the United States, 75 percent of children complete all those six recommended vaccines by the age of 18 months of age, and some of them are undervaccinated which is also pretty significant.

And then if you think about developing countries, this issue of course most likely is much bigger.

So then the question is then why do infants need multiple doses when one vaccine dose is sufficient to elicit protective immune response in adults?

So we became interested in this question. And we started to study the mechanism of dysregulated T-dependent response to vaccines in early age.

So in order to be able to understand that, we need to focus on how T-dependent response happens in adults, because it's very well studied.

We know that in the T cell zone, the antigen is captured by dendritic cells and then presented to CD 4, which then relocate the follicles and become T follicular helper cells.

And then they then help the dendritic helper B cells, which leads to memory and plasma cells. And this reaction is very tightly regulated. And there are stimulators and inhibitors.

For example, IL 6 is a subject cytokine. It's very important in the generation of TFHs. In addition to IL 6, there's IL 21. But there's also the inhibitor, cytokine IL 2.

This is mostly likely needed because excess TFH leads to autoimmune disease and also it has to do with clone expansion and affinity maturations.

And then as I said, the IL 2 inhibits -- and how IL 2 inhibits TFH is very well studied through phosphoryl step five, which then blocks BCF six, which is the transcription factor for TFHs.

But how IL 6 improved TFHs was not known until 2019, where (inaudible) lab showed that in a very I think neat study, they showed that IL 6 signals through phosphoryl step three, and then inhibits IL 2 receptor beta, which then removes these receptors and the IL 2 doesn't have sufficient receptors to induce the inhibitory BCR inhibitory signal. And that's how it appears like this IL 6 mediated stimulation of TFH generation happens. And it's also very well established that newborns don't generate TFHs as good as adults, which then explains why that you need multiple immunizations.

And typically TFHs are marked by PD1 and CXCR 5, and many labs have shown this. And we also presented the same data. You can see how small the TFH population in newborn mice.

And this is the kinetics and this dramatic difference explains why they don't respond well. And since based on these findings about IL 6 and IL 2, then we wanted to focus on their physiology in newborns.

So we immunized newborn nice, five to seven day old mouse, and adult mice with pneumococcal TT conjugate vaccine, and then analyzed their cells.

And we first looked at IL 6 production, IL 2 production, IL 2 receptor expression, and then step five maturation.

The first thing we saw was very surprising to us. We saw that after immunization by day one, neonates in the spleen had a lot more IL 6 than the adults.

So here, the IL 6, which is a signature cytokine that stimulates TFHs in adults, you have even more in neonates. You can see that it continues until day three and then goes down.

You see in one example, you see the C cells, they are cells, but we saw them in B cells, even T cells for this IL 6 after immunization with PTS 14.

Next, we looked at IL 2. Here, I need to focus on -- present this data indicating that we exclude TFRs. And these cells are PD 1 post, 6

CR posted, and then fox B3 negative. And you can see that IL 2 actually has produced much more in neonatal TFH compared to adult TFH. And not only IL 2 has produced more, but also IL 2 receptors, both alpha and beta produced more in neonates compared to adults.

So now here we have a situation where in adults, IL 6 inhibits IL 2 receptor beta, but after immunization, not only we have more IL 6 in neonates, but then also this leads to more IL 2 production and also more IL 2 receptor production, which I didn't show the data, but there's more phosphoryl step five also.

So this is the working hypothesis on how neonates TFHs are not generated as good as adults. So we wanted to do additional experiments to prove if this is true or not.

So one way to prove it is to give excess IL 6. So if IL 6 is inhibitory, so we gave the vaccine again with the PTS 14 with IL 6. It's like an adjuvant, or without IL 6.

In adults, what we saw is that IL 6

induces more TFH, whereas neonates, already low TFH goes even lower, if we have excess IL 6.

And this is the quantification. And not only the TFHs are affected with excess IL6, in adults, we see more IGG and in neonates, already low IGG response against TPS 14 decreases further.

And then when we look at cytokines, IL 2 inhibitory cytokines, IL 2, now with IL 6, we have more IL 2 in neonates. We have less IL 2 in adults.

And what about the receptors? Also receptors are increased in neonates, and only IL 2 receptor beta is decreased, just like (inaudible) lab showed, which IL 6 targets receptor beta in adults.

So this is the summary of what I have shown so far with the immunized mice and mice immunized with IL 6 containing vaccine. It is inhibitory for TFHs. IL 6 induces more IL 2, more receptors, and more step five.

And then the question is that again, is IL 6 responsible? Then the final experiment with

it was in IL 6 knockout mouse. So if IL 6 is really responsible, then we should see a phenotype reversing this effect.

And IL 6 knockout mouse has been used in the field within adults in numerous labs. And here is one example.

You can see that in IL 6 knockout mouse, TFH population decreases significantly, underscoring the importance of IL6 and generating TFHs.

So in newborns, first we looked at antibody response. You have more antibodies when you don't have IL 6.

You have better TFH when you don't have IL6. And you have less IL 2 when you don't have IL 6 compared to wild type.

And then also the receptor levels go down, both alpha and beta. So this is the situation. Then in wild type mouse, you have the lower IGG, TFH, germinal center B, germinal center B cells. I didn't show the data there also.

Same as TFH. And that is correlated

with the receptor expression, IL 2 expression, and also step three.

But when we remove IL 6, then everything reverses and we improve the vaccine. And then we know that IL 6 signals through step three, you can see that here, ex vivo staining for step three shows more step three phosphorylation when we have excess IL 6.

But then how is it possible that in adults, you have this opposite effect on IL 2 and IL 2 receptors? And we are now trying to understand that.

The challenge here is that the subpopulation is very small. So we are developing an in vitro system to generate TFHs, which can respond to IL 6.

And I can say that I had a poster today. We were able to succeed in that, which means that in vitro, we can give IL 6 to human T cells and improve TFHs in neonatal T cells. We can suppress TFH with IL 6. So hopefully, we will be able to interrogate those cells and try to get the answer

we are looking for.

Yes, this is the summary of what I just said. But what does this mean? The implications of all these studies show that in response to vaccines, adults and newborns in this system substantially differently.

And then the delineation of these differences between adult and neonatal in the system can help improve pediatric vaccines by devising strategies tailored for neonatal immune system.

And with that, I want to thank people who did the work. The initial TACI work was done by Sunita Anora and (inaudible) led the TFH work. And we had great support from the Core facility. And thanks to Richard Brown for TACI knockout mouse. And we were generously supported by the Administration and also from the Perinatal Health Center of Excellence Grant. Thank you.

DR. KURTZ: Thanks, Moustafa. So you guys are going to take questions at the end together. So, if we could ask Dr. Weiss to come

up for the last talk of the session, and then they'll take joint questions. Thank you.

DR. WEISS: Okay, so good afternoon, everyone. And thank you to those of you in the audience and online who have made it here through the end of the symposium. This is the final talk.

So, also, I want to mention that the important work that you saw earlier today by Dr. Satte and Dr. Montefiori has provided a great background for some of the topics that I'm going to touch on in my talk, which is Antigenic Assessments of Recent SARS CoV-2 Variants for Guiding COVID-19 Vaccine Variant Composition Updates.

So I'll begin my talk with a very brief review of SARS CoV-2 variant evolution, and then I'll go through some of our laboratory data that informs variant composition updates for COVID-19 vaccines.

And this will include neutralization of new variants by post-vaccination serum as well as antigenic assessments of new variants using

primary infection sera.

And what I mean by primary infection sera, it's convalescent sera from people that have been infected by their first virus, SARS CoV-2 virus, the first variant, and there's no background immunity from vaccination.

So, this graph from the CDC makes the important point that there has been a divergence of SARS CoV-2 test positivity, number of cases shown by this orange line on the graph, and COVID-

Tests shown by the blue bars on this graph over time.

And this is really primarily due to the increasing population immunity. So somewhere around about 2022, you can see that the test positivity cases continue.

So you can see that the test positive cases continue, but the deaths fortunately do not increase at the same rate.

And so this is due again primarily to the impact of immunity, and as you heard from Dr. Sette, cellular immunity helps clear virus

infected cells, which mitigates disease.

And as you heard from Dr. Montefiori, neutralizing antibodies can help prevent virus infection of cells.

Nonetheless, we still see that these waves continue, and actually our most recent wave of test positive cases was high, not quite as high as the first Omicron variant.

So why do these waves continue? Well, clearly, it's because SARS CoV-2 is evolving quickly, as we know too well.

And so what we're seeing in this graph, as I'm point -- what I'm pointing out here is that each of these peaks in the test positivity cases shown in the orange actually is associated with new variants that dominate that wave.

And so, what is believed then is that these -- and there's data that I'll show you and has already been shown, is that these new variants are escaping antibody neutralizers.

So believe then that vaccines that can match the circulating variants can improve

protection against those variants as well as variants that are similar to them, and there is plenty of data to support that.

So during the course of the pandemic, my lab has been involved in measuring post-vaccination and primary infection serum neutralization of new variants as they've occurred.

And in the context of high population immunity, there's really several important considerations.

And that is, and Dr. Montefiori pointed this out as well as Dr. Sette, serum neutralization titers reflect past antigenic exposures from vaccinations, infections, or both.

And then as well, the responses to COVID- vaccines may also be influenced by prior SARS CoV infections and vaccinations. So for our studies, our serum sources come from two large prospective cohort studies. One is called the PASS study, and this is our major source of COVID-19 post vaccination sera.

This comes from healthcare workers at Walter Reed National Military Medical Center and this clinical study is led by Edward Mitre.

The EPICC study also is our primary source of primary infection sera, and this comes from Adult U.S. Military Healthcare Beneficiaries. And that clinical trial is led by Simon Pollett.

And the methods that we use in our lab are very similar to the ones that you heard from Dr. Montefiori. We use a lentiviral pseudovirus neutralization assay.

And I think the main difference in our assays is that we've created a stable cell line expressing both ACE 2 and Tempris 2.

We've also done antigenic analysis using a variety of methods. And that's done in collaboration with Leah Katzelmek at MIAID.

So, in this first data slide, I'm showing you that new variants evade neutralization by post vaccination sera.

So, we looked at sera from different groups and have had different histories of

vaccinations. So this dark purple line is the group that had just three vaccinations with the original or prototype vaccine based on the ancestral variant.

And what we see is that we have very good titers obviously to the max strain and pretty good titers against the later Delta strain.

But as you know in 2021, and 2022, the first Omicron variant developed a very large wave to be a one variant. And we saw that titers dropped four fold.

And then a later Omicron variant, I'm calling here BA4/5, those are related variants that have the same spike but different internal genes.

But you can see that that led to more than tenfold drop in neutralization titers against that variant.

People that got four doses of the original vaccine had a similar drop in titers to those new Omicron variants at the time. But the titers were higher if you had that fourth dose.

So in the spring of 2022, there was decisions being made about the next booster and whether the variant composition should be updated.

And at the time, the BA 4/5 variants or the BA 5 variant was replacing the first Omicron variant. And so a decision was made that an Omicron antigen should be included in the next vaccine booster.

And because this was really just the first time that the variant composition had been updated and there was no clinical data for this new change, a decision was made to make it a bivalent vaccine that included both the original ancestral antigen for which there was clinical data as well as improved coverage over this Omicron variant.

That vaccine contained a half dose of the original ME Omicron antigens.

So that is what I'm showing you here now in this blue line. And actually, it turns out that there was really only a modest increase in titers against the matched variant, BA 4/5, about

two-fold, if you compare it to someone who got four doses of the original vaccine.

But the following year, when in the spring when decisions were being made about again whether to update the variant composition of the vaccine for the fall campaign, obviously the virus had continued to evolve.

In thi1s case, it was a whole new lineage. It was a recombinant virus called XBB.1.5. And so, when we looked at post-vaccination sera for those that had gotten already three doses of the original and the bivalent, you can still see that there was a great drop off of titers of more than 13-fold.

So at that point, a decision was made then to include -- to update the vaccine to include the XBB.1.5 variant. And this is now a monovalent with the full dose.

So this graph then shows in the green bar a sera from the work then that got the booster with the XBB.1.5. And what you can see is just GMP titers, comparing it against all the other

vaccine groups.

They're not all highly controlled the same way, but you can clearly see that those that did get the XBB.1.5 booster had an increased titer to all the variants.

So here's the match variant here, the XBB.1.5, quite a bit higher. We even did four doses of the original vaccine and the bivalent, so they're an equal number of vaccinations, and still the XBB.1 gave higher titers. I left that off here to not have too many lines on the graph.

So, and however, the virus obviously has continued to evolve. And by the time we were making decisions about potential updates, a brand new lineage had emerged, and this is now called the JN1 lineage.

And then some sublineages called KP2 and KP3, which are somewhat related to the JN1 lineage, emerged around the time that a decision was being made this past spring.

So as you can see, there was a five-fold drop in titers relative to the match strain here

for the JN1, more than nine-fold for the KP2, and then more than tenfold for the KP3. And the KP3.1 actually is already evolved a little bit more. It's even slightly more evasive as actually David showed you in his slides.

So, the other important point that I want to point out here is that this was a monovalent against XBB.1.5, but interestingly, we did get some boosting, what we call this back boosting, to titers to earlier variants that were seen by people in this group.

So it back boosted the BA, the BA 1, and some of these earlier variants. And so that message then said that for people that had earlier exposures, there was no need to continue to include some of these earlier antigens in order to get the benefit of the updated vaccine.

So, as you know, recently, the updated JN1 or KP2, that's what's in the current updated vaccines, those are monovalent, it's KP2 for the MRNA vaccines and JN1 for the protein based vaccines, and that's just based on the

capabilities of the manufacturing.

We don't have that sera against the updated vaccine, but what we do have is primary infection sera made for the JN1 infection.

And this was done in the hamster model, which is one of the preferred models for SARS CoV-2. So this will give us an idea that if you're exposed to a JN1 antigen, what does it look like against some of the recent variants.

So we have very high titers against the match strain here, against the JN1 variant, against now the KP2 and the KP3 variants.

We get pretty good cross neutralization, really not more than twofold drop in neutralization titer.

And these are pretty related. You can see there's just a few mutations that are different between the JN1 and these KP2 and KP3.

And that actually most of the immune evasive properties is due to this 4/5/6 substitution that's actually present in both of them.

The other interesting point I want to -- so here is that they're unlike what I just showed you with the post-vaccination sera, there's no cross -- relatively no cross neutralization to the earlier variant.

And so this is kind of a feature of primary infection sera, that it tends to be very focused mostly on the infecting variant or variants that are somewhat related.

And in this next slide, I provide more examples of that. So we have primary infection sera here now against many different variants.

And what I want to point out here is that for the infecting variant shown in red, the highest titers tend to be against the infecting variant.

Now, we do see some cross neutralization among different variants, but they tend to be variants that have the same kinds of RBD mutations.

So for the beta infection, we see we get the cross neutralization to the gamma variant, the

iota and the mu variance, and they all have this immunodominant E484K mutation.

And similarly, for this epsilon infection, primary infection sera, we get the highest titers, but we also see pretty high titers against the delta and the lambda variants, which also share this L452R mutation.

And the final point I want to make on this slide is that while we do have good cross neutralization among some of these pre-Omicron variants, these primary infection sera from pre-Omicrons do not tend to cross neutralize the Omicron variants at all.

And most of them are really below the level of detection. And the exact opposite is true. We have a few sera for people that have been infected only with the BA1 variant. And we do not see good cross neutralization to the pre-Omicron variant.

So because the primary infection sera tends to be more specific to the infecting variant and more focused, it's really well suited to help

us distinguish some antigenic differences among the variants.

And so one of the visualization tools that we use now is called antigenic cartography, where we generate an antigenic map and we think primary infection sera is going to give us the best discrimination among these variants.

So antigenic cartography is simply a visualization tool that shows the antigenic relatedness among the variants. And this is done by applying two-dimensional scaling to the neutralization titers that I just showed you. So it's not new data, it's just a new analysis of the same titers that I just showed you.

And these squares, I'm not sure if they show up in the projection, there's little grid squares here, that corresponds to two-fold dilutions and the serum titer for neutralization, and that translate into antigenic distances.

And so the main point I want to show here is that we do have a clustering of these pre-Omicron variants forming one sort of broad

antigenic cluster.

But then as you can see, the Omicron, especially this first one, and then that's the only one we had true sera against, forming a completely separate kind of antigenic cluster.

So, if we want to continue making antigenic maps, we're running into a problem because it's very hard now to get primary infection sera from humans because of the high population immunity due to people having repeated vaccinations and infections.

So the obvious question then is can we use animal primary infection sera as a suitable alternative to human primary infection sera for generating these antigenic maps.

So we looked into that question, and the serum sources we used were from hamster primary infection sera. And these were generously provided by some collaborators that includes Michael Holbrook's lab at NIH.

He gave us primary infection for the early variants. And Tony Wang here in NDDP. His

lab provided us primary infection sera for some of the more recent variants.

So obviously, we simply compared the neutralization titers to hamster and human primary infection sera using our same assays and then applied antigenic cartography and an additional analysis.

So this shows the comparison of the hamster and the human GMTs against the panel of variants that we tested. We just looked at the variants that we had match set for in both hamsters and humans.

The red trace is the GMTs for the hamster titers against the different variants, and the black is against the humans. And sorry for the colorblind out there. I'm just realizing.

But anyway, you can see that the traces are very similar in both titers and specificity, and they correlate quite well.

So that gave us confidence then to go ahead and generate an antigenic map using the hamster sera. And when we compare it to the human

map that we made, and I showed you earlier, is they're pretty similar.

So we're getting a cluster, pre-Omicron cluster, where the beta variant is a little bit of an outlier in the upper part of the antigenic space and the delta is in the opposite direction, and then the BA 1 forms a separate cluster.

But now with the hamster sera, we have additional primary infection sera for some of these newer variants.

And what we're seeing is now a new antigenic cluster up here with a lot of the XBB variants as well.

So we think this is going to be helpful and just doing some of these kinds of antigenic analyses going forward.

So, with more complex sera from humans that have been a result of multiple antigen exposures from vaccines or infections or both, we use a different kind of visualization tool, and that's called a landscape analysis.

So landscapes are generated by plotting

the neutralization titer of this complex sera corresponding to the primary antigenic map that I just showed you.

So the antigenic map, this X/Y plain, kind of gives you the whole antigenic space of the different viruses and then the landscape shows you roughly the antibody coverage across that space.

So you can see that the serum group that had the XBB.1.5 booster has a more flat landscape corresponding to higher titers to all the variants and these happen to be the Omicron and the XBB variants in this part of the space where the pre-Omicron variants are here, but the groups that did not see that XBB.1.5 booster have low titers.

This can also be useful if your primary antigenic map has a lot more variants and you haven't really tested your complex sera.

You might be able to interpolate what the titers and coverage might be. So these are just tools again to help us think about the antigenic differences in coverage across the antigenic space.

So in summary, continued variant surveillance and assessments of post-vaccination sera against new variants help inform COVID-19 vaccine composition updates.

Hamster primary infection sera may be a good substitute for human primary infection sera for assessing antigenic differences among new variants.

Antigenic cartography aids the visualization of antigenic differences among the variants. And the landscape analysis model serum neutralization coverage of the variants across the antigenic space.

And so efforts are now underway by many public health groups to establish more of a coordinated network, framework for assessing antigenic changes in new variants to aid decisions about the updates.

And with that, all credit goes to my dedicated, hardworking lab, including Russell Way, Sabrina, Sabri, Rachel, Richard, Stephanie, and Brittany.

Several of these people have moved on to medical school. And my collaborators, I mentioned. And all of the investigators over at the Uniformed Services University and Military Facilities have really made the collection of these valuable serum possible.

And with that, I'm done.

DR. KURTZ: I'm going to turn over the table here to our two final speakers, and they can take any questions and we can see if there's any online.

I'll start off with a question for Carol. So I'm assuming this is knowable. Has mutation rate changed in SARS CoV-2 between the introduction of the vaccination pre and post? So has vaccination actually ramped up the mutation rate or selecting for or against it?

DR. WEISS: I can't quote you any references for that, but I don't think so. I mean, I think most of the world is not vaccinated, probably, and we get these big global sweeps.

There's some geographic variation, but,

yes, I think -- early on, pre-Omicron, I mean, there was some sampling with these mutations going on where the virus was trying to -- was clearly escaping.

But early on, it was just a lot of adaptation as well. But now with the highly immune population, there's a lot of selection.

DR. KURTZ: So actually as one other quick follow up, so it seems like there's, with all these new lineages, there's a pretty quick expansion and then contraction or evolutionary death.

And so, and then on to the next lineage and the next lineage, and some are related and some aren't. But in terms of thinking about a timeline between the expansion and contraction of a lineage that's so different you're losing neutralization, antibody potential.

What does that also mean in terms of sort of the timeline of the expansion and contraction of the average lineage and the escape variants and the timeline of how we can pivot the

manufacturers to picking a new strain?

I mean, it seems like these strains are coming and going within a few months. Maybe the average lifespan of one of these lineages is maybe on the order of three to six months, and that seems like a really quick timeframe to be able to pivot to a new variant for a vaccine.

DR. WEISS: Yes, so there's a complicated answer. I mean, there are many considerations going into whether vaccines get updated.

Market demand, infection capability, pathogenesis, epidemiology and all those things. So it's true that it seems that the virus is evolving, in my opinion, about three times faster than flu, where we're trying to do an update every year.

And we may never get there. If you want a perfect match, obviously, what's important is this less severe disease over time, obviously.

And so a lot of these updates may only be taken by people at high risk, for example, or

by choice or whatever.

But there's a limit to what the market will bear for how many times things are going to get updated to be quite honest about it.

But if we can increase durability, and maybe as people get more and more immune, that durability is going to be better, different types of vaccines.

So I think we're still trying to figure out the variant waves, if they're going to continue at the same rate or not. They might, but certainly, hopefully the disease will not be as great.

DR. RAJASAGI: One message to follow up on the mutation rates, whatever, how much has been driven by vaccine or not, and I don't know the answer, that's why I'm asking.

Has there been an increase on non-spike mutations as a function of the evolution or not? Do you know? I mean --

DR. WEISS: I would ask you that for this order. I think there's selection going on in

some of the internal teams a little bit in terms of escape maybe from host innate responses and things like that. But that's more adaptation, not really escape.

So ask your question again. I just got distracted there.

DR. RAJASAGI: Other antigens. Whether there has been a change over time in how frequently we see mutations outside of spike.

DR. WEISS: Yes, okay. So I'm not an expert on that. It's not changing as much. Spike is clearly changing the most.

But I think most of those changes you see going on is probably not necessarily immune pressure, per se, but the cat and mouse of the virus adapting to maybe innate responses and things going on.

Because we do see some recurrent changes in like the morph state and some other interesting genes that I know a little bit less about.

But I would really be interested in what you're seeing from this pressure from the cellular

level. Okay.

DR. RAJASAGI: And one question for him. Again, highlighting my ignorance. I think that there are examples of vaccines that, like BCG, my recollection is works better in preventing in childhood than in adults.

And I was wondering, is there any difference with IL 6 business that you were highlighting in terms of BCG or not?

DR. AKKOYUNLU: No, we haven't studied BCG. But I think this is only valid for antibody responses, CD 4. So BCG, we have, yes, it's another mechanism.

So it's worth looking at it. I mean, we are expanding it to look at other components of an active immune system to see if there are other differences.

So it's just like scratching the surface so far. And just to remind you, when you're talking about MRNA vaccines, there is a very nice paper showing that its immunogenicity highly dependent on the lipid particles inducing IL 6.

So as we know that MRNA vaccines are only approved about six months. So we don't know. Maybe that's why there was some observation that it's not working very well. But I don't know, just speculation.

DR. GOLDING: Yes, thanks. I have a question for each. So, what I'm learning from you, neonatal immune response is significantly different, not only against polysaccharides in terms of the need for inability to recognize the independent antigens.

But even the response to the dependent is not the same. How do you think it should impact our regulation of vaccines for children and design of -- because normally we -- even vaccines that are trying to target childhood diseases, we test in young adults and then we do de-escalation.

And the assumption is that it will show safety and immunogenicity. But would you suggest from your studies, based on either the different role of IL 6 and germinal center, that you need to have a totally different approach to generating

vaccines for the very young?

DR. AKKOYUNLU: Yes, I think that's the take home message from our studies so far. There's still much to do.

But I also would like to emphasize that current vaccines have mostly have all of them, right? So the addition of adjuvants has been a bit slow so far trying different adjuvants.

Experimentally, there were a lot of trials. But what people do is trial and error. It's empirical. You just add certain adjuvant and see if it's working or not, rather than designing an adjuvant that would target those pathways that are beneficial.

For in this case, if you're inducing IL 6, it wouldn't be good. Right? So I think our data clearly shows that.

But it needs to be seen if it's also with different adjuvants, which adjuvants induce IL 6, which adjuvants don't induce IL 6, in vivo after immunization.

So those are the I think take home

messages. And as I showed the vaccination schedule for all the T-dependent, you have to get three to four vaccines in order to elicit protective response.

Otherwise, people try to truncate that and it doesn't work with the current formulations. In our field for the pneumococcal, we know there's another phenomenon. Maybe we're diverting from the thing, but immunological creeping in pneumococcal vaccines where it started with 7 valiant and then 13 valiant and now we have 20 and then there are even up to 30 coming.

And we know that with every vaccine, antibody responses and (inaudible) activity against the common sero types are decreasing. And that's called immunological creeping.

So, now the companies are coming up with strategies to improve their vaccines by adding adjuvants for the first time.

All of them had alum or nothing before. So I think the field really needs more knowledge to be able to tailor vaccines for different age

groups.

Older age is another issue that Letterman from U.K. has done really nice work on showing that there are differences there also.

And most vaccines of course are tested in adults. And the mechanisms are also revealed in adults. So I think there's a gap there.

DR. GOLDING: Thank you. So, Carol, I'm thinking again about all the last couple of slides that you showed.

So what struck me is when you showed the cartography. You really emphasized the fact that after XBB.1.5, it sort of was latent, which suggested there is a decent cross reactivity against new variants.

When you showed the data from the hamster that was just infected let's say with JN.1, there was very little cross reactivity against XBB.1.5.

So I'm starting to get a sense that what the hamster tells us and what the human post-vaccination tells us may not be the same, even

though when you do the cartography they look sort of similar.

What do you think really should be the more relevant measurement to decide on strain change? And it's a little bit like what happened with influenza, right, when we take the ferrets they give you sometimes for differences and human panels give you less.

So I think we are at the same crossroads now with COVID. And just your own personal --

DR. WEISS: Yes, maybe to frame it optimally. So, I mean, I think we use data from many different sources.

We are interested in the post-vaccination sera which reflects the human population. But when you have to -- if you want to find out whether some of the new variants -- you want to optimize your ability to distinguish how different they are, if you're going to update it, the vaccine, right now, why not making it closer to one that is circulating.

But how different is it from something

else? So I guess what I'm trying to say is, the primary infection sera gives you more antigenic discrimination. It's just another piece of information.

And then if you kind of do that landscape analysis, it kind of lets you sort of see what your complex sera looks like over this antigenic space.

But both are important. And I guess the last thing I would say is, we still have to worry about the pediatric population that hasn't seen anything.

So it's not completely irrelevant looking at this primary infection sera as it pertains to the pediatric population that hasn't seen it.

But, so it's not one or the other. I think the post-vaccination sera is clearly the most important, actually.

But there's quite a bit of value in helping to distinguish these variants using the primary infection sera. So, that's -- I didn't

mean to say one is better than the other. They're all useful.

DR. GOLDING: Yes, the last comment that I wanted to make is that this point, we really don't know what fall decrease in cross reactivity predict major breakthrough infections.

What struck me again, based on your slides, that after the XBB.1.5, you only see maybe one or twofold reduction towards later Omicron, yet we know right now that we have lots of infections.

So maybe we should pay attention for just twofold decrease in cross reactivity. Is it the fall decrease from last year or is it the -- or do you think we will identify a GMP threshold?

DR. WEISS: Yes, we don't know. Again, why keep with the old one if you're going to boost again? But, yes, so we don't know that threshold. And I think it could change over time, too, again, based on sort of like the durability of the responses.

So part of it is what I showed you, and

I forgot to point it out, but those were samples taken at the peak time post-immunization.

So six months later, not everyone's titers are going to be at that level, and they may be well below what could be enough to protect against infection.

So durability is a big question. A lot of people are looking into that now as we get more and more exposures, you're getting better durability.

DR. GOLDING: Thanks. Beautiful talk.

DR. ELKINS: We want to do justice to the people online. So for Moustafa, I think you've already addressed this, but the question has to do with the state of the situation with adjuvants to make polysaccharide vaccines effective in infants. Is there anything more you want to say about that?

DR. AKKOYUNLU: For polysaccharides, if I understand correctly, yes, I mean, that should be tried, but at the same time, the conjugate vaccines are working pretty good if you give them

repetitively.

So, I think one has to try and see if it's better or not. But I doubt that it will be better because the memory part still be, you need T-cells I think.

DR. ELKINS: And then the other one asks about your thoughts about whether those higher IL levels that you see in neonates are serving another functional purpose, such that if you manipulated that, you may have some unintended consequences, and maybe you learn something about that from the IL 6 knockout mice.

DR. AKKOYUNLU: Yes. No, that's important. I'm sure there is a function for the development of the babies.

And then if you elevate that, would there be consequences adverse? I doubt it, because it would be temporary during vaccination that there will be long-term consequences, but that needs to be bear in mind so that -- yes, I agree, that's a good point.

DR. BURTS YOUNG: Coach from the back

row here. So I think we'll just thank our speakers now as we wrap up. So let's give everybody a round of applause.

And our ADR receiver, Karen Elkins, is going to give us just a few wrap up comments.

DR. ELKINS: So I know it's late, but some attention must be paid to a few important points. First, I really want to thank all of our external speakers who joined us for our first hybrid meeting post-pandemic and who took the trouble and the time to come and to enrich us with their science and with their presence. We are really very grateful.

And we thank everybody here and online. And there have been hundreds and hundreds for joining and making this symposium what it is.

I hope you all learned something. I hope you all made a connection or a collaboration. And I hope you enjoyed some of the wonder and magic of biological science in the last couple of days.

But the meeting itself is not a product

of magic. It happens by incredibly hard work. And I particularly want to call out the planning committee who has worked for the better part of a year on the scientific content and the format.

Ronna, David, Muhamma, Marisabella, Yen, Ronit, Daniel, Hana, Daron, Katie, Alex, Emily, thank you ever so much.

Please, when you see these people, give them your appreciation. Not listed here are a cast of thousands who were involved in the logistics who got our speakers traveled, who got our rooms arranged, who did our publicity, and our AV team who made it all work, which I find borderline miraculous. They all deserve a debt of thanks.

And not listed on this slide, because I really have to call out Monica. Monica has done thousands and thousands of details and hours of work on this, not only kept track of those thousands of details but integrated them all.

The neural networks involved are remarkable. Thank you, Monica. And, yes, right,

she's back there somewhere. And we are in your debt, as ever.

And with that, thank you all for coming, and we are adjourned.

(Whereupon, at 4:33 p.m., the PROCEEDINGS were adjourned.)

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CERTIFICATE OF NOTARY PUBLIC

DISTRICT OF COLUMBIA

I, Michelle Begley, notary public in and for the District of Columbia, do hereby certify that the forgoing PROCEEDING was duly recorded and thereafter reduced to print under my direction; that the witnesses were sworn to tell the truth under penalty of perjury; that said transcript is a true record of the testimony given by witnesses; that I am neither counsel for, related to, nor employed by any of the parties to the action in which this proceeding was called; and, furthermore, that I am not a relative or employee of any attorney or counsel employed by the parties hereto, nor financially or otherwise interested in the outcome of this action.

(Signature and Seal on File)

Notary Public, in and for the District of Columbia