Transcript

Beth Linas:

Good afternoon. Welcome to the RECOVER Research Review or the R3 Seminar. My name is Beth Linas and I'm an infectious disease epidemiologist with the RECOVER Administrative Coordinating Center and the moderator of today's seminar. The goal of the seminar series is to catalyze a shared understanding of the research within the RECOVER consortium. I want to start by thanking everyone who submitted questions in advance. Please submit any questions that arise during today's presentation using the Q&A feature in Zoom. After the presentation, we will answer as many questions as possible. A Q&A document will be posted with the recording of the seminar on recoverCOVID.org. It will include the answers for submitted questions relevant to today's presentation.

Questions about other scientific topics will be addressed in future seminars and answers to broader questions about RECOVER will be available in the FAQs at recoverCOVID.org. And as a reminder, we cannot answer individual questions about clinical care.

The topic of today's seminar is Persistent SARS-CoV-2 Antigens in Correlation with Long COVID Symptoms: Findings from a Multi Cohort Study. Our presenters today are Dr. Zoe Newell Swank, who obtained her PhD in bioengineering from the Swiss Federal Institute of Technology and is currently a postdoctoral research fellow in Dr. David Walt's lab, where her research focuses on detecting biomarkers linked with infectious diseases and neurodegenerative disorders.

Dr. David R. Walt is the Hansjörg Wyss Professor of Bioinspired Engineering at Harvard Medical School, Professor of Pathology at the Brigham and Women's Hospital and Harvard Medical School, core faculty member of the Wyss Institute at Harvard University, associate member at the Broad Institute, and is a Howard Hughes Medical Institute professor. His lab's research focuses on creating and using novel technologies to solve unmet clinical diagnostics problems.

And Dr. Beth Karlson, a Professor of Medicine at Harvard Medical School, Vice President of Mass General Brigham Personalized Medicine, and a rheumatologist and epidemiologist at Brigham and Women's Hospital. Dr. Karlson obtained her MD degree from Johns Hopkins School of Medicine. She completed her medical residency at the Brigham and Women's Hospital, followed by a clinical and research rheumatology fellowship also at the Brigham.

Today's speakers will share our current understanding the gaps in our knowledge and how RECOVER will contribute to filling these knowledge gaps. And with that, I'll pass it over to Dr. David Walt.

Dr. David Walt:

Thank you. Thank you Beth. Next slide please. Next slide. So as most of the audience is aware, approximately one in four individuals experienced symptoms up to five weeks after SARS-CoV-2 diagnosis. And nearly 10% of individuals have continuing symptoms after 12 weeks, which is considered to be Long COVID. It's also known as post acute sequelae of COVID, which is abbreviated PASC. But Long

COVID is a poorly characterized syndrome with variable symptoms and it's highly subjective with unknown etiology.

It's characterized by a wide range of symptoms affecting a number of organs and the CDC, the WHO, and also the National Academies have agreed that 12 weeks of symptoms is what defines Long COVID. So if symptoms persist after that timeframe, it's considered Long COVID. But it's also worth noting that symptoms do not need to be persistent but can comprise new symptoms that occur even after 12 weeks of infection. For example, many individuals experience post-exertional malaise or dysautonomia. Next slide.

While a lot is known about acute COVID-19, much less is known about the pathogenesis of Long COVID making it very difficult to implement effective treatment strategies. There's many potential mechanisms that have been proposed including persistent viral reservoirs, gastrointestinal dysbiosis, and inflammation, just to name a few. And some of the others are shown on the slide. Discovering the underlying mechanism remains difficult primarily because many studies include very heterogeneous patient cohorts, which has led to Long COVID being classified according to different criteria. So there's a lot of heterogeneity in what is defined as Long COVID and the community has worked hard to come up with consistent definition. Next slide please.

So if we could identify biomarkers as shown at the bottom of the slide, that are specific to Long COVID or different Long COVID phenotypes, which would be even better, then the Long COVID patients could more easily be classified and potential treatment strategies could be evaluated. Next slide.

So in an early study from my group during the initial phase of the pandemic, we investigated the mechanism of something called multi-system inflammatory syndrome in children or MIS-C. We found that there was viral RNA in stool specimens and also we discovered the viral spike protein in the blood of many of these young children, which led us to that viral persistence in the gut led to the viral antigen leaking into the bloodstream, which caused the post-acute symptoms of MIS-C. So we asked ourselves could viral antigens and inflammatory markers also be linked with persistent viral reservoirs that cause Long COVID?

There was an autopsy study by Stein and coworkers from NIH that first revealed the presence of viral RNA and proteins in dozens of tissue types up to one year post-infection. So over 50% of cases had persistent RNA in lymph nodes from the head and neck, from the thorax, the sciatic nerve, ocular tissue, and also the central nervous system. So in 2022, we examined a small cohort of Long COVID patients and we published a preliminary study showing that we could detect antigen in 65% of these Long COVID patients. Next slide.

So since our initial report, there has been a lot of evidence suggesting that viral persistence is present in a subset of those with Long COVID, but many of these studies were not designed to measure the symptoms, but they provided evidence that SARS-CoV-2 is capable of persistence in numerous reservoir sites. The SARS-CoV-2 RNAs or proteins have been identified in tissue months after acute infection despite not being able to detect the nasopharyngeal RNA by the traditional PCR testing.

So even though there's a lack of concordance between the nasopharyngeal test and the presence of persistent antigen, it's important to recognize that there has not been a direct link between

those two. But I need to make it clear that the link between both the viral RNA and the protein in these tissues and the protein in blood, which we're going to talk about today, has yet to be demonstrated. So there's never been sort of a study that shows that an infected, this viral persistence actually is concordant with the presence of these protein antigens that we're measuring. Multiple studies, including our own preliminary work though identified the SARS-CoV-2 proteins in the blood up to one year, now even two years after the initial infection. So next slide please.

Before we get to our results, which I'm going to turn over to Dr. Swank, it's important for me to emphasize that we're measuring components of the virus. These are the protein antigens. So as many of you know, the RNA codes for the protein and what we're measuring is the protein that is produced by the virus. These are the protein antigens and not the virus itself. And so we don't know whether the proteins are coming from the translation of the RNA into the protein or they are remnants of the actual intact virus. More research is needed to understand if the SARS-CoV-2 RNA that's identified in Long COVID tissue samples is actively transcribed, translated, replicated, or whether the infectious SARS-CoV-2 protein is linked with a replicating virus in these tissues, or it's just the viral RNA, which is the precursor to the protein. So that's important to recognize that there has not been a connection between the actual virus and the presence of the antigen. So I can't stress that strongly enough. So I'm going to turn this over to Dr. Swank and then I'll wrap up later after Dr. Karlson.

Dr. Zoe Newell Swank:

Sorry. Okay, thank you. So in order to see if we could detect these viral proteins in the plasma or serum collected from individuals that had been infected with SARS-CoV-2, we wanted to gather more cohorts so that we could look at independent collection sites because we had studied a small cohort initially, but we definitely wanted to see if that study could be replicated. So we went about looking for different cohorts that sampled blood longitudinally approximately every three to four months and up to around a year after SARS-CoV-2 infection from a sort of diverse cohort of individuals. And then over time, each of these cohorts had their own sort of way of recording post-acute symptoms. But we also were able to get information about the demographics of the cohort when they were infected as well as whether or not they were vaccinated. And then our primary measurement was to detect the SARS-CoV-2 antigens. And then once we had all of this information, we wanted to see if there was an association of symptoms and the presence of antigen. Next slide please.

So we were able to find four different cohorts of individuals that enrolled in different SARS-CoV-2 studies. The first was the LIINC study, the long-term impact of novel coronavirus. And from our colleagues who started this study at UCSF, we were able to obtain samples from 171 individuals across over 600 time points up to 14 months post-infection. This cohort was mostly recruited in the early stages of the pandemic, and so most of them were infected before receiving any COVID vaccine. And no reinfections were documented among this cohort. And they collected symptoms at each collection time point based on interview or administer like a healthcare professional interviewed each participant to understand which symptoms they had and they had a list of symptoms they were most interested in.

At Mass General Brigham, we had a small cohort of individuals. It was also early on in the pandemic. So before these, most people were infected before receiving a vaccine. There were no documented reinfections in this cohort either. And this cohort was slightly biased towards individuals that went to an infectious disease clinic because they had ongoing symptoms. So it's most likely that

most were enrolled after they had an acute infection and their symptoms were collected based on chart reviews.

Then we had a cohort from the Allen Institute in Seattle, which included 80 individuals and at 80 time points up to seven months post-infection. And they were also all infected before vaccination. And they also used interview or administered surveys to record the symptoms.

And then finally we had a large cohort from RECOVER, which probably more of you are much more familiar with. And this included 392 individuals across over 600 time points. And here we had mostly three and six month time points post-infection. In this case, most of them were vaccinated before being infected because this study started later on in the pandemic and we automatically excluded any individuals that had had documented reinfections during the study period. And these were, they used self surveys to record a number of symptoms. Next slide please.

So here you can see just in a more pictorial way, the sort of span of these studies. So the LIINC study took place over the longest period of time. So basically you can see that LIINC and MGB and Allen cohort started their studies in the ancestral waves. And then depending for how long participants were collect, like how long they enrolled participants and how many time points they got, they also spanned into the Delta and Omicron waves. And then RECOVER was the latest study which includes mostly Omicron variant infections. And if you look on the map we had, where the samples were collected were from very geographically different places across the US in a lot thanks to RECOVER, which recruited from many sites across the US. Next slide please.

So if we look at the demographics of the cohorts, we see that most cohorts were more biased towards female participants, which is basically in line with the fact that we see Long COVID predominantly or more predominantly occurring among women. And we had different levels of diversity in the cohorts with the most diverse cohorts being RECOVER and LIINC. And then the vaccination rate, as I mentioned before, was different. So LIINC, MGB, and Allen were mostly unvaccinated prior to their first infection or prior to their infection, whereas in RECOVER they were mostly all vaccinated prior to infection.

And you can also see that the hospitalization rate was slightly different for the different cohorts. So for Allen and RECOVER, the rate of hospitalization during acute infection was very low, whereas in MGB and LIINC it was around 20%. So that also affects maybe the severity of symptoms they had initially during the acute phase. And that also brings me to the fact that participants were enrolled either during the acute phase or the post-acute phase, which has some bias towards whether or not they had persistent symptoms. Next slide please.

So the symptoms that we decided to focus on were 34 symptoms that were found to have higher frequencies among people with Long COVID. So not necessarily people with Long COVID, but a RECOVER study which compared infected and uninfected individuals. These 34 symptoms had rates higher than 2.5% among the RECOVER cohort. So we found these to be a set of symptoms which are more commonly associated with Long COVID. So we thought it would be a good idea to look at these symptoms in particular to also just have a general idea of how antigen might be associated with COVID symptoms or Long COVID symptoms.

And you can see here that based on the cohort, we did not always explicitly record a certain symptom. So RECOVER obviously had the study was based on the RECOVER. So all the symptoms were explicitly questioned in the RECOVER survey. And Allen also, the survey used for the Allen Institute cohort also included most if not all of these symptoms. Whereas LIINC had their own survey, which did not include all of them, but included most of them. And since the MGB cohort used chart reviews, basically if any symptom was ever recorded in a chart review, then we considered it to have been reported. But if it was never reported, then we could not necessarily determine whether or not the person had it for sure or not. So we can't say the presence or absence with certainty. Next slide please.

So now I'll talk about how we detect the viral protein, and some of you may be familiar with the ELISA assay or the enzyme-linked immunosorbent assay. And this is a way to detect proteins using a sandwich of two antibodies. So normally you have a capture antibody which binds to a protein, and then you have a second antibody to detect the same protein, which is then linked with an enzyme which can turn over a substrate and create a signal. And if you'd run this reaction in bulk, then it takes so many proteins until you can actually observe a quantifiable signal. And this is known as an analog reaction.

However, the Walt Lab specializes in creating digital assays where you can measure single molecules. So you can think if you instead make such an assay digital by for instance, capturing a protein by using antibody-coated beads, then you can detect single proteins, contain them in a micro compartment and then count single molecules directly. And using this method you can have better sensitivity, sometimes thousandfold or higher sensitivity, more sensitivity compared with standard ELISA.

And especially if we're looking for something that might be rare in the blood, like we said, if the hypothesis is that you have a persistent reservoir somewhere in the body, then the viral protein coming from this or coming from RNA or wherever it's coming from, will probably be dilute in the bloodstream. So you actually might need a much more sensitive method to detect a small concentration of protein in the blood, which is why we think that using these digital assays is very important. Next slide please.

So in the Walt Lab, the technology known as Single Molecular Array or SIMOA was developed and it works by running basically an ELISA on a small bead. So you have a magnetic bead which is coated with antibodies, and these antibodies are specific to a certain protein and in our case they're specific to a SARS-CoV-2 antigen. Then you can use a second antibody which is linked with the molecule known as biotin, which will then bind to your antigen as well. And then you will have the biotin which can link with the streptavidin-linked enzyme, which then turns over a substrate to create the signal.

And normally you dilute your samples such that you'll have either no antigen molecules or one antigen molecule per bead. And then when you load these beads into a microwell array where only one bead fits into the well and seal this well with oil, then you can start to see the signal created in the wells. And since you would have either no antigen or one antigen per bead based on the sample dilution, you can then count individual protein targets or antigen targets inside the wells based on the presence of a signal or not. Next slide please.

So we developed these SIMOA assays for SARS-CoV-2 antigens and we developed assays that detect the full spike protein. So that's done by using a capture antibody that detects the S2 subunit and a detector against the S1 subunit so that you know both subunits of the full spike are present. And then

we also have an assay against the S1 subunit by itself since the S1 subunit often gets cleaved upon viral entry into the cell.

And then we also have an assay against the nucleocapsid protein. And it works as I described before, where you incubate your sample with the magnetic coated beads, then you add your detector antibody and then that links with your enzyme. And when you add substrate and load it into the microwell arrays, you can then count individual antigen particles. Okay, next slide please.

So this is what we did for all of the samples in our cohort and we found that we could detect any antigen in approximately 21% of all individuals at any time point post-infection. But just to break it down, since we had collected samples approximately every three to four months, we also wanted to look at how much antigen we were detecting in those periods over time. So we can see that in the acute phase before less than one month after infection, around three to 5% of individuals were positive with antigen. And then we saw actually the greatest rate of antigen detection for spike and the highest percent was during the four to seven month time period. Next slide please.

We can see here, if you look at the overall detection of antigen in any one of our cohorts at any time point, the rates were definitely variable. So for a LIINC we saw the lowest rate of antigen detection around 10%, whereas for Mass General Brigham cohort we saw 44% of the samples were positive for antigen at any given point. And we think this is like we've sort of mentioned before, that this cohort was biased towards individuals that came to an infectious disease clinic because they had recurring symptoms and these symptoms could not be basically attributed to anything else. So they were probably more enriched for persistent symptoms. Next slide, please.

And we also looked at, because RECOVER had the nice added variable where you could tell if participants were enrolled during their acute infection or during the post-acute phase, and we saw that in the case for RECOVER, there was actually no significant difference between the amount of antigen we detected in people that enrolled during acute infection versus people that enrolled during the post-acute phase. Which is also interesting because in this case it wasn't biased towards people that enrolled post-acute. And then I think I'll turn it over to Beth now for the next slide.

Dr. Beth Karlson:

Thank you. Okay, so I'd like to show a couple of slides. Results of our symptoms on antigen detection, but let's go to the next slide where I tried to expand it to make it a little more readable. And I'd like to take you through this. It's a complicated slide. So the first thing to look at here in each of these groups, these are each of the cohorts. And on this side here I have listed the symptoms. And then the next slide have the rest of the symptoms. So we have 34 symptoms that we looked at. Okay, let's go back. Thank you.

And across each row of symptoms, what you have is the number of participants in the cohort and then whether they were positive, the percent who were positive on each of these three antigen tests and this summary variable we made, which is any antigen positivity. So I find the easiest way to look at this figure is to look down the column one first in each cohort because this tells you what percent of each group had these symptoms. So you can see that the LIINC cohort that Zoe described before, where people were interviewed about their symptoms, 58% reported fatigue, 57% reported

brain fog. Another common symptom was shortness of breath, chest pain, muscle pain, joint pain. A lot of symptoms in this cohort.

The MGB cohort, which was the small chart review cohort from patients being seen in an infectious disease clinic, had lower rates of some of these symptoms. The highest rate was shortness of breath, and then quite a few of the symptoms weren't even measured because they didn't appear in the chart. So because it wasn't a systematic survey, we don't have data on all of the symptoms.

Allen looks very similar to LIINC in terms of the frequency. If you look down this first column, the high frequency of symptoms is seen here and similar symptoms. So a lot of fatigue, brain fog, dizziness, palpitation, shortness of breath.

And then the RECOVER cohort, which as Zoe mentioned had mostly vaccinated participants. Their rate of symptoms is lower. And also these were self-reported symptoms on a questionnaire, not with an interviewer asking about the symptoms, but strikingly very high fatigue and high rates of musculoskeletal symptoms in the RECOVER cohort.

So the second aspect to notice here is I would look at this any antigen column for each cohort because the darker colors show you the people who have antigen in 10%, 10% are this darker blue color or higher in each of these symptoms. So you can see some of the really common symptoms here. Chest pain, palpitations, shortness of breath, and cough also have 10% or higher antigen. In MGB, we see it in palpitations and shortness of breath. And Allen, we see this high rate in palpitations and shortness of breath. We don't see that as much in the RECOVER cohort.

So this is one of the nice things about studying different cohorts because we're looking for consistency across the cohorts. You can see consistently very high reports of fatigue and the rates of antigen positivity in the fatigue group, 16%, 18%, 28%, 19%, pretty high rates of antigen in the fatigue group. So if we go to the next slide, those are the other half of the symptoms.

Oops, I can just advance. There we go. Oops, the next slide you can see headache, very high frequency in the LIINC and the Allen cohorts and pretty high rates of antigen positivity, over 10% here. The Allen cohort has people with high rates of vision complaints, 12% of symptoms, GI 42% in these two cohorts and high rates of antigen positivity, relatively high rates of antigen positivity in the GI category of symptoms.

Okay, so let's go on to the next slide. So when you look at this, you wonder, well, what about people who didn't have any symptoms? So we were able to take our symptom results and classify people in each cohort as participants who had no symptoms or asymptomatic. So LIINC only had 17 of those, MGB 26, Allen 21, RECOVER had more participants with no symptoms. This is the percent among each cohort. So 10% of LIINC were in the no symptom group, 27% of Allen, 41% of MGB.

Then we plotted out how many were positive on the spike assay. And you can see something really striking here that these three cohorts, very few people were positive people who had no symptoms were positive on spike, but a lot of the RECOVER participants, so 19 of these 64 or 5% of the overall cohort had no symptoms and spike positivity, and 24 of the overall cohort or 6% of the overall cohort were positive with any antigen. So in this RECOVER group, it looked like antigen positivity was

found even in people with no symptoms. We don't really know why that is. It could be because these participants are vaccinated and so it reduces their symptom rate, but they still have evidence that there's persistent antigen from pieces of the virus or proteins from the virus are still found in the blood. Okay, next slide.

So we wanted to look at various ways of classifying Long COVID. And this analysis was done before there were a lot of publications regarding Long COVID symptoms. So what we did was we developed these categories of having at least one symptom or at least two symptoms or at least three symptoms. And we ran logistic regression models to calculate odds ratios to see what the risk would be of having a symptom if the antigen was detected in these different categories. Next slide.

So right here you see the at least one symptom category. And One thing I'd like to note here is that definition of Long COVID was published by NASEM Group, the National Academy of Science Engineering and Math, saying that anybody with a single persistent symptom can be classified as Long COVID. So we have results here showing that 220 of these people had one symptom and had antigen. And in our model, our unadjusted straightforward model, that was a twofold increased risk of having one symptom if you had the antigen. Then we adjusted our model for other features that might predict that someone would be positive for antigen. So we did an adjusted odds ratio where we accounted for age, sex, how long it had been since their infection, and which cohort they were from because we saw different positivity rates across the cohorts. And this is roughly the same, it's a 1.8 odds ratio close to twofold. And this is what we call our confidence interval, which is that the true result lies somewhere between 1.4 and 2.2.

When we classify people as having at least two symptoms. Out of those 34, we had 186 who were positive on the antigen. Our unadjusted result was a 1.7 fold increased risk, and then the adjusted models showed a 2.0 or twofold increased risk of having two symptoms if you have the antigen. And having three symptoms was slightly lower in the unadjusted model, but once you account for all these other factors, again, it's about twofold. So if you look down this column, were showing approximately a two times increased risk of having these symptoms if you have the antigen. Next slide.

These are the other factors that were in our model and we just wanted to show you how the antigen itself increases the risk of these different patterns of symptoms. Females were more likely to be antigen positive. There was really not a strong association between age and being antigen positive. Being longer since the infection had a higher risk of being antigen positive. And amongst these cohorts, the highest risk we saw were the MGB cohort and the Allen cohort. Was only slightly elevated results in the LIINC and the RECOVER cohorts. Next slide.

So the other thing we looked at was just the spike, to see if spike could be a biomarker that we could use by itself. And spike also had high rates amongst lots of people in this yes, yes category. A high rates of spike with one symptom group, the two symptom group, and the three symptom group. Our odds ratios were slightly lower, so a little less than twofold, increased risk if you had the spike. Here's the unadjusted results and then these are the adjusted results adjusted for all the same factors that we did in the main model. Okay, next slide.

So the other thing we did was look at the symptoms according to organ systems. So this is something that some groups are starting to do is think about whether your biomarker is a marker of

having symptoms that are localized in one area of the body or one type of syndrome. So we looked at ME-CFS, which is characterized by fatigue and post-exertional malaise. And what we required for this to put people in this category is they had to have both fatigue and post-exertional malaise and have either dizziness or brain fog. And that was from a CDC definition of ME-CFS. But just recall that the fatigue itself was very strongly associated with antigen. This is a more strict definition, so it would remove some people if they don't meet these criteria from this ME-CFS category.

For dysautonomia, we collapsed symptoms into one group, symptoms of dizziness, dry mouth, GI, and bladder symptoms. And again, as I mentioned, we did some organ systems. So cardiopulmonary symptoms, musculoskeletal, urologic, head, eyes, ears, nose, and throat is what this stands for. And this is mostly people who have smell or taste problems, but also some people reported headache and vision problems and so forth.

So now I want to show you next slide is another one of these heat map slides. And this is looking at people who had post-acute. So after the acute infection of COVID, looking for symptoms and antigen detection in the same format as before, but now we have collapsed into all these groups of symptoms, the ME-CFS, dysautonomia, and the organ system groups. We found that 43% of individuals who reported cardiopulmonary musculoskeletal or neurologic symptoms were antigen positive. And if you go to the next slide, I've blown this up a little bit to make it a little easier to see.

So here's cardiopulmonary as a group. And we see the same thing that I mentioned when we were looking at individual symptoms that both LIINC and Allen cohorts had really high frequency of these symptoms. Now that we group them, it's a 64% frequency in the Allen cohort, 26% were spike positive. In cardiopulmonary and LIINC only 18% were positive, MGB 25%, RECOVER 12% were antigen positive. But must recall that RECOVER had a lot of people with musculoskeletal symptoms, joint pain, back pain, muscle pain. 17% were spike positive, 20% were antigen positive, any antigen.

We also saw very high rates of antigen positivity in the neurologic system. Some rates in the HEENT, which is mostly smell and taste problems, some higher than 10% in GI. ME-CFS is 13% in this cohort, but we don't see that high rate of antigen in the other cohorts. So we're starting to see some patterns here focused on some of these organ systems as well as ME-CFS in this one cohort. Whereas fatigue by itself had much higher rates. Next slide.

So the other way to look at, so here's our model where we took those symptom groups, the two syndromes and the organ system groups and said and asked the question, is the antigen more common if you have symptoms across these two or more groups? And the answer is only modestly. Really not very striking results. Our unadjusted model wasn't significant. The adjusted model had an odds ratio of 1.5, whereas our results before were closer to 2.0. So looking across the groups didn't seem to help us. The antigen didn't seem to detect people who had symptoms across multiple groups. And next slide.

Another way to look at Long COVID is using what's called the RECOVER PASC score or the research index. And this was published in a JAMA paper where the RECOVER investigators looked for symptoms that distinguished people who were infected from people who were not infected and created this weighted score. Here are the scores and it weights certain symptoms more strongly than other symptoms. While it doesn't include all 34 symptoms, there is a nice correlation chart in that publication showing that many of these symptoms are correlated or associated. So they're markers of other

symptoms. And you can see some of the symptoms are the ones that I've been talking about like the cardiovascular symptoms, smell and taste symptoms, post-exertional malaise, fatigue is in the score.

And what they decided in this research index was to classify people as PASC positive post-acute sequelae of COVID, another term for Long COVID, if the score was more than 12 and if it was less than 12, these are possible PASC or indeterminate but not classifiable by this research index. So using this research index, which is a combination of symptoms which is sort of similar to our more than two symptom groups, we found an odds ratio of 1.5, but it wasn't significant. So this research classification was not significant and wasn't helpful in looking at an association between the antigen positivity and this research score. Next slide.

So in conclusion, we found that one in five individuals were antigen positive up to one year after infection. The majority of our individuals reported cardiopulmonary musculoskeletal or neurologic symptoms. And of those groups, 43% with antigen positive, the presence of antigen was associated with a twofold higher odds of having Long COVID symptoms. And these findings highlight the need to better understand the source of persistent antigen and whether circulating antigen correlates with viral RNA and antigen found in tissue reservoirs needs to be studied. Okay, next slide.

So I just wanted to give you some things to think about in terms of what our limitations are of the study and some of the open questions that hopefully we'll have time to get to. So we have a lot of cohort variability as we've shown you, the types of symptoms that were reported and how the symptoms were recorded varied. The participant enrollment time was quite different where three of the cohorts started during the acute infection and followed people also during earlier waves of the virus, whereas the RECOVER cohort was more from the Omicron wave and later. We don't really know why spike as opposed to nucleocapsid or the S1 piece of the spike was prominently detected. We don't know if these antigen levels will correlate with people getting better. Do people who improve over time do the antigen levels go down? And we don't know yet if the blood level of antigen is associated with having tissue levels of antigen.

There also are lots of other possibilities for other biomarkers that can be used to better understand Long COVID. And we're really looking forward to understanding those and working with other groups on these other biomarkers. And I think that I'd like to turn over to Dr. Walt for some more discussion. Thank you.

Dr. David Walt:

Okay, thank you Dr. Karlson. So there there's many potential mechanisms that link these viral reservoirs to Long COVID and our study raises a number of questions, they're outlined here on this slide. I'll just focus on a few of them. For example, there's evidence of both viral RNA and viral protein persistence in the CNS. And as we know, a large proportion of individuals suffer from neurologic symptoms. So the question is can we combine our antigen data from neurologic symptoms with downstream clinical test results to understand if the antigen is not only linked with these symptoms, but also with these clinical measurements.

To better understand the pathobiology of Long COVID, are there other biomarkers, for example, cytokines or the antibodies to the virus? Can those be combined with our antigen measurements to

better predict the presence of Long COVID symptoms? Will biomarker panels help us predict different Long COVID phenotypes? And then finally, and I know this is of interest to a number of people with certainly cardiopulmonary symptoms, the spike protein is thought to contribute to the formation of these pro-inflammatory blood clots and therefore is there a connection between the presence of circulating spike antigen and microclot formation or does microclot formation mask the presence of circulating spike? So I'm going to go through a few of these in detail with respect to some of the studies that we're involved in to help address these questions. Next slide please.

So we're pursuing additional studies to answer many of these questions. For example, the LIINC study that was part of this cohort is continuing to follow participants for up to two years post-infection to determine how long the antigen persists. The neurologic symptoms have been linked with pro-inflammatory microclots, as I mentioned in the previous slide. So within the LIINC cohort, we're collaborating with Dr. Michael Peluso and Dr. Deeks to determine if neurologic symptoms correlate with both microclotting and persistent antigens. And they're recording neurologic symptoms during this study as well as other clinical measurements such as the MRI so that we can evaluate these clinical neurologic pathologies.

We're carrying out another recovered cohort study in which we expanded our biomarker panel to understand if antigens but also inflammatory markers and antibody profiles can better predict the symptoms. And I'll talk about that in a minute. And we're also participating in the RECOVER-VITAL study to determine if antiviral treatment is effective in eliminating the viral reservoir. So I'm going to describe some of these in the next few slides. Next slide please.

So drawing samples from the RECOVER cohort, we measured an expanded biomarker panel as well as antibody profiles. This is through the pathobiology arm of the RECOVER effort. Next slide.

This cohort includes, and we've already run many of these samples. We're just in the process of analyzing the data now. Includes 216 individuals that were infected with SARS-CoV-2, and the samples were collected zero, that is at baseline, three months and six months post-infection. And these are all characterized as Long COVID individuals. And our primary measurements include antiviral, antibacterial, and self-reactive antibodies being measured in conjunction with Steve Elledge's lab here at Harvard using his VirScan technology. Next slide.

So as I said, we measured the antigens, the Anti-SARS-CoV-2 antibodies against spike, S1, RBD, and the nucleocapsid, and we also measured a panel of 10 cytokines with our multiplexed assay as well as CRP and IL-17. These are other inflammatory markers that have been discovered based on previous work that link these markers with Long COVID. Next slide.

And then Dr. Elledge's lab is running the VirScan library, which includes all human viruses, all other known coronaviruses and all previously identified bacterial epitopes present in the immune epitope database. And then he also runs something called AI scan, which doesn't stand for artificial intelligence. It stands for autoimmunity and that covers the entire human proteome. And the hope is that by combining these antibody profiles with our cytokine and antigen and SARS-CoV-2 antibody studies, we hope to be able to have a more comprehensive picture of the syndrome. Next slide, please.

As I said, we're collecting these data and we're employing a variety of computational methods to determine which of these biomarkers can discriminate between PASC negative and PASC positive individuals and also whether we can subtype some of the different categories of PASC using these biomarker panels. We're comparing these markers over time just to see whether longitudinal measurements actually help us stratify individuals. And then as I mentioned, we're also incorporating other clinical information such as sex, age, and comorbidity. So our plan is to analyze these biomarker data and hope that we can come up with a way to subphenotype these particular individuals based on these biochemical measurements. Next slide.

There were a couple of comments in the Q&A about stop classifying, just figure out a treatment. And so I'd like to address that because the RECOVER study is also actively engaged in trying to find appropriate therapies, one of which is the RECOVER-VITAL study which we're engaged in. And this is a highly diverse cohort. It's from 69 sites across the country. We have 984 total participants, a highly diverse population that broadly represents the nation. And the goal of this is really to evaluate Long COVID treatments that target potential Long COVID mechanisms. And we're involved in this particular study, which is to study whether we can use the presence of viral persistence as a key marker for a targeted therapy. Next slide please.

So here's the, I'm not going to read this, but this is basically aimed at testing whether a persistent viral infection can be treated with an antiviral agent. So can the antigen that we're detecting be used as an effective biomarker to evaluate whether that antiviral treatment is efficacious? Next slide please.

So the viral persistence are of RECOVER test the effect of the antiviral drug PAXLOVID. So this is a well-known antiviral agent, I'm sure you've all heard of it. And we're testing it on three symptom clusters, which are shown here, the exercise intolerance, the cognitive dysfunction, and the autonomic dysfunction. Those are the three symptom clusters that have been included in this particular study. Within each of the three clusters, there are three treatment groups and you can see those. They're a 25-day regimen of PAXLOVID, a 15-day regimen of PAXLOVID, and then there's a 25 day of a control arm. So the next slide actually describes this I think in a little bit more detail.

So as I outlined on the previous slide, the total cohort includes three groups of individuals with Long COVID who belong to those three different symptom clusters. And within each symptom cluster, the participants are randomized such that there's equal proportions of each group given each treatment, the 25 day, the 15 day, and what we're calling the placebo.

Now the placebo actually is Ritonavir, which is an HIV antiviral and it's being given because it has a bitter taste. And as many of you who've had taken PAXLOVID know, you can tell that when you're on PAXLOVID. And so this is being done simply to prevent individuals from knowing that they're on a placebo. Otherwise, the placebo will be recognized by participants. Next slide please.

So the primary outcomes are based on the patient symptom surveys that are being given 90 days after they have completed treatment. The secondary outcomes are based on the specifics for each of the symptom clusters and the treatment efficacy will be determined based on a predetermined change from baseline for those treated versus the control groups. And the exploratory outcome includes these antigen measurements which were carrying out over time. So our main interest is to determine if

a decrease of antigen from the baseline level to the 90-day post-treatment level correlates with an effective antiviral treatment and/or symptom resolution. So let me just sort of rephrase that. What we're trying to do is test whether those individuals who have antigen positive detection in their blood are responding to the antiviral by having the antigen levels decrease over time. That's our expectation. That's the exploratory hypothesis. Next slide.

So what we've described today is how our antigen assay works and how it can be applied to understanding the underlying mechanisms of some aspects of Long COVID. The presence of antigen suggests that there is a persistent viral reservoir that may, may, be the underlying cause of Long COVID in a subset of individuals. We also see antigen individuals who do not exhibit symptoms. So there's a mystery there. Maybe they're just controlling their symptoms better because they have a better immune response. The RECOVER-VITAL study is testing whether regimen of an antiviral drug can eliminate this reservoir resulting in improvement or even a resolution of symptoms. And we should know the answers to some of these questions by late spring. So I encourage all of you to stay tuned.

I want to thank all the wonderful collaborators I've mentioned. We've mentioned some of them who are listed on this slide, and I also thank NIH RECOVER for funding our work and all the dedicated participants who provided samples to enable us to carry out these studies. Dr. Swank, Karlson, and I will now entertain questions from the audience and thank you very much.

Beth Linas:

Thank you so much. So we'll have some time for some Q&A. There's questions to individual people and then there's sort of general questions and feel free to jump in if you have a response to a question that wasn't asked to you. So I will start with Dr. Waltz. This is actually one of the first questions that came in, so we're going to back up a little bit. Have similar studies been conducted for other viruses, CMV or EBV, showing viral persistence in tissues? If yes, do other viruses show similar persistence? If no, is it possible this persistence is not unique to COVID virus?

Dr. David Walt:

I think I answered this question to the person who asked the question. There definitely have been other studies that have postulated viral persistence. We have not applied our methodology to any of those other persistent syndromes. I think it's obviously something that's worth doing because of the ultra sensitivity of our assay, but I think that there have been a number of studies, including influenza, where folks have postulated and actually measured some viral persistence in individuals who have post viral infections syndromes. Maybe Dr. Karlson has more detailed information about this, but I'm not...

Dr. Beth Karlson:

Just for mononucleosis. I think for Epstein-Barr virus that there have been studies that shown that Epstein-Barr virus can reactivate and produce more symptoms but not with your technology. So it's a different technology. They look for very specific antibodies and sometimes they look for circulating virus but not looking for the antigen particles yet.

Dr. Zoe Newell ...: I would also add that Ebola and Zika viruses are like they function, they're similar RNA type viruses that function in a similar way. And they've also, although they are less well studied, there's also evidence that they persist for longer and people also have symptoms for longer. And there's been some animal studies that show that the virus can persist in tissue as well.

Beth Linas:

Great, thank you. And actually Dr. Swank question for you. Can you infer anything meaningful based on the highest antigen detection being four to seven months after infection or do you attribute this to a measurement artifact?

Dr. Zoe Newell Swank:

Yeah, sorry, I didn't get to answer that question before, but yeah, I think we can't really infer anything meaningful at this moment. Also, just because it was like all the cohorts were together and they were heterogeneous, it was just what we happened to see. But I wouldn't also know if it's quite significantly different from the other months also, because we have different number of time points at each month. Which is why I think moving forward we're trying to stay within a single cohort where we have a better control over all the parameters that were measured in terms of symptoms and the timing of the sampling. Because I think we were mostly just interested to understand if we see antigen in more than one cohort that's collected independently. And I think in terms of that, we definitely see that we're detecting it, but I don't know if the timing is meaningful at this moment, basically.

Beth Linas:

Great, thank you. And Dr. Karlson, there was a question for you. Techniques like IVIG are being studied to combat persistent reservoirs, early results are mixed. Could the location of the reservoir impact the effectiveness of IVIG?

Dr. Beth Karlson:

Well, theoretically IVIG should diffuse into all the tissues except for the brain. So I'm not quite sure of the data on this, but there is a blood-brain barrier and it's hard to get therapies into the brain, so I think it really depends on where the viral reservoir is. Hopefully we'll have more data on that over time. So it would be really helpful to assess viral reservoirs in tissues that are easy to test, such as the GI tract would be a pretty easy place to do a sample, measure the antigen level, measure presence of antigen, give IVIG, measure antigen after a trial and see if it goes down and if the symptoms are better.

But as far as I know, there aren't any controlled trials trying to treat the antigen level in the tissue yet. The RECOVER-VITAL is the first one that I know of that's using the antigen assay in the blood to look for response. David, do you know of anybody who's doing tissue antigen measures in a trial setting?

Dr. David Walt:

No.

Dr. Beth Karlson:

No.

Beth Linas:

Great. Just a general question for everyone. Were pregnant individuals represented in your study?

Dr. Zoe Newell Swank:

No, I don't think so. Well actually there might've been some in the RECOVER, but I don't...

Dr. Beth Karlson:

Yeah, RECOVER does include pregnant individuals and I don't think we did anything specific to look for that. So there may have been a handful of pregnant people in the RECOVER analysis. It's something we can look at, but we haven't looked yet.

Beth Linas:

Great. I'm having internet issues. I apologize you guys. General question, what significance, if any, do rapid tests have in Long COVID? For instance, I continued to test positive on several brands of rapid tests for almost two years, negative on PCR.

Dr. David Walt:

Yeah, I saw that question posted. I think it's a bit puzzling because the antigen test is testing for the nucleocapsid protein and what that's saying is that this particular individual has some sort of presence of antigen viral protein, the nucleocapsid protein in their nasal swab, but that they are negative for PCR. So that's quite puzzling. I think either there's that individual either is producing lots of nucleocapsid that's circulating or they're just getting a false positive on the antigen test. That's the only explanations I could come up with.

Beth Linas:

Okay. Does Ritonavir have any biological effects that could invalidate it as a control?

Dr. David Walt:

Beth, could you repeat the question please?

Beth Linas:

Yeah, yeah. Does Ritonavir have any biological effects that could invalidate it as a control?

Dr. David Walt:

I was not involved with the design of the study. My understanding is that Ritonavir has no effect as an antiviral on coronaviruses. So I would expect the answer, I would suspect the answer is no.

Beth Linas:

Another question for everyone. I thought PAXLOVID was already disregarded as a possibility to treat Long COVID or was this another study and are you studying it in another way?

Dr. David Walt:

There's certainly significant amount of anecdotal data that suggests that individuals who have a five-day, who get reinfected and are put on PAXLOVID observe that their Long COVID symptoms diminish while they're on the five-day regimen of PAXLOVID, but they return once the PAXLOVID has been discontinued. I think that was sort of one clue that suggested that suppressing viral replication was an effective strategy for perhaps curing some of these individuals. But the issue was that five days was inadequate to clear the virus. And so that's why we're testing the 15 and the 25-day regimens to be able to access viral reservoirs that may be very difficult to access simply because of bio-distribution of the drug.

Beth Linas:

Anyone else?

Dr. Beth Karlson:

Also, I would point out that sometimes when you're designing a study and recruiting for a study, some other group will publish a publication showing a negative result and there could be some trials published on PAXLOVID that don't show a benefit, but generally you need a preponderance of evidence, so you need more than one study to sort of get at the true answer. And so I think that even though it's taken a long time, it seems, to get the study launched and to get it people recruited and to reach the end time point. We will be getting answers from this study pretty soon and it will sort of add to the evidence and then scientists can look at all the studies that have been published together and decide if PAXLOVID looks effective, what the dose should be, what the duration should be, et cetera. Or if it isn't effective at all.

And having the biomarker will add to the evidence. If this antigen level does go down in the people who feel better, then that's really great biologic evidence of the efficacy of the drug, but it could come back negative. So we don't know until the results are analyzed.

Dr. Zoe Newell Swank:

Yeah, I was also going to say since Long COVID is so heterogeneous, I think at least some of the preliminary antiviral studies I've seen have not exactly addressed the different phenotype groups. So I think it's possible that depending on the type of symptoms you have, which is probably due to the different mechanism of Long COVID affecting an individual, it could be more prone to have one drug work over the other. So I think seeing the three different phenotype groups could also be very interesting because maybe it tells you something more than just an overall Long COVID trial with antivirals.

Dr. David Walt:

Yeah, and Zoe, you make a great point. The fact is, as you just heard, the presence of antigen in our cohorts is on the order of 20 to 25% approximately. And so if one just lumped all these individuals without knowing who has a persistent antigen, then the statistics are probably just not going to be there because 80% of the individuals for whom you're trying to clear a virus might not be responsive. And so you might get a small but insignificant signal out of that.

Beth Linas:

Great. Also to the group. In addition to plasma samples, will you be looking at antigen presence in various tissues, especially GI?

Dr. David Walt:

I think I responded to that one, but the answer is no. We're not. Being able to obtain tissues is a challenge, so I'm not sure it would be particularly worthwhile. I mean, we obviously can test stool in these individuals, but we think that our marker is sensitive enough to pick up antigen in blood. So that's been our go-to sample.

Beth Linas:

Sorry about that. Dr. Swank, is there a path to making, or anyone really, to making these assays, whether analog or digital more widely available for researchers and clinicians?

Dr. Zoe Newell Swank:

Well, so I guess for the RECOVER trial, we did validate this assay in a clinically certified lab. So now the clinical Translational Biomarker Core Lab at Brigham and Women's is running those samples according to clinical guidelines for the RECOVER trial. And then in terms of adapting this assay to make it more widely available, we're also trying to translate it to another platform which uses flow cytometry as a readout. So you would not need the instrumentation that's associated with the SAMOA assays. You can do everything by hand and then read out with flow cytometry. And this has also the benefit that it can be more sensitive as well. It was developed in our lab and there's a few of us working on trying to

translate this assay, which could then be used more readily by everyone because flow cytometers are more widely available, I would say.

Dr. David Walt:

But just want to make sure, because I know that there's a lot of patient representatives on this line, that this is not right now a clinically approved test. This is for research use only. We've transferred it to the CLIA Lab at Brigham. As Zoe has just mentioned, our hope is that if the RECOVER study shows that the presence of antigen is a... If the VITAL study shows that PAXLOVID is effective at reducing the levels of antigen and that it is not effective with individuals who do not have antigen, then the expectation would be that eligibility for a long regimen of PAXLOVID would require the antigen test that we've developed to be run for inclusion in a follow-up therapeutic regimen. And so if that's the case, then we will definitely be translating this and transferring it to other laboratories, other clinical laboratories for widespread use of the assay. So I assure you that our goal is to get this out there, but we have to show that it's actually measuring something that's meaningful first.

Beth Linas:

Great. I have a question for you Dr. Karlson. It's a little bit long. For the RECOVER cohort, when reporting percent of cohort with symptoms or with antigen, what denominator was used? Was it the full cohort or recognizing your 64 patients with no symptoms but antigen were those 64 patients removed from the denominator? EG is a denominator for heat map only symptomatic patients.

Dr. Zoe Newell Swank:

You're muted. You're muted.

Dr. Beth Karlson:

Sorry. We use the total number of participants in each cohort as the denominator. So for example, RECOVER had 392 individuals, so our percentages reported in those heat map figures are out of the 292. We didn't remove the asymptomatic. So it's percent positive among 392. And then also in the asymptomatic, the table in the paper and that I showed on the screen among the asymptomatics, we use the total number in each cohort there so that we have the same denominators across all of the figures and the table.

Beth Linas:

Great. Another question for you, but I think can be answered by anyone. What do you make of patients with SARS-CoV-2 antibodies spike quantitative results that keep rising after infection. Can anyone address an autoantibody theory? Any results greater than 2000 units per mil have any significance?

Dr. Beth Karlson:

I can talk about that because actually a rheumatologist, and I'm working on a separate paper where we're looking at autoimmune diseases that develop after COVID after infection. There are actually probably six or seven papers now from around the world showing excess development of autoimmune diseases. Most of these analyses are done in electronic health records. So we have looked in RECOVER and don't have enough cases yet to say whether we're seeing any evidence in RECOVER of excess autoantibody related diseases.

We have one investigator who's looking at antibodies in a sample set from RECOVER. The results are not available yet, but we have distributed samples to a lab in the RECOVER investigator group to test for autoantibodies. So we might see something in the next few months and you might read about it in a paper in the next year or so. But I would say that it does appear that autoimmune diseases are more common in people who have had SARS-CoV-2 infection.

Beth Linas:

Great. Question for everyone. To your knowledge, are there any studies looking at differences, symptoms, recovery rate, et cetera, between patients with Long COVID from different COVID strains and years of initial infection?

Dr. Beth Karlson:

So the RECOVER cohort JAMA publication that was published in 2023 did look at people infected before Omicron versus people infected after Omicron came around. And the rates of Long COVID were higher in the pre-Omicron group versus the post-Omicron group. There also are some publications from around the world showing less frequent development of Long COVID with Omicron. So I think we're starting to see that evidence. As I mentioned before, you'd like to see it in multiple papers before you're sure that that's a trend, but it appears that, and certainly the RECOVER cohort that we had in this paper had lower rates of antigen and lower rates of symptoms and lower rates of organ system manifestations in the three other cohorts. And it's mostly from the Omicron or later era. So I do think we're starting to see lower rates.

Dr. Zoe Newell Swank:

Yeah, but it's also, I guess I would add, it's hard to understand if the rates are different due to the variant itself or due to the vaccination because...

Dr. Beth Karlson:

Exactly, exactly.

Dr. Zoe Newell Swank:

Vaccination also became more readily available and it's also hard to know. It's also something which is, we also relied on participants to report that if they had been infected before or if they were reinfected. But a number of times we also saw people who were supposedly uninfected had antibodies against nucleocapsid. So even if they were vaccinated, which they would have spike antibodies, but we saw also nucleocapsid antibodies. So I think it's quite difficult as the pandemic moves on to actually find good controls that were never infected, first of all, or you know...

Dr. Beth Karlson:

And never vaccinated. The best control would be people who are never vaccinated, never infected, and since up to a third of people have asymptomatic infections, even people who think they've never been infected, about a third... Have never been infected, about a third of them have been infected. So it's really hard, as Zoe said, to really answer that question.

Dr. David Walt:

Zoe's tested individuals who have claimed they've never been infected. We test, I think Zoe, we're what, 99.4% of all samples have anti-nucleocapsid at this point. So pretty much everybody's been infected whether they know it or not.

Beth Linas:

Great. Well thank you so much. Thanks to our presenters and thank you to our audience for attending the seminar and engaging with the Q&A. As a reminder, a recording of today's seminar will be available on recoverCOVID.org within a few weeks. We'll also be posting a Q&A document that has responses to the questions we received today, including some that we did not have time to address.

Before we conclude, a reminder that researchers both within and beyond the RECOVER initiative can now apply to use RECOVER data for ancillary studies. This data from the three RECOVER cohort studies, adults including pregnant individuals, pediatric, and autopsy and biospecimens collected from cohort study participants. Interested researchers must submit an ancillary study proposal and receive approval. Researchers must also have independent funding to conduct the proposed study. To learn more and apply visit recoverCOVID.org/ancillary. And we can put up the next slide.

This slide lists the topics for future sessions. We have some exciting topics coming up and hope to see you at future sessions. Additionally, you'll see a short survey come up on your screen, which asks you for your feedback on the seminar. We would appreciate if you could take a minute to fill out this brief survey. Thanks so much and have a great day.