# Transcript

## Dr. Nedra Whitehead

Hello, everyone, and welcome to today's R3 seminar on understanding the biomarkers of PASC. I'm going to move to the next slide, Shane. The goal of the R3 seminar series is to catalyze a shared understanding of the research of the scientific stakeholder community within the RECOVER consortium. All of these sessions are recorded and posted to recovercovid.org if you missed one or want to refer back to one in the future. Thank you. Next slide.

Our panelists today are Dr. Michael Peluso, Dr. Mohamed Abdel-Mohsen, Dr. David Walt. Our discussion is Dr. Grace McComsey, and I am Dr. Nedra Whitehead. I am with the RECOVER Administrative Coordinating Center at RTI International. Dr. Peluso is an infectious disease physician at the University of California San Francisco. And prior to COVID, his research was focused on the chronic sequelae of HIV infection. When the SARS-CoV-2 pandemic emerged, Dr. Peluso led the efforts to implement the long-term impact of infection with novel coronavirus for LIINC study at San Francisco General Hospital based on the hypothesis that COVID could have a long-term impact on health and wellbeing. Dr. Peluso leads projects within LIINC that are aimed at understanding the biological mechanisms that drive Long COVID. He's also responsible for the implementation of the UCFS enrolling sites for RECOVER.

Dr. Abdel-Mohsen has contributed to over 70 peer-reviewed studies that advance the understanding of how the host immune system responds to viral infections. His research at the Wistar Institute focuses on the role of host glycans in regulating immunological and inflammatory response during viral infections. His laboratory's recent discoveries have shown that the previously unappreciated factor of host glycosylation impacts chronic inflammation and viral persistence during viral infections. These studies set a stage for broader scale studies to understand the upstream mechanisms and downstream consequences of these observations and to design novel strategies to manipulate glycosylation, to reduce viral persistence, or prevent or delay the development of viral associated comorbidities.

Dr. David Walt's academic and clinical appointments include being the Hansjörg Wyss Professor of Bio-Inspired Engineering at Harvard Medical School and the Co-Director of the Massachusetts General Brigham Center for COVID Innovation. He's received numerous national and international awards and honors for his fundamental and applied work in the field of optical microwell arrays and single molecules including the 2021 Kabiller Prize in Nanoscience and Nanomedicine. He's a fellow or member of several academies, including the National Institute for Medical and Biological Engineering and the National Academy of Inventors, and he is inducted into the US National Inventors Hall of Fame.

Dr. Grace McComsey is an infectious disease physician also, Associate Chief Scientific Vice President of Research and Associate Chief Scientific Officer at University Hospital's Health System and a Professor of Pediatrics

and Medicine at Case Western Reserve University. Dr. McComsey is an internationally known researcher in the field of HIV. She's the author of over 300 peer-reviewed publications, mostly in the area of metabolic and cardiovascular complications of HIV infection and the role of systemic inflammation and immune activation. Since the COVID-19 pandemic emerged, Dr. McComsey has been very involved in efforts to encourage clinical research at Case Western and the University Hospital System to treat and prevent a COVID and Long COVID. And she's a contact IPI for the NIH Impact Study and the Case Western Reserve contact PI for RECOVER. Thank you.

I thank all of our panels for being here today. We have an outstanding panel. As I said, the seminar today is on the biomarkers of PASC. The first talk is by Dr. Peluso, who's going to talk to us about biomarkers of PASC, why they are needed, and early immunologic findings. The next talk is going to be Ultrasensitive Viral Antigen Measurements in PASC Patients by Dr. Walt. The last talk is going to be on markers of fungal translocation and whether they're elevated in PASC, and the induction of NF-κB signaling. And that talk is by Dr. Abdel-Mohsen. And Dr. McComsey is going to tie everything together for us and wrap things up. We are now going to Dr. Peluso.

## Dr. Michael Peluso

Hey, everybody. It's a pleasure to be here today to talk with you a little bit about where the field is at in terms of biomarkers for PASC and for Long COVID. I've been charged with giving an overview of where we are in November 2020, some of the early immunologic findings really focused on mechanistic biomarkers of PASC, and then I think setting up the framework for the subsequent talks by Dr. Walt and Dr. Abdel-Mohsen. Here are my disclosures.

These are some news headlines from the last year about acute COVID, Studies Point to Big Drop in COVID Death Rates, A gamble pays off in spectacular success: How the leading coronavirus vaccines made it to the finish line, Pfizer's COVID Pill Works Well, Company Confirms in Final Analysis. This is all great news, super positive, very exciting. In contrast, these are some headlines about Long COVID from the last year, "There's no one Long COVID" Expert struggle to make sense of the continuing mystery, she went to one doctor, then another and another, and Long COVID patients, in search of relief, turn to private company.

And so, how did we get here? Why is there such a disconnect between everything that's been accomplished in acute COVID and the struggles that we face with understanding and managing long? I'm coming at this from the perspective of our local research study at UCSF, which is called LIINC, and stands for Long-Term Impact of Infection with Novel Coronavirus. Our study opened in the earliest days of the pandemic, in April 2020, and our responsibility in the study was really to characterize the post-acute immune response in people who had COVID, made it through the acute infection, and recovered. So, we started seeing these people in person in April of 2020, and we quickly learned within a month that many people coming to give blood samples for our study were saying, "I had COVID four or six weeks ago, and I don't really feel back to normal."

And so, what we did was we began to systematically record everything that people were telling us, asking everybody the same questions in the same way, and we ended up unintentionally being one of the earliest studies of Long COVID in the US and in the world. Since that time, we've enrolled hundreds of individuals with and without Long COVID, seeing them through all of the various waves of the pandemic, which has been ongoing, and really done a deep dive into trying to characterize them clinically and in terms of what's going on immunologically.

Importantly, most of our participants in LIINC have now rolled over into the RECOVER program, and for that reason, the network now has access to all of these historical samples that we've been collecting since way back in April of 2020. And so, we're excited to share those specimens and those data with RECOVER.

One of our earliest findings in LIINC was that patient-reported outcomes, although probably the most important outcomes for Long COVID, are really challenging to measure. This is a really difficult clinical condition to track over time. There are several reasons for this. There are multiple different Long COVID endotypes, which I'm sure everybody on this call knows. So, we think that there are different clinical subtypes of Long COVID which may have different etiologies. There's a lot of variability between individuals who are experiencing long, and there can also be variability over time within an individual who's experiencing Long COVID, where some symptoms can disappear and then come back, some symptoms can disappear and stay away, and other symptoms may take a while to emerge, and then maybe they're quite consistently. And so, this makes it really challenging to exclusively use patient-reported measures as core outcomes for Long COVID.

And so, the result of this is that we really desperately and urgently need other measurements that we can make to supplement the clinical data that people are telling us. And so, these are some quotes from Dr. Fauci over the last couple of years that I think emphasize this issue, "You don't really know what to do about something until you know what the lesion is, and we don't know what the lesion is yet." "We don't even know what the target of treatment would be." And Long COVID trial endpoints, which we're all motivated to launch Long COVID clinical trials, these endpoints absolutely have to be standardized.

And so, there are a number of different types of biomarkers that might be useful for Long COVID, and I want to review those before I delve into what's been going on. The first type, which I think most of us will talk about today, are mechanistic biomarkers of Long COVID. These are the types of markers where we can take a measurement to figure out what is the actual underlying pathophysiology of PASC or of Long COVID. The second type of biomarker that would be really useful are diagnostic biomarkers. So, this is to confirm that symptoms that a person is reporting are actually due to Long COVID and not something else. And so, an example of that is there are all sorts of causes of shortness of breath, and a person who had COVID may have shortness of breath due to Long COVID or they may have shortness of breath due to another medical condition like asthma or COPD. And so, it'd be really useful to be able to do a test to say, "This person's symptom is specifically related to COVID."

The third type of biomarker that would be really useful or predictive or prognostic biomarkers, so this can predict who is at risk for a certain outcome, you can imagine a predictive marker being measured during acute

COVID when somebody just gets infected to determine their likelihood of developing Long COVID weeks or months later, and you can also imagine a biomarker like this being useful in somebody who already has Long COVID to predict whether they're likely to improve with their symptoms getting better or resolving spontaneously or whether they're likely to have ongoing issues. And then finally, the holy grail of biomarkers are really surrogate markers. What a surrogate marker means is you identify the marker, you identify the level of the marker, and then you do something to change that. And changing the level of the biomarker results in a change in the clinical outcome. And so, surrogate markers are really going to be critical if we want to develop endpoints that can be targeted in clinical trials, and this can also greatly increase the speed at which clinical trials can be implemented and we can get results from clinical trials. And so, all of these efforts are ultimately hoping to identify surrogate markers.

I want to couch this in a different pandemic, the HIV epidemic, which has been, as you know, ongoing for 40 years, because I think the lessons from HIV really inform a lot of this work in PASC. The key point is that for most of the '80s and part of the '90s, diagnostics and therapeutics for HIV really struggled because there were not good biomarkers yet. And these are some news headlines from the archives from the early '90s. You can see here lots of scientific efforts at scientific conferences for HIV and AIDS, but there's a daunting maze, people are frustrated. And many of the story of the Dallas Buyers Club where so many individuals, who themselves were experiencing HIV/AIDS or who had friends or loved ones experiencing this, were really desperate for treatment to get people better to prevent bad outcomes.

think that's sort of where we're at now with PASC. There is a lot of data being generated, but there are many, many more questions than answers, and people are really hoping that we can get to therapeutic soon. And then another nice parallel of all of the intense HIV advocacy that drove the field forward in the '80s and '90s, I think, is being mirrored now with all of the community efforts and advocacy efforts around PASC. Plasma HIV RNA, the measurement of the virus in the blood, is really the key measurement in people with HIV now. But before this biomarker was discovered, clinical trials for HIV treatments relied on the types of endpoints that we're thinking about now for Long COVID. So, whether people had reductions in CD4 T cell counts, which can take months or years to develop, whether they developed HIV/AIDS related adverse events, which again take years to develop, or whether people died. This all happens over long periods of time.

So, in order to study different treatments without a biomarker like plasma HIV RNA, these studies had to be huge. They required thousands of people and years of follow up to even see small effects. And then in the mid-90s, plasma HIV RNA was identified as a predictive biomarker. And so, what this paper by John Mellors is showing was that the measurement of the amount of HIV in a person's blood now would determine whether they developed AIDS-related events or whether they survived years later. And so, this allowed us to identify people who were at the highest risk of bad outcomes without having to wait to see if those outcomes developed.

And then very shortly after that, HIV RNA was identified as a surrogate marker, where decreasing the amount of HIV RNA in a person's blood would totally change the clinical outcome months or years later. And so, a reduction in the amount of HIV in a person's blood resulted in a decreased risk of AIDS-related events later on. This meant that changing the marker could change the outcome, and now we had a target for clinical trials. The validation of plasma HIV RNA is a surrogate marker transformed the field, all of the clinical endpoints vanished, and this really accelerated the development of treatments. And you can see here, there wasn't a lot that was developed in the '80s and '90s, and then there was an explosion of HIV therapy in the late '90s, which has continued for 20-plus years now. Our focus now is really just on optimizing therapy.

And so, we are at the very beginning of this for PASC. We've identified, we think, some mechanistic markers and are trying to figure out how they're all related to one another, but we don't really have diagnostic biomarkers that have clear clinical use yet. We don't have predictive biomarkers, and we certainly don't have surrogate biomarkers yet, and that's where we need to get.

I'm going to shift gears now and then the second half of this talk about some of the mechanistic biomarkers. This is a schematic showing an individual with acute COVID who gets through the acute infection and either recovers fully or develops Long COVID and has persistent symptoms. The bottom of the slide shows some of the hypothetical mechanisms that are being investigated for what drives Long COVID. Many of the R3 seminars this year have explored in depth some of these mechanisms. There was a great one last month on microvascular dysregulation and clotting issues. The talks today are really going to focus on the three mechanisms outlined in the boxes here today. And so, we'll talk about biomarkers of these mechanisms in more detail.

The first marker, which Dr. Walt will talk about in much more detail, is persistent SARS-CoV-2 virus in a person's body. I just want to highlight this biomarker study from our cohort in San Francisco, the LIINC study, where we studied individuals who had COVID and either did or did not have neurological symptoms about three months after they had COVID. We did this in collaboration with Ed Goetzl at UCSF. He measured exosomes, which are little membrane-bound pieces of cells that you can collect in blood and that were measured from neuronal cells and from astrocytes, which are the support cells of the nervous system. He made this really surprising finding that months after people had COVID, we were able to detect SARS-CoV-2 proteins in these little vesicles that were derived from cells and were circulating in their blood.

In addition to that, people who had neurologic symptoms had the highest levels of proteins in these neuronal and astrocytic exosomes, whereas people who had non neurological symptoms but still had PASC, so they had shortness of breath for example, had slightly lower levels, and people who had recovered from COVID and did not have any Long COVID symptoms had even lower levels. And so, this was one of the earlier clues that there might be persistent virus at play. There are some complimentary observations listed in these other studies on the side, and Dr. Walt will talk about these types of biomarkers in much more detail.

What about inflammation? There's been a lot of effort over the last couple of years to characterize post-COVID inflammation. To me, one of the most interesting early studies, actually not shown here, was a study of convalescent plasma donors who were donating blood after they had COVID to, in theory, help people who had acute COVID. This study identified that individuals who felt well enough to donate blood after COVID had higher levels of inflammation in comparison to historical blood donors. So, when we saw that, we began to think, "Well, we wonder whether people who are more symptomatic may have higher levels of inflammation than people whose COVID symptoms have resolved." And so, in LIINC, we did a biomarker study looking at 120 people who all had COVID, with and without Long COVID, using an ultrasensitive assay to measure protein in the blood. What we found was that people who had Long COVID had higher levels of certain biomarkers like interleukin 6, TNF-alpha and IP-10, suggesting that there was some inflammatory signal related to Long COVID.

We also found that the differences in IL-6 levels persisted over months after people had COVID. And so, there are a number of really high-quality studies, some of which are shown on the bottom of the slide, that have made very complimentary observations suggesting that similar pathways are pretty reliable in predicting who has Long COVID and who might develop Long COVID. This is another area of great interest right now.

One of the criticisms of this study was, "Well, you took everybody who had all sorts of symptoms with Long COVID, but what if you looked at just certain types of symptoms? Would these observations really hold up?" And so, in response to that criticism, we did another study studying a couple of hundred people with and without Long COVID who had neurological symptoms. What we basically found was the same thing. In fact, a lot of these observations and associations were even stronger. We found that in individuals who had neurocognitive symptoms like brain fog, headache, et cetera, after they had COVID compared to people who didn't have those symptoms, there were higher levels of the same markers throughout the recovery period in the first few months after they had COVID. These held up for months.

And then, we also looked at certain biomarkers of neurologic turnover, so GFAP, which is an astrocytic marker, these are the supporter cells of the nervous system, and we found that elevations in GFAP early on predicted whether people would have Long COVID a few months later. And then what about cardiopulmonary symptoms? I just showed you some neurologic findings. Cardiac symptoms, we found in our group are actually associated with decreased exercise capacity. A lot of this work was led by one of our cardiologists, Matt Durstenfeld. On this graph here, you can see that people who were reporting symptoms related to shortness of breath, chest pain, et cetera, have lower than expected exercise capacity on cardiopulmonary exercise testing.

And then, so what Dr. Durstenfeld did was he said, "Well, how does this relate to the markers of inflammation that we measured previously?" He was able to show that higher levels of inflammation with those same markers that I just talked about a few months after somebody had COVID predicted whether they would have lower exercise capacity related to COVID symptoms months after that. And so, we're beginning now to tie

measurements that are made with biomarkers in the blood to both symptoms and also objective physiologic measurements in people experiencing Long COVID.

There are lots of other proposed mechanisms of PASC, some of them are outlined here. If you think that Long COVID is caused by irreversible tissue damage in the early days of infection, there are certain biomarkers, many of which are being measured in real time by RECOVER to look at kidney, liver, heart, brain function. If you believe that persistent viral infection is causing Long COVID, there are biomarkers related to that that Dr. Walt will talk about in more detail. We already talked about some of the inflammatory markers. If you think that microbial translocation from a leaky gut is contributing to Long COVID, there are markers of that, which Dr. Abdel-Mohsen will talk about. There's a lot of effort into identifying auto antibodies. We had a great biomarker talk about microvascular disease last month, and many of these are also being measured in RECOVER. Many of you will also be aware of a really excellent pre-print from the group at Sinai and Yale identifying serum cortisol as a biomarker of PASC.

And so, the reason I'm giving you this list is that each of these biomarkers can potentially be studied in therapeutic interventions of Long COVID to figure out whether changing the level of a marker has an effect on how somebody feels. And that's ultimately what we're trying to get to. Where are we in November 2022? Biomarkers are providing clues as to the mechanisms of Long COVID. They're potentially beginning to help with diagnostics, although the clinical utility isn't so cut and dry yet. I think these could theoretically be helpful in terms of entry into clinical trials and monitoring therapy in clinical trials. But we're still at the early phases of this. This is the timeline of HIV where it took 15 years to develop disease markers, predictive markers, to get to a surrogate marker. And after that surrogate marker was identified, there was an explosion in therapeutics. We're at the beginning of this timeline for PASC right now. I am hopeful that all of these types of biomarkers will be developed and validated in short order. I think that once that happens, we're going to see the same explosion in therapeutics and really begin to get answers for people.

In conclusion, right now, most of the work is focused on mechanistic biomarkers of Long COVID. I think these studies are really going to increase in complexity over the next year, start to explore relationships between markers and delineate pathways that need to be disrupted for Long COVID treatment. Diagnostic biomarkers are under development right now, but the clinical utility isn't clear yet. Predictive biomarkers are needed in the short term as we begin to move toward therapeutic studies. And ultimately, we are going to need good surrogate biomarkers to jumpstart the therapeutic interventions and get people the answers they need. I'm going to end there by acknowledging my research team, and we'll turn it over to Dr. Walt. Thanks so much.

## Dr. Nedra Whitehead

Thank you, Dr. Peluso. Dr. Walt is going to speak now on ultrasensitive viral antigen measurements in past patients. Dr. Walt.

## Dr. David Walt

Thank you, Dr. Whitehead. I want to commend Dr. Peluso on an excellent presentation, really helps set things up. I'm going to talk about ultrasensitive antigen measurements in PASC patients. A lot of you probably have seen some of the work that I'll describe later, and I'll give you an update on some of the more recent work that we've done. But I want to also take you back a little bit because I think it's important that we provide some context for where things are today relative to where they were previously.

Before I get started, I just want to describe the methodology that we employ in our studies. This all arose as a consequence of the need to really drive sensitivity for markers, particularly proteins, that are undetectable. Standard Elisa has a bottom limit of about one picomolar in terms of its limit of detection, whereas, the method that I'll describe, and actually Dr. Peluso mentioned it earlier, Simoa, is a technology that was developed in my laboratory that allowed us to digitize the signals from single protein molecules so that instead of having this continuum of intensity when you look at a measurement, you actually can literally count the number of molecules. What that does is it allows us to get away from lots of background signal that is present in some of these larger volume assays. A much lower limit of detection is a consequence of this digitization.

The typical limits of detection are in the femtomolar concentration range, which is about 1,000 times or even 10,000 times more sensitive than a standard Elisa. The technology is a B-based Elisa system that simply uses many more beads than there are molecules in a sample. At very low concentrations of protein, we have many more beads than we have molecules, and so most of the beads will have nothing bound to them. The beads that end up with something bound to them, as you can see this upper section of the slide, a protein molecule that binds to one of these beads gets labeled with an enzyme. The bead then gets loaded into this microwell array. One bead fits into each well. We then add a substrate that allows the conversion of a non-fluorescent substrate molecule into many molecules, a protein fluorescent product per second. And so, within a few seconds you get these bright spots lighting up where there is an immuno complex. And where there is nothing bound, it stays black. And so, this is the way that we literally count the number of protein molecules that are present in a sample. As I say, it's much more sensitive.

The instrument that's used is an instrument that's commercialized by Quanterix. This is my disclosure. My lab developed this technology. We licensed it to Quanterix. I'm on the board of directors. I hold equity in the company. The workhorse instrument, and again, my lab gets no benefit, we don't get an academic discount, we just pay the normal retail rates for the instrument as well as the reagents that are used, but because my lab has developed the technology, we can hack the system. We can develop our own assays that get run on these plastic discs that are the consumable part. Again, this is the workhorse technology. This particular instrument's capable of running four plates in about five hours. And so, for clinical samples, that's what we do. It's a sample processing robot with an imaging system that's built into it to look at the single molecule binding events.

In the early days of the pandemic, we developed both an antigen assay and an antibody assays. I think you all recall that the first assays that came out for any kind of detection of infection were the antibody assays. These were just serological assays that were measuring various components of antibodies that developed against the SARS-CoV-2 virus. We developed these both antigen and antibody assays. Again, I'm not going to go into the details of these, but we use these to look at archival samples pre-pandemic. This was pre-October 2019 samples as well as PCR negative samples. I think you out recall that there were some false negatives that were occurring in some of the earlier PCR data that were coming in.

As you can see, as patients were infected, these are people who self-reported coming into the emergency department at the hospitals, the Mass General Brigham system, as they, again, self-reported from first appearance of symptoms, their antibody levels increased over time, over a period of several weeks. We also observed the presence of circulating S1 protein. This is the S1 fragment of the spike protein. We also measured the presence of the nucleocapsid protein, again pre- and post-pandemic.

So, for an individual patient, you could see that their IgM levels would increase, their IgA levels would increase, and then their IgG levels would increase. And as soon as a [inaudible 00:33:17] converted, you saw almost instantaneous conversion of or removal of the antigen and return to baseline of the S1 protein. In a few of the patients, basically the more severe patients, we occasionally would measure the full spike protein. But this is really just to set the stage for the next part of the talk, which is really looking at the presence of SARS-CoV-2 spike, the full spike, in patients with PASC. The pre-print came out in July and then was published in September. It did garner a lot of attention. This pre-print I think was downloaded about 70,000 times. And so, the idea is that what we'd like to do is, as Dr. Peluso really beautifully described earlier, is to follow the course of patients as they go from acute infection to either full recovery or if they now have exhibited symptoms of long COVID, if we can come up with biomarkers that not only can show the presence of and use it for diagnostic purposes for long COVID, but also look at these biomarkers as a vehicle for monitoring whether we have therapeutic efficacy and these patients can fully recover and use that biomarker in the context of a clinical trial, which I'll describe a little bit later in the talk.

We took a relatively small sample. This was a sample of opportunity. These were all obtained from Ash General Hospital. We had 37 patients that had been diagnosed with PASC. These were all pre-Omicron. And then we had 26 patients that we selected as controls who had acute COVID-19 and followed those patients. I've had some pushback in terms of whether we should have used a different cohort for our control, but we measured what we got. We looked at longitudinal plasma samples, and we measured all three of the antigens. Well, there are really two antigens here, nucleocapsid spike, but we also measured the presence of S1. We measured, and Dr. Peluso also mentioned this, using the Simoa assays, these are ultrasensitive assays of these 10 cytokines, just to correlate them with presence or absence of these proteins. I'll show some other measurements that we made a little bit later.

And so, again, this is just recapitulated what I've already showed you here, taking patient plasma, exposing them to assay with different specificities, capturing antibodies on these for either the S1, the nucleocapsid or to spike the detector antibody. The S1 case was simply a different epitope that was looking at different epitope of S1. In the case of spike, we used a capture antibody that was S2 and used an S1 detector antibody. So that's how we were able to detect full spike and then nucleocapsid was standard and then ran the single molecule assays in the standard way.

To our surprise, what we found was that we measured, as others have, some nucleocapsid persisting in some of the past patients. Occasionally, we'd measure S1 in some of the past patients. But in the control, so these are the acute infected controls, we monitored them over, in some cases, a period of a few months. Most of them or none of them actually converted into long COVID, at least in the group that we had selected. Of course, these were retrospective samples, so they were selected not to have long COVID.

But you can see that in the case of these individuals, we never measured spike protein. Their spike protein levels were always at baseline. In the case of S1, as we observed previously, S1 appeared in the acute phase of infection, but, in fact, disappeared as soon as these patients zero converted and remained at baseline months after their infection had left. The same goes for nucleocapsid.

Again, these were samples of opportunity, so this was not a deliberate retrospective study or prospective study that was done where particular time points were selected from all patients. These were just samples of opportunity. These patients eventually were diagnosed with PASC. We were able to obtain longitudinal samples from these individuals at various time points. What I can tell you is that roughly 65% of these patients had presented with spike protein during and up to 12 months during which we were able to obtain samples. We did not measure it in 35% of the patients.

The conclusion here was that there must be a persistent viral reservoir of active virus that is producing the spike protein and putting it into the plasma. What it also tells us is that there is a subset of these individuals, of these PASC patients who probably do not have a persistent viral infection and their symptoms could be due to the acute damage that occurred during the acute phase of infection. But again, most of these patients were not hospitalized. They were reasonably mild cases of COVID in the acute phase. But just to drill down a little bit more to show you some of the temporal profiles here, so what you can see here, and I want you to focus mostly on the blue dots here, the spike protein in this particular patient is present both at three months and also at seven months. Here, this patient had many different time points. Again, elevated levels of the spike protein. They did have some S1 that was present here. It was after vaccination, so this was their first dose of vaccine, their second dose of vaccine. You can see that the S1 goes up after vaccination, another observation that we have made with mRNA vaccines.

But you also can see that in some of these patients with PASC, there's measurable spike protein here. It goes down to baseline for a period of time, and then it comes up. It goes down, it goes up. And so, we really don't

know what's happening here. We know that this is not necessarily something where every time you take a measurement in these patients you're going to get a positive measurement of spike. In contrast, these are the acute patients. You can see again, they exhibit S1. This patient actually had a measurement of nucleocapsid here that goes to baseline, but this is measured in weeks, not in months. And so, you can see that when these patients zero convert, their measurements go down to baseline, which are the dotted lines that are over here for the three different assays.

We were curious about whether these patients that have PASC may have compromised neutralization capacity. And so again, I'm not going to go into the assay, it's a surrogate assay that's a competition for the two receptors. Others have published similar format assays. Again, this is an ultrasensitive assay. The spike levels are in blue, the neutralization capacity is in red. That's the NT-50 over here. You can see that even patients that have good neutralization capacity over long periods of time, so again, here's vaccination dose one, dose two, there's perhaps a little bit of a drop off, but again, this individual has significant amount of neutralizing capacity. Yet, at this late time point, about 12 months after their acute infection, they still have a measurable spike that's appearing here.

In contrast, this patient has no neutralization capacity and presents with spike at both of the measurements. I don't want people to draw too much conclusion here and get too much in the weeds with the ups and downs here, but the point is that neutralization capacity does not seem to give any indication of whether an individual is going to be PASC positive or PASC negative. We also measured the cytokines, I mentioned that as well. We may have seen similar kinds of slight increases in the mean of things like IL-6 as Dr. Peluso described earlier, but there's nothing that sort of jumps out in terms of the difference between the PASC individuals and the acutely infected individuals. And again, looking at this over time, there's nothing that predicts, at least from eyeballing this, whether a patient has PASC or not. There's no discernible difference from what we can tell.

We did look a little bit more; we did a deeper dive with respect to the correlation of presence of spike with patient symptoms. You can see that these are acute symptoms, these are the persistent symptoms. We then looked at the presence of spike as a fraction of the population here. Again, about 65% of the neuro patients present with spike protein, that's the red bar here. Again, the pulmonary patients are antigen positive 61% of the time. And you can go across the board, you can see that the numbers are quite small. Remember that the total number of patients here is 37, so statistically, this is not particularly significant, but again, it's just interesting post-study analysis of whether there was anything that jumped out with respect to the symptom clusters and the persistence of spike protein.

We ran a second study from the MGH Cardiac Clinic. These were provided by Dan Baruch. We noticed a similar measurement of the spike protein in these patients over time. I can't remember the exact... oh, six months. This was actually a prospective study where T zero was baseline three months and six months. These are omicron patients by the way, and so you can see now that 78% of these patients actually exhibited spike. We have some

unanswered questions here. We always get the question, "Why don't we detect S1?" Well, we think that the particular antibodies that we're using, when the S2 binds to the [inaudible 00:47:17] antibody, it prevents S1 from being detected with the two different epitopes.

And then what is the source of the full spike? I think Dr. Peluso covered this very nicely. We think it's probably present in some membrane fragment. It's unlikely that it's circulated virus particles, but there's the possibility that that's there. We know that there have been culture-negative plasma run on some of these samples, and so it's unlikely to be circulating viral particles, but it could be EVs or membrane fragments because we know that the full spike must be membrane associated. I should mention that we just completed last week running roughly 600 samples from the Mayo bio repository for the RECOVER study. It showed many fewer spike positive samples, and we don't really understand this. So right now, I think we are going to have to wait and see. We're just not sure if there's something different about post-omicron samples versus pre-omicron samples. All the samples that we got from Mayo were post-omicron. We don't know if it has to do with our reagents having just a slightly different specificity or if, in fact, post-omicron patients, in fact, PASC presents differently. And maybe the number of spike positive patients with omicron PASC might actually be many fewer.

Again, as Dr. Peluso mentioned earlier, whether this kind of marker can be used in a clinical study, I think there's plans right now to measure spike in patients who are going to be enrolled in a clinical study with an antiviral. Whether the spike protein will be used as inclusion criteria I think is under discussion. But in my opinion, I think we should probably take all comers. We should take those patients who are spike positive and then put them into the two groups with the antiviral regimen and the placebo arm and then take the spike negative patients and put those in placebo and antiviral arm. And then the controls, which are to be determined, would be to do the same with them and see what the outcomes are. This would be a true control group of non-PASC patients.

The conclusions are detection of the spike in the majority of these patients provides support for its use as a biomarker. Stay tuned on the pre- versus post-omicron study. We should have some answers by the end of next week based on some experiments that we're presently running. We also have obtained some studies from Dr. Peluso and Dr. Deeks from UCSF. I believe that those are all pre-omicron. So that will give us some clues as to whether the pre- versus post-omicron is something that needs to be looked at more carefully. And then the fact that it persists up to 12 months post diagnosis suggests that there must be, at least in some patients, these persistent viral reservoirs. We're presently working on also trying to identify the nature of the spike protein through isolation of extracellular vesicles followed by mass spectral studies. And then again, I mentioned the clinical trial that will employ this assay as one of many assays. The people who run this who are running this persistence study, Zoe Swank is the postdoc, Yasmeen Senussi, the RA who's running lots and lots of samples, and Galit Alter, who provided some of these samples, Dan Barouch and support from Mass Consortium for Pathogen Readiness. I'll pass this back to Dr. Whitehead for introduction of our next speaker.

## Dr. Nedra Whitehead

Thank you, Dr. Walt. Our next speaker is Dr. Abdel-Mohsen who's going to speak about markers of fungal translocation elevation in PASC and NF-κB signaling.

## Dr. Mohamed Abded-Mohsen

Thank you very much. Today, my presentation will focus on this publication we recently published earlier this year in June that focus on trying to understand the rule of gut-lung axis during long COVID or PASC. But before I do so, I just want to spend a minute or two telling you what is gut-lung axis and why we think it's relevant to PASC.

A couple of years before the COVID-19 pandemic, there was a robust body of scientific literature describing how any lung infection or damage that is severe enough to cause systemic inflammation and comes with a cytokine storm, which is a sudden increase in many of those pro-inflammatory molecule called cytokine, how some of those cytokine, in particular TNFα and IFN-γ, can injure the gut and cause increasing in intestinal permeability or what also called leaky gut, which allow microbial translocation, which mean that is microbes, both bacteria and fungi, that are in our gut and lung but should not penetrate to the blood do now penetrate to the blood and being recognized by our immune system, in particular by our myeloid cells. This recognition further increases inflammation, which in its own merit can worsen disease outcome.

But also, this immune inflammation that is caused by microbial translocation can lead to several metabolic alterations that can lead to the accumulation of several metabolites with toxic properties to many distant organs, including the lung itself and the brain and whatnot. This increase in systemic inflammation and this metabolic alteration can, as I mentioned, worsen the disease outcome. So that is one way how severe COVID-19 can indirectly impact the gut and causing leaky gut through cytokine. But in particular, in the case of severe COVID-19, we also know that the virus itself can directly infect the gut cells, may be feeding this vicious cycle.

Earlier in March or April 2020, at the beginning of the pandemic, we ask as a question, "Is there a potential link between the lung inflammation and gut during severe COVID-19?" And taking the longest story short, because that is not the focus of this presentation and it was published in a different publication, we did a multi-omic approach on samples from individual who are not having SARS-CoV-2, our control, mild COVID-19, moderate COVID-19, and severe COVID-19. And we did this multi-omic approach in order to identify gut-related biomarker of COVID-19 severity. And indeed, doing so, we found that a severe COVID-19 is associated with significant increases in marker of tight junction permeability. So tight junction are the proteins in our gut or the lung that prevent bacteria and fungi to penetrate to our blood. And as you can see here, this marker for an example called zonulin, which is not just a marker of tight junction permeability, it's actually a targetable driver of it, is significantly increased during severe COVID-19.

So, this opening in tight junction and increasing in tight junction permeability should allow both bacteria and fungi to translocate from our gut or lung to the blood. And indeed, we found several evidence that is during severe COVID-19 there is increases in marker of bacterial translocation measured by several markers, including this marker called LPS-binding protein. LPS are sugars that cover the bacteria that can be recognized by our immune system through our receptor called TLR4. And the binding between LPS and TLR4 causes immune inflammation and cytokine production.

As you can see here, we found evidence of bacterial translocation during severe COVID-19. We also found evidence that severe COVID-19 is associated with a state of fungal translocation. And here we measure fungal translocation through this marker called beta-glucan, which is, again, sugar, polysaccharide that's covering fungi, and again can be recognized by a receptor in our immune system called Dectin-1. And the binding between beta-glucan and Dectin-1 significantly causes immune inflammation.

And indeed, we found a strong positive correlation between the level of bacterial translocation here in the top x-axis or fungal translocation in the bottom x-axis and many markers of immune inflammation. Here I'm just showing you an example of IL-6 as an example. And as I mentioned too, this immune inflammation caused by microbial translocation can lead to metabolic alterations. And indeed, when we did a full metabolic analysis on the plasma sample from these individuals, we found that is the metabolome of the individual suffering from severe or moderate COVID-19 cluster distinctly from the metabolome of the control. And that is due to dysregulation of several metabolic pathway that can lead to the accumulation of metabolite with toxic properties to many organs.

Here I just will show you one example of this, which is the best way responsible for the degradation of an amino acid called tryptophan. Tryptophan is an essential amino acid upon microbial translocation can be degraded to multiple metabolites with toxic properties like quinoline and quinic acid. And indeed, we found that during severe COVID-19 there is an acceleration or higher level of tryptophan catabolism, degradation of tryptophan, in a manner that is associated with higher inflammation during acute COVID-19. And this is important because accelerated or higher level of tryptophan catabolism has been associated with long-term complications during many other viral infections, including HIV infection.

Since we published this data, there were a robust body of literature confirming it and validating it and using stool sample and gut biopsies and show that severe COVID-19 is indeed associated with a state of gut microbial dysbiosis, which is changing in the composition of the bacteria and fungi in the gut and microbial translocation, both bacterial and fungal, in a manner that is associated with higher inflammation and worse disease outcome, with the idea that is if the initial infection lead to leaky gut, this will lead to uncontrolled inflammatory responses that can have detrimental consequences on multiple organs in the body as opposite of if this initial infection maintain or lift the gut healthy and maintain controlled inflammatory responses, that the skin prevents this detrimental effect on multiple organs.

Obviously, when it became clear that many individuals are suffering from long COVID or PASC, we ask this question, "Does microbial translocation contribute at least partially to inflammation during PASC?" In order to answer this question, we analyzed sample from two independent cohort of PASC, the first one which is the UCSF LIINC cohort that Michael introduced and talked about earlier in his presentation, and we work it closely with Michael, Tim Henrich, Steve Deeks to select samples from 117 individuals three to four months after their recovery from acute COVID-19. 56 of those individuals did not suffer from any resistant symptoms while 61 of them suffered from two to 18 different symptoms after recovery from acute COVID-19. This was our primary cohort, but we validated some of the data using another cohort from Rush University working with Alan Landay, Ali Kesh, and others. This cohort contained 50 individual that are experiencing PASC three to four months after recovery from acute COVID-19 and 50 age, gender, and ethnicity match SARS-CoV-2 negative control.

What we did, we collected plasma samples, and we measured marker of tight junction permeability and microbial translocation, markers of inflammation and cytokine. And we also, as I mentioned to you, that is this immune inflammation caused by microbial translocation can lead to metabolic alteration, so we did also a full metabolic analysis on those samples. Before I show you some of the result, I just want to show you some clinical and demographic confounder that could exist in the samples we analyze from the uses of LIINC cohort, so when we compare the individual experiencing or not PASC, we found no difference in age. However, we found a difference in BMI, body mass index, that is individual experiencing PASC had the higher BMI than non-PASC. So, we know that his BMI is a potential co-founder in our result that is we need to adjust for.

We also didn't find a difference in sex, ethnicity, and the existence of pre-existing medical conditions except hypertension. Again, hypertension was higher in individual experiencing PASC compared to non-PASC. So, from this we know that is BMI and pre-existing hypertension are confounder in our analysis, that is we will need to adjust for, which we did, and I will show you the result. We started by looking at marker of tight junction permeability, and I mentioned to you this marker before, zonulin, and indeed, we found evidence that is individual experiencing PASC have higher level of zonulin or tight junction permeability compared to individual not experiencing PASC.

So, this opening in tight junction would suggest that we should see either fungal and/or bacterial translocation. And while we didn't see or we didn't find clear evidence of bacterial translocation during PASC, we indeed found evidence of a fungal translocation, again measured using this marker called beta-glucan. As you can see here, many individuals with PASC have higher level of fungal translocation compared to individual with no PASC. You might note that we drew a line at 40 picogram per ml of beta-glucan in the plasma because this was a clinical cutoff that is recently been associated with a level of fungal translocation that can cause inflammation and lead to critical illness during other respiratory related infection as shown in this publication.

And interestingly, we found a correlation between the level fungal translocation here and the number of symptoms that is individual that are experiencing PASC are experiencing. As I mentioned too, individual

experiencing PASC in this cohort experience up to 18 symptoms, and the more fungal translocation they had, the more symptoms they are experiencing. There was a negative correlation between the level of fungal translocation and quality of life score, which is a self-reported score for quality of life. Again, the higher level of fungal translocation, the lower quality of life the individuals are experiencing.

I mentioned to you that BMI and hypertension were potential cofounders, so we needed to adjust for them in a multi-variable logistic model. Doing so, as you can see here, even after adjusting for BMI and hypertension, we still found that there's level of zonulin but much better level of beta-glucan still can distinguish individual who are experiencing PASC compared to the non-PASC. We also took advantage of having sample from another independent cohort from Chicago from Rush University to confirm this result, and indeed, in this cohort, we found that it's individual experiencing PASC here in dark blue have much higher level of fungal translocation compared to SARS-CoV-2 negative control.

And again, you can see here, a lot of those individual experiencing PASC have higher level of fungal translocation above this clinical cutoff of 40 picogram per ml while you rarely see that in individual who are SARS-CoV-2 negative as a control. From this, we concluded that this PASC is associated with higher level of fungal translocation, and our next question was, "Does this level of fungal translocation mechanistically contribute to inflammation during PASC?" As I mentioned to you, we do measure fungal translocation by this molecule called beta-glucan, which is a polysaccharide that is on the surface of fungi. This beta-glucan have a receptor on the surface of myeloid cells or immune cells called Dectin-1. And upon the binding between the fungus to Dectin-1, it activates a signaling PASC way called syk inside the cells that eventually activate a very important signaling PASC way called NF-κB, that can lead to the production of pro-inflammatory cytokine.

So, one could hypothesize that is possible that following recovery from acute COVID-19, some level of fungal translocation persists in some individuals, and this fungal translocation, in particular beta-glucan, contribute to PASC or long COVID by inducing inflammation. We have some observational evidence that this might be true from our cohort. We found that the level of fungal translocation here in the x-axis correlate strongly with many markers of inflammation, including TNF-alpha or IL-6. But this is just a correlation obviously, they don't infer causality, and we wanted to find more direct evidence that this level of fungal translocation in the plasma of individual experiencing PASC can indeed cause inflammation.

We use this reporter system, cell line that express high level of the receptor of beta-glucan called Dectin-1 that can activate syk signaling, can activate NF-κB, that will lead to pro-inflammatory cytokine, and this cell only respond to fungal translocation. The good thing, there is a small molecule inhibitor that can inhibit syk, so we can use this to make sure the specificity of our result. As you can see, when we treat these cells with a pure beta-glucan that we can commercially buy, it can lead to high level of NF-κB signaling that can be reduced when we add this inhibitor called [inaudible 01:06:54].

So, what we did, we treated this cell line with a plasma sample from individual experiencing PASC or individual who are SARS-CoV-2 negative, with the idea that the plasma samples from individual experiencing PASC will have higher level of fungal translocation that can lead to immune inflammation more than the plasma from individual who are SARS-CoV-2 negative, which is indeed what we see here. You can see that plasma from individual experiencing PASC can mechanistically cause more immune inflammation, NF-κB signaling, compared to plasma from SARS-CoV-2 negative control. And this was specific for this signaling PASC way because when we use the inhibitor [inaudible 01:07:35], this signaling will reduce to the background.

We also found that the level of beta-glucan or fungal translocation we measure in the plasma of this individual correlate with the ability of this plasma to activate NF-κB. So, from that we concluded that the level of fungal translocation in the plasma during PASC indeed capable of inducing inflammation. To what degree that contribute to the overall inflammation during PASC, obviously that is still unknown.

We are currently thinking of this model that during PASC there is an opening in the tight junction in the gut or in the lung that allow fungal translocation to move to the blood. And this fungal translocation can be recognized by our immune system to cause inflammation. And obviously, this inflammation in its own merit is bad, but as I mentioned to you, this inflammation of these immune cells called myeloid cells can also lead to metabolic alteration that can eventually lead to the accumulation of metabolite with toxic properties, including this metabolite, for an example, called quinolinic acid, which is a byproduct of tryptophan catabolism. As I mentioned to you, tryptophan is an essential amino acid that when degrading can lead to the accumulation of multiple metabolites with toxic property. Including quinolinic acid, which have a toxic property to the brain.

And indeed, when we examine the metabolome of those individual experiencing PASC and compare it to individual with no PASC, we did find evidence of increasing in the level of quinolinic acid compared to the control. And even so, this difference is modest at best. There are two things interesting about this. Since we published this in June, it seems like this observation is quite reproducible, and there are at least two reports reported the same thing.

This is in a paper from Ireland, I believe, that also reported higher level of quinolinic acid in individual experiencing PASC compared to control. I believe this is actually a [inaudible 01:09:28] brand from Australia that show it also, that individual experiencing PASC specifically with neurological related symptoms are also having higher level of quinolinic acid and higher level of tryptophan catabolism. So that's interesting. The other thing that is interesting is that this quinolinic acid is neurotoxin because it's activating a receptor in the brain called NMDA. The activation of this receptor can cause multiple problem in multiple diseases. And when we looked at the rest of the metabolite, we found many other metabolites that is also capable of activating the same receptor to be elevated in the plasma of individuals with PASC compared to control, including this metabolite called S-sulfocysteine.

Again, this is important because the activation of this receptor has been highlighted in many other diseases with neurological symptoms, including Alzheimer's and other, and because of that, there are a lot of work in designing an antagonist that can inhibit the activation of this receptor. This might provide a mechanistic functional best way that should be exploited during PASC, at least the PASC involving neurological related symptoms. And indeed, when we actually divided the individual who are experiencing PASC with and without several neurological symptoms like neuropathic pain or vision problem, we found that these neurotoxic metabolites are higher in particular in individual who are suffering from PASC with these neurological symptoms.

So just to conclude, in our study, we found higher level of fungal translocation in the plasma of individual experiencing PASC compared to non-PASC or SARS-CoV negative control. This higher level of fungal translocation correlated with higher level of inflammation and elevated level of host metabolic agonist of brain receptor with established neuro-toxic properties. Mechanistically, also we found that this level of fungal translocation can directly and mechanistically induce immune inflammation by a specific best way that we have identified. And of course, together this data suggests that targetable mechanism by which fungal translocation may contribute to inflammation during PASC. And we are working with many other in current and future studies to investigate both the diagnostic and prognostic potential as a biomarker or function as a mechanism of these marker of intestinal permeability, microbial translocation, and also host metabolite during PASX.

With that, I will go to my acknowledgement of slide. First and foremost, thanking all of the volunteer participants in our studies, thanking all folks in our lab, in particular Leila Giron who led this work, and our collaborators, Qin Liu, Aaron Goldman in Worcester, our collaborator at UCSF, Michael Peluso, Steve Deeks, Tim Henrich, and others and Rush University Ali Kesh, and Alan Landay, and others, our funders from Campbell Foundation, PA Pennsylvania, and NIH. Thank you for your attention. Thank you.

## Dr. Grace McComsey

All right, well, thank you Dr. Abdel-Mohsen, and thank you for all three speakers. I think these were amazing talks. I only have a few slides, and then we can get to a couple questions. We learned a lot today. It's a good start for biomarkers field. We learned that there's persistent circulating spike protein up to 12 months even though, obviously, Dr. Walt pointed out that they have different phenotype and a small sample size. But I think it's a great, actually, study and a very good virologic biomarker. We heard about elevated markers of intestinal permeability and the fungal translocation and nicely about the association of fungal translocation with key cytokines. So, it brings the gut and the inflammation that we see in patients with PASC together. We heard from Dr. Peluso about these biomarkers in different PASC phenotypes. Next slide. Next slide, Shane.

So, definitely biomarkers are important. And like Dr. Peluso, I've lived the HIV from almost start to end. It changed the HIV disease. And like it or not, long COVID is the new pandemic, so unfortunately, a lot of people are suffering. We do need biomarker to understand mechanism, understand the phenotypes, do preventive studies.

So, it's very important during the acute phase or even before to be able to predict who will end up with PASC so we can do those preemptive preventive studies as well as treatment studies. Next slide.

I guarantee you it's not going to be that easy because, obviously, we've done a lot of work in HIV to get to the biomarkers that predict that we can use in clinical trials. The biomarkers should be stable, reproducible. They should predict the outcome not in one study, they should predict it with accuracy, with generalizability among studies. And since the PASC mechanism is not likely multifaceted, it's unlikely that one single biomarker is going to predict all the phenotypes. We may need to combine multiple biomarkers or even have some scores with weighted clinical factors in addition to a combination of biomarkers that we can use in treatment studies. And then the change should predict clinical benefit, obviously if we are going to use those markers for clinical trials. But I even say more that we have to have biomarkers that respond very quickly to the intervention. We can't use something like IMT that take years and years to change. Next slide.

On the IMT thing, I wanted to show you a different kind of biomarker in one slide, something that we did here in Cleveland. This is called EndoPAT. It's an FDA-approved machine that can measure endothelial function as well as arterial elasticity. What you're seeing here, we have three groups of people, some who had no COVID, and some who had COVID and either recovered fully, no symptoms, these are the COVID positive, no PASC, or have PASC. Median symptom number was six. I mean, some people had up to 20, so they were pretty symptomatic.

What you're seeing on the left side is that endothelial function as measured by RHI was almost the same in all three groups. So, we could not pick up any difference in endothelial function. However, looking at arterial elasticity, you see a big difference actually between the PASC group and the other group. What that mean is people who had made an apparent recovery post COVID had the same arterial elasticity than people who never had COVID. However, those with PASC have very abnormal number signal and arterial stiffness. Why they have that stiffness even though age and all the other things were pretty balanced and controlled for, we found that several markers of inflammation, like you see in red, IL-6, TNFs, VCAM, as well as markers of gut integrity or gut alteration, LBP that you heard about, and another marker called IFABP were independently associated with arterial stiffness. Next slide. Next slide, Shane.

My group does a lot of work even on nutrition and how that's affecting COVID and PASC, and I wanted to highlight some preliminary work we have but just to show you that we have to think beyond just the typical biomarkers. We've done work on vitamin D and K and had shown that in acute COVID, vitamin K in particular predicted outcome. So, if you control for everything else, people who had COVID and had very poor vitamin K status ended up with much more severe disease than those who had good vitamin K levels.

What we did in this PASC study is, again, split the group by three COVID positive no PASC, PASC, or no COVID, and we looked at a marker of vitamin D and vitamin K. I can tell you, the D, there was nothing. There's no hint that vitamin D is any different by COVID or PASC status. It didn't correlate at all with arterial elasticity. However, vitamin K results are pretty interesting. We found that poor vitamin K status, so increase in the marker

you see called MGP, so poor vitamin K status was associated with PASC. Very different between the PASC group and the other two groups. And even more, that red line you see, so within the PASC group, there was significant correlation between vitamin K. So poor K level, [inaudible 01:19:52] status was associated with worse arterial stiffness. Just gives us something that we're working at and we're looking at other investigations to see if that could be something good for prevention and/or for treatment. Next slide.

## Dr. Nedra Whitehead

Grace, can we go and see if we can get in a couple of audience questions? We've only got a few minutes left.

## Dr. Grace McComsey

Sure. I know a couple questions were an antibodies, so if you want-

## Dr. Nedra Whitehead

That's [inaudible 01:20:26].

## Dr. Grace McComsey

Right. I can cover that because that's the question.

## Dr. Nedra Whitehead

Yeah, go ahead. And just so people know, there was one question about, "Any antibodies associated with COVID?" And then there's one more generally about whether there are clinically relevant auto antibodies for post autoimmune or inflammatory encephalopathies.

## Dr. Grace McComsey

Yeah, so antibodies, yes, I picked a couple studies. I think we have studies now show mostly anti-nuclear antibodies, which are the antibodies you see in autoimmune disease and lupus. This study in particular showed them to be really high level at three months after COVID, and then they waned although they remained positive at 12. In this study, they correlated with phenotypes of fatigue and dyspnea, although there are other studies that correlated some of these auto antibodies with neurologic phenotype. So, definitely the answer is yes, and two studies have shown them to be present in a significant number of people who ended up with auto antibodies. They're present at the time of acute illness. So, telling you that it may have been a subclinical present even before

COVID in a subset of patients. And maybe this is the group that we're seeing the auto antibodies on as manifestation of PASC.

## Dr. Nedra Whitehead

Thank you. I had a couple of questions that came in specifically for Dr. Abdel-Mohsen. First question was, "How would you explain that fungi but not bacteria are translocating?"

## Dr. Mohamed Abded-Mohsen

I don't think fungi but not bacteria translocated, I think we just did not find evidence of the bacterial translocation. This could be due to the sensitivity or the kinetic of the marker we use to identify bacterial translocation compared to fungal translocation. For an example, LBB has been shown to be more sensitive to circadian rhythm when the sample were collected after meals while the markers for fungal translocation are more stable. I don't think there is a possibility that she will have a fungal translocation without a bacterial translocation. It's just like a classic case of lack of evidence is not evidence of lack. I think that both happen, but we just need to find a better way to identify the bacterial translocation, which we are working on.

## Dr. Nedra Whitehead

Thank you. The other question that was specific to your presentation was, "Do you think your findings are specific to SARS-CoV-2 infection or if they may apply to other post-viral syndromes?"

## Dr. Mohamed Abded-Mohsen

No, I do think that the back or long COVID is a great example of actually post-acute syndromes associated with many other viral infections. There is a great review recently written by Akiko Wisaki and her group describing this. I do think that microbial translocation and gut inflammation in general could contribute to the post-acute syndrome of many other viral infection. We know that it does contribute to HIV for an example. That is a common theme during I even after long-term suppressive [inaudible 01:23:50]. It seems like it also happens with PASC, and I will bet it happens also with many other viral infections that lead to chronic inflammation.

## Dr. Nedra Whitehead

Anybody else want to comment on this question?

## Dr. Grace McComsey

Yeah, actually, I had that same question I was going to ask you, Mohamed. Could it be that you picked up more fungal because you selected for people who are hospitalized? So, remember, if they're hospitalized with COVID, they get steroids, they get the antibiotics, so you could see that they'll have more fungal translocation rather than bacterial. Was that the case with your population?

## Dr. Mohamed Abded-Mohsen

No, actually, Michael here actually can comment on that, most of the individual in the LIINC cohort were non-hospitalized during their acute COVID or PASC in order to, in particular, avoid this potential bias. Personally, but again, that is to be proven, I do think it has something to do with the kinetics of the marker and when we measured it and how we measured it that we missed the bacterial translocation more than the fibrillation itself. I think both are there, just we didn't detect it yet. We're working on that.

## Dr. Nedra Whitehead

I'm going to get in one more question that I found really interesting. The remainder we'll answer in the Q&A that'll be posted on the website. But the last question is, "Do any of the speaker see any probability of overlap between some of the biomarkers, for example, the presence of persistent virus that might affect [inaudible 01:25:30] properties and produce micro clotting?"

## Dr. Michael Peluso

Yes, I think the schematic that I showed was the most simplistic schematic that we could make right now where everything's in its own silo. But I think over the next year what we're going to find, and I think that RECOVER certainly will contribute a great deal to, is that there are a million arrows connecting all of those different mechanisms. Those arrows might be different for different endotypes of PASC, but it's highly likely that many of these things are interrelated. We will eventually figure that out.

## Dr. David Walt

Yeah, I agree. I think that one of the benefits of RECOVER is that because we're collecting large volumes of samples on many patients, we can run all these assays on the same patient cohort and do exactly that, make those correlations and really understand the relationships between the biomarkers, which can't be done right now simply because small sample aliquots obtained from a multitude of biobanks on different patients, and so there's no way to compare each patient with another.

## Dr. Mohamed Abded-Mohsen

I will just add one thing. I do agree that it's different. Most likely, many of those markers are interconnected to each other, but even if they are not interconnected, it's most likely for something that complicated as PASC or long COVID, that is we will need any way a... It's unlikely that one marker or a couple of markers can predict this complicated clinical syndrome, and it's likely both viral and host marker need to be combined together and trained together to identify those even if they are not interconnected, which is I do believe they are, but even if they are not.

## Dr. Nedra Whitehead

Thank you all for coming today. We have one more seminar in 2022, which is on vascular pathophysiology. I also want to ask you to take a minute to answer our survey on the usefulness of today's seminar and how well you liked, and we might improve in the future. It's only a three-question survey, it shouldn't take you very long. Thank you all again for coming today. We really enjoyed.

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