Transcript

Lisa Newman

Hello everyone and welcome. I'm Lisa Newman, the contact principal investigator for the RECOVER Observational Cohort studies at the Administrative Coordinating Center and the moderator of today's session. Welcome to the RECOVER Research Reviewer (R3) Seminar. The goal of this series is to catalyze the formation of a scientific stakeholder community within and beyond the RECOVER consortium and foster a shared understanding of the state of the science and to provide an enduring educational resource for RECOVER investigators, the broader scientific community, clinicians, patients, and other public stakeholders. I want to start by thanking everyone for attending and to those who submitted questions in advance. Please submit any questions that arise today using the Q&A feature in Zoom. After the presentation, we will answer as many questions as possible about today's presentations. Some questions may also be answered within the Q&A during the webinar. We will not answer questions about individual clinical care.

Today we'll present on the mechanistic pathways of post-acute Sequelae of COVID session one. The purpose of this session is to provide a high-level overview of the mechanistic pathways, including viral persistence and viral reservoirs, residual tissue and organ damage and injury, immune response, inflammation, and autoimmunity and reactivation of other viruses, and secondary damage and reprogramming of host tissues and organs. Subsequent seminar sessions will do a deep dive on these different pathways. We have an impressive speaker panel today. Our presenters are Dr. Akiko Iwasaki, Dr. James Stone, and Dr. Amy Proal. Dr. Marrah Lachowicz-Scroggins, the moderator, will synthesize the information presented by these panelists and guide initial discussions with them.

So, let me start by introducing our panelists. Dr. Iwasaki is a professor of immunobiology, and molecular, cellular, and developmental biology at Yale University and an investigator of the Howard Hughes Medical Institute. Her researcher focuses on the mechanisms of immune defense against viruses at the mucosal surfaces and the development of mucosal vaccine strategies. She is the co-lead investigator of the Yale COVID-19 Recovery Study, which aims to determine the changes in the immune response of people with Long COVID after vaccination and is the director of the new Yale Center for Infection and Immunity. Dr. Iwasaki is at the forefront of the COVID-19 pandemic with respect to research, science, communication, and public service. She's quoted in numerous media outlets with expert insights and is considered one of the top 50 experts to trust during the pandemic. She is also well known for advocacy on women and underrepresented minorities in the science and medicine fields and has a large follower base in social media. Dr. Iwasaki will present the immunology of Long COVID today.

Our next presenter will be Dr. James Stone. Dr. Stone is director of the Autopsy Service and head of the Cardiovascular Pathology Service at Massachusetts General Hospital, and associate professor of pathology at Harvard Medical School. He is a site principal investigator for the RECOVER Autopsy Cohort and member of the

RECOVER Steering Committee. He is the past president of the Society for Cardiovascular Pathology. He has published multiple papers regarding the pathology of COVID-19, including an international multi-center study describing the spectrum of cardiac changes associated with SARS-CoV-2 infection. Dr. Stone will discuss the pathology of SARS-CoV-2 infection and implications for the biologic mechanisms underlying post-acute sequelae of COVID.

Finally, Dr. Amy Proal is a microbiologist who serves as president of PolyBio Research Foundation and directs the organization's Long COVID research consortium. Her work examines the molecular mechanisms by which viral, bacterial, and fungal pathogens dysregulate human gene expression, immunity, and metabolism. In her work with PolyBio Research and the Long COVID Research Consortium, she conceptualizes and coordinates large-scale collaborative research projects among research teams studying infection-associated chronic illnesses such as Post-acute Sequelae of COVID-19, or PASC, ME/CFS, and long line. She has written multiple review articles that delineate core biological drivers of both the PASCs and ME/CFS disease processes. Dr. Proal will present an overview of SARS-CoV-2 reservoir in Long COVID or PASC.

Our discussant today is Dr. Marrah Lachowicz-Scroggins. Dr. Scroggins is program director at the National Institutes of Health, at the National Heart Lung and Blood Institute Division of Lung Diseases, and the Airway Biology and Disease Branch. Her portfolio areas of focus are respiratory medicine, pulmonary pathophysiology, and immunology with a concentration in diseases such as cystic fibrosis, disorders of mucociliary clearance, including ciliopathies, and other rare lung diseases. Her current portfolio also includes respiratory tract infections and susceptibility, mucins and mucus biology, mucosal immunology, epithelial cell biology, gene delivery and editing technologies, and women's health. Before joining NHLBI in 2018, she was the assistant research professor at University of California San Francisco and assisted in Airway Clinical Research Center. She is the current chair of the NIH RECOVER pathobiology working groups and scientific program lead of the Recover Pathobiology research portfolio.

Dr. Lachowicz-Scroggins, as I mentioned, will serve as the seminar discussant. Following the presentation, she will conclude by synthesizing them and asking a few questions to initiate the discussion, then we will open it up to questions from the audience. We'll try to answer as many questions as possible related to today's presentations. Please welcome me in welcoming all of our speakers. Now I'll turn it over to Dr. Iwasaki.

Dr. Akiko Iwasaki

Thank you very much for the kind introduction. I'm very excited to be here today. So, in my talk I'm going to give a sort of broad overview of what we understand about the immunology of Long COVID and then to give you some of the recent insights that we're gaining from our own studies and sort of conclude with some of the key questions that need to be addressed in this field.

So, before I begin, I want to emphasize that Long COVID is not the only post-acute infection syndrome that happens after a so-called acute infection with viruses and bacteria and parasites, which have been long known to cause these types of sequelae. SARS-CoV-2 is the newest member to join this list of post-acute infection syndromes and we really do need to understand the underlying mechanisms of these diseases. And there may be something that's shared, the ultimate sort of disease phenotypes and symptoms converged to something very similar between each other. And so, whether some of the pathogenesis that we can think about for Long COVID may have a shared biological sort of processes with other pathogens is something that we should keep in mind.

So, I'm going to start with a potential hypothesis. This is not at all a comprehensive list of different hypotheses but some major ones that are kind of coming out. One is that there is viral reservoir that is established within a person with Long COVID and persistent viral reservoir or viral pathogenesis sort of molecule patterns such as the RNA itself or the antigen itself can trigger a chronic inflammation of both innate and adaptive immune system and that can trigger some of these sequelae of acute COVID. There are numerous papers that are coming out. I listed just a few of them here, but there's definitely evidence of viral antigens and RNA found in various tissues months post infection, and you'll hear a lot more about this from Jim and Amy later.

We also think that some of the Long COVID pathogenesis could be driven by autoimmunity. This can be mediated either by autoreactive T-cells or B-cells that secrete antibodies against cell antigens. And a very nice paper by Jim Heath's group that was published in Cell last year identified the early presence of lupus-related autoantibody as one of the four predictive factors for developing Long COVID. There are a couple of other studies, including our own, which I will describe in more detail, that demonstrated that at least autoantibody against type-one interferon for example or other antigens, intracellular antigens are not found to be significantly elevated, but there may be other types of drivers that are related to T-cells.

There's also dysbiosis of microbiome and potentially latent virus reactivation that could be contributing to Long COVID. And these are, again, some of the papers that demonstrated that EBV reactivation may be happening in Long COVID patients. And the Jim Heath paper demonstrated EBV viremia at the time of acute COVID is one of the four predictive factors for developing Long COVID. And tissue damage, which you will hear more from Jim's talk, I'm sure, is something that can occur during Long COVID potentially after a severe acute COVID phase. But we've also seen in an animal model with Dr. Michelle Monje's laboratory that even a mild respiratory restricted COVID infection in mice can result in chronic changes in the brain that is seen after seven weeks post infection.

And this study is interesting. They use dogs that are trained to sniff out SARS-CoV-2 infected cell supernatant and these dogs were able to identify about 50% of the long-haulers, whereas 0% of the control groups were identified providing a pretty unbiased sort of analysis of potential viral antigens or viral antigen-driven organic compounds that are released by the Long COVID patients. So, there are all these hypotheses and Long COVID is not one disease, it probably is a combination of these types of pathologies that occur. Some long haulers may be suffering from one of these while others may have a combination. And we also have to think about the

temporal relationship of each one of these things driving each other. So, it's a very complex picture, but actually intense research has now identified very key insights into these hypotheses.

So today I'd like to talk about our own research that we're doing with Dr. David Putrino's group at Mount Sinai School of Medicine, where he is treating thousands of patients with Long COVID, and we've started collaboration as soon as Long COVID became known. And his group consists of Jamie Wood, Laura Tabacof, and Dayna McCarth. Together, we've been working over two and a half years on this disease to try to understand the immune phenotypes and these are the first authors that contributed to that study. It's currently posted on medRxiv if anyone is interested.

So, the way in which we approach this is to recruit participants that have Long COVID, which are the purple people here. And then the convalescent controls, these are people who acquired COVID around the same time but have fully recovered from COVID. And the healthy control, these are the people who hadn't been infected with SARS-CoV-2. And we also had healthcare workers that provided their serum for antibody analyses. From the biospecimen collected from these patients, which are peripheral blood cells, we did a flow cytometry to look at the cell types in the blood as well as their activation status. We also looked at antibodies against human exoproteome, which are transmembrane and secreted proteins. We also did SARS-CoV-2 antibody profiling against the viral antigens. We also did peptide display library with Serimmune, which detects antibody reactivity against linear epitopes found in virtually any pathogens or humans. And plasma proteomics to look at the cytokines and hormones. And we also did a lot of the symptom survey and EMR, which I'm not going to have time to talk about today.

So, the Long COVID participants in purple, compared to the convalescent control were fairly well-matched with respect to age, sex, and acute COVID severity. And I want to emphasize that we try to focus on non-hospitalized patients who then went on to develop Long COVID. And as with many other studies, this is a female-dominated disease as you can see. And then these from acute COVID was around 400 days plus or minus, meaning that this is well over a year after the initial infection phase, so we're looking at a pretty late phase of Long COVID.

I'm just going to give you some highlights of what we've discovered so far. So, for instance, when we looked at different types of cell subsets using the flow cytometry and then the healthy control is orange, the convalescent control is yellow, and Long COVID patients are in purple. What we found was that there is an elevation of non-conventional monocytes, both percentagewise and activated phenotypes, as well as reduction in the conventional dendritic cell type one. These are the cells that are known to be important for inducing T-cell immunity against the intracellular pathogens. We also see elevated levels of activated B-cells and double negative B-cells in the Long COVID participants.

With respect to T-cells, we saw that there is a reduction in the CD4 T-cell central memory T-cell type in the Long COVID patients and elevation in the exhausted T-cell subsets, both CD4 and CDA T-cells. So, this suggested to us that there may be something chronic or persistent that the T-cells are recognizing in the Long

COVID participants, and we are trying to now understand what these T-cells might be reactive to. We also looked at intracellular cytokines that are produced by these CD4 T-cells and CDA T-cells and found that the Long COVID patients had elevated levels of IL-2, IL-4, and IL-6. And when we focused on the IL4, IL6 double positive CD4 T-cells, we saw that they were pretty much only found in the Long COVID participants. And I'll come back to these cell types in a little bit because there's an interesting correlation between these cells and EBV.

We also looked at antibody response against the nucleocapsid and the spike protein, either the total spike, the S1, and the receptor-binding domain of the spike protein. And we controlled for the number of vaccines that the recipients received, which is two doses, and looked at their ability to bind to these antigens. And what we found was that the Long COVID participants had elevated levels of the anti-spike, anti-S1, and anti-RBD antibodies. And this, we don't know how that's coming about, but potentially it may be driven by persistent antigen that's driving the elevation of these antibodies, but that's speculation.

Next, we wanted to understand what circulating factors are most distinct between Long COVID and non-Long COVID participants. And what we found was that the cortisol was the number one most differential expressed or differential levels found in Long COVID versus the non-Long COVID participants. And what we found was that their cortisol level was about half of the healthy control participants level. This is a circulating plasma cortisol level and cortisol is a very important hormone that's diurnal, regulated during the day, and we wanted to make sure that the collection time is about the same between the three groups, which you can see the collection time is roughly around the same time.

And so cortisol is a highly regulated hormone that's induced by the hypothalamus-pituitary-adrenal axis and the fact that we have lower levels of cortisol, we thought that perhaps there will be a compensatory increase in the adrenocorticotropic hormone from the pituitary to restore that level, in which case we did not see such an elevation in the Long COVID patients, suggesting that there may be some central dysregulation of the cortisol levels in these participants. In addition to cortisol, there were many other cytokines, and complement factors, and chemokines that were found to be differentially expressed in Long COVID patients.

What about the dysbiosis or reactivation? Well, we haven't been able to do a microbiome analysis yet, but we have been looking at potential signs of latent virus reactivation and we used three different orthogonal methods to look at these issues. The first method that we used was the Rapid Extracellular Antigen Profiling, which was developed by Dr. Aaron Ring's laboratory to detect antibody reactivity to viral surface proteins and human exoproteome. Using this strategy, as I mentioned to you already, the Long COVID participants had elevated levels of antibody reactivity to the receptor binding domain of the spike protein throughout different variants of concern.

And in addition, very interestingly we saw elevated levels of IgG against Epstein-Barr, glycoprotein 42, as well as p23, and early antigen diffuse. So, these elevation in IgG levels suggested that there must have been a sort of recent reactivation of EBV that happened in the participants with Long COVID but not the other groups. We also saw an elevation in the VCV glycoprotein E reactivity potentially indicating again reactivation of VCV. All the other

antigens that were on the panel, there was either no difference or slightly reduced levels, but most of them were not significant.

So, I told you that we use three different orthogonal strategies. The other method that we used is the Serimmune panel where we are looking at linear epitope against viral antigens. In this case, as I mentioned, REAP score for the glycoprotein 42 is elevated in Long COVID participants. But looking at the Serimmune data, we also saw quite a significant elevation in the Long COVID participants for a linear epitope that is found within the middle of this GP42 viral glycoprotein. And interestingly, this GP42 is used specifically by the virus to enter B-cells. And when we looked at the relationship between the antibody reactivity to EBV antigens on the x-axis and sort of look at the IL-4, IL-6 double positive CD4 T-cell levels, there was a positive correlation between these two factors.

Again, there's just a suggestion of there's a link between EBV reactivation and the double positive Th2 cells. And there's been a lot of literature in this field that demonstrates that EBV, because the GP42 sort of interferes with the MHC class two TCR engagement tend to drive a Th2 response as opposed to Th1 response, which is the more appropriate antiviral response. And this relationship may be indicating that there's a link between EBV reactivation, GP42 expression, and the development of the Th2, but that still needs to be, the link has to be solidified with more research.

And the autoimmunity, so we were very excited about looking at autoimmunity because during the severe acute phase of COVID we found multiple functional autoantibodies against the human exoproteome against cytokines, including interferon alpha and many other cell surface markers that were sort of correlating with disease severity. So, we use the same REAP approach to identify whether there are autoantibodies against different surface antigens in humans. And this result demonstrates that there really aren't significant difference between people with Long COVID versus those without with respect to the type of autoantibodies that they have and also the antibody reactivity per patient. And looking at number of reactivity does not correlate with Long COVID propensity scores within any of these groups. So again, we're not dismissing the importance of autoimmunity, but at least within this cohort we don't find significant evidence of autoantibodies and symptoms or Long COVID status.

So, taking into account all these factors that we measured, we then wanted to identify, using machine learning, if there's a way of classifying the patients based only on the immunophenotype between Long COVID and non-Long COVID. And that's essentially what we were able to demonstrate with 96% confidence that we were able to distinguish people with Long COVID based only on the immunophenotype. And when David Van Dijk, our collaborator, who is a computational person, and Raul and his lab analyzed various different factors contributing to such prediction, we saw that autoantibody had very little contribution to the prediction of Long COVID, whereas antibody against COVID antigens, viral epitopes, these are the EBV reactive antibodies, cytokines, and hormones, and flow cytometry features were all contributing to this separation of Long COVID versus non-Long COVID groups.

And there were specific immunological perturbations that were defining Long COVID through this machine learning algorithm. And what we found was that the cortisol was the number one factor that was the lowest in the Long COVID followed by things like the circulating TCM levels being lower and some chemokines and CDA T-cell levels, whereas what was higher in the Long COVID were the exhausted T-cells, both CD4 four and CDAs activated B-cells and EBV reactive antibody levels and so on. So, we're beginning to see some features associated with Long COVID. And again, because there is likely multiple endotypes, we are now seeing the whole picture in a flat surface here, but we'd like to be able to dissociate in each subset and seeing what the root cause driver of these diseases might be.

So, to summarize, our key findings are that first the patient-reported outcomes alone were actually also significant and sufficient to identify Long COVID patients, which I didn't have time to talk about, but I'd really encourage you to look at our preprint. As I mentioned, the immunophenotyping does reveal increases in exhausted T-cells, these Th2 type of T-cells as well as activated B-cells and non-classical monocytes. SARS-CoV-2 specific antibody responses are elevated in Long COVID patients. Now, that doesn't mean that these antibodies are necessarily neutralizing or functional, all we're saying is that there's an elevation of these antibody levels.

We also saw evidence of herpes virus reactivation, particularly EBV, and there were no significant differences in autoantibodies to extracellular proteome using REAP. The machine learning really is telling us Long COVID can be efficiently predicted from immunological data alone and low cortisol levels were the strongest predictor of both defining Long COVID as well as disease severity.

So, putting all this together with many other papers that are emerging, we feel that so far, the autoantibody may or may not be playing a role, but we don't see a significant signature of this yet, at least in our own studies, in a couple of other papers. We also see that there may be this viral reservoir or viral antigens or PAMPs that are remaining in some tissues. Now, we only look at the peripheral blood, so we cannot talk anything about the tissue itself, but it may be reporting on some of these. Consistent with that idea is the elevated antibody levels and these sort of chronic levels of some of these inflammatory cytokines that are elevated. We also see latent virus reactivation of EBV and potentially VCV, and this may be happening in a subset of patients. And tissue damage, again, we didn't look at this in our study, but I'm sure you'll hear a lot more from Jim about this.

So, I'm just going to end by talking about what research is needed. There's so much that's needed, but we don't really have a good grasp of global epidemiology of Long COVID, which is really important and necessary. We need to identify risk factors; we need to understand the endotypes. Once we understand the endotypes and the driver of diseases, then we can look at a biomarker for these endotypes as well as potentially rational treatment options that target the root cause of these diseases. And even if that's not possible, we can target the downstream pathologies such as microclots, and platelet activation, and vascular dysfunction that's occurring in many of these patients.

So, I'll end here by thanking the people who are involved. There are just so many people involved, I'm just the messenger here. The first authors I've already kind of highlighted before from my own lab as well as many other laboratories throughout Yale. David Putrino was a key contributor to the study that I mentioned today. We also have another study ongoing called Yale LISTEN Study that Harlan Krumholz and I are leading. And then all the people who are sort of critical for the analysis part of the study that I mentioned, Aaron Ring, Ruslan Medzhitov, David Van Dijk, Rachel from Serimmune. And Amy and I, we are collaborating in multiple fronts including ME/CFS research and lots of folks that contributed to the mouse work that I briefly mentioned. So, thank you so much for listening.

Dr. James Stone

My name is Jim Stone and I'm thrilled to talk to you today about the pathology of SARS-CoV-2 infection and the implications for the biologic mechanisms underlying PASC. So PASC basically involves symptoms that the patients are feeling following the acute phase of SARS-CoV-2 infection. And these of course can involve many different organ systems and I'm going to try to go through some of these organ systems and show what we know, in some cases what we don't know about the pathology in these organs. Next slide. So clearly a major site of involvement of the virus is the lungs, and in the acute phase the primary pathology is what's called diffuse alveolar damage. And you see this in the top row of images, particularly in the middle where the astrocytes are highlighting these red rings which are hyaline membranes characteristic of diffuse alveolar damage.

There’re also neutrophilic infiltrates, which you can see in the middle row on the left. These are typically seen when you have coexistent bacterial infections or sometimes fungal infections. On the far right in the middle, you can have mucin plugs plugging up the airways in the lungs. In the bottom left there's chronic inflammatory infiltrates, and in the bottom right we see a microthrombus, and microthrombi are certainly a common feature in the acute phase of the disease, particularly in the lungs. In the center you see an immunohistochemical stain showing the presence of virus. Early in the pandemic, this was all we had were existing antibodies to the virus. Fortunately, we've been able to largely move on from immunohistochemistry. Next slide.

So, in-situ hybridization is really the gold standard for localizing virus within the tissues now, this was one of the very early studies out of Mass General showing in-situ hybridization for the virus within the lung tissue, there's a hyaline membrane, the red area on the left. And then on the right, all of those red dots basically represent amplified viral RNA within a Hyaline membrane. Next slide.

So many of the major hospitals in the Boston area contributed to a single Cell Atlas basically of pulmonary infection in the acute phase of SARS-CoV-2. And what came from this single cell analysis, which was led by the Broad Institute, was that there were many cell types that actually contained virus within the lung tissue. The most frequent cell type to harbor virus were myeloid cells, but this may have been in part due to the fact that the epithelial cells were largely wiped out in many of these patients. But certainly, there's many different cell types,

even within the myeloid lineage there are many different types of myeloid cells can contain virus. And endothelial cells as well contain virus within the lungs including many different types of endothelial cells. Next slide.

So as the diffuse alveolar damage starts to organize for patients that are able to survive that acute phase, that's really severe acute phase, initially you'll go through a state of organizing diffuse alveolar damage, and that's what you see in the top right. And I should say this is a study out of the Mayo Clinic, and in some cases that organizing diffuse alveolar damage where you have a granulation tissue phenomenon forming, in some cases that will progress to scarring. And in the bottom right you see basically dense scar tissue within the lungs, pulmonary fibrosis. And unfortunately, this is largely irreversible. And certainly, some patients who have PASC, actually who survive severe acute phase disease, may actually have scarring in the lungs that explain their symptoms. So certainly, one subset of PASC patients are suffering from scar tissue in the lungs. Next slide.

So, the question is how long can virus remain in the lungs? And there's been several studies now that address this. This is one of the earlier ones that came out last year from Spain showing that virus can be detected in lung tissue at autopsy up to 108 days from the onset of infection. In the right half of the boxes, each box represents a different lobe of the lung. And notice that the colored boxes are lobes where virus was detected. And not every lobe necessarily had detectable virus in a given patient and it really underscores the need to do extensive sampling by PCR when you're trying to identify how many patients actually have residual virus. Next slide.

So, we launched a multicenter study to look at the heart very early in the pandemic and this was a study through the Society of Cardiovascular Pathology and the European Association for Cardiovascular Pathology. And we were quickly able to identify in patients dying from severe acute phase COVID-19, that there were multiple pathologies in the heart explaining the troponin elevations in these patients. Some of the patients did have myocarditis, a number did have microvascular thrombi giving rise to ischemic injury, and there were other pathologies such as right ventricular strain injury, where the right myocytes in the right ventricle were actually dying because of the elevated pulmonary pressures of all the lung disease. Now, we'll talk about some of these in more detail. Next slide.

So, this is an example of the myocarditis. Now, this is an uncommon finding. Certainly, the majority of patients do not develop myocarditis by any means, but you see basically in some patients, these diffuse lymphocytic infiltrates which are damaging the muscle. Next slide. At higher power, you can see this foci of inflammation composed of lymphocytes and macrophages, and there's disruption of the muscle in several areas. Now, in COVID, this is usually a CD4-predominant process, and it is usually multifocal and not diffuse, and it's one of the reasons why a myocardial biopsy often is not very successful in identifying the myocarditis in these patients. Next slide.

There's also diffuse inflammation in most of these patients, even if it doesn't rise to the level of myocarditis. In this particular example, we're seeing a diffuse macrophage infiltration with CD68 positive

macrophages. In severe acute phases, most of the patients will have an increase in diffuse inflammation in the heart even if it's not myocarditis. Next slide. There's also pericarditis in a fraction of patients. And interestingly, sometimes this is actually CD8 predominant in contrast to the typical CD4 predominance of the lymphocytes in the myocarditis in these patients. Next slide.

So early in the pandemic, there was a big question about whether the virus actually could involve organs outside of the lungs. We knew that patients were undergoing viremia. In fact, about two thirds of patients who had severe acute phase disease had virus detected in the plasma. We also knew that if you looked at heart tissue, fresh heart tissue in patients succumbing to the acute phase of the disease, about two-thirds of them had virus detectable in the heart tissue, but it really wasn't clear if this was just because it was in the blood or actually making it into the tissue. We should keep in mind that the virus is a single-stranded RNA virus as you see on the left and it's really the positive strand that's incorporated into the intact virion. And when it enters the cell... Sorry, go back. When it enters the cell, it will make a reverse strand or a negative strand, and it can also make sub-genomic sequences. And either the reverse strand or the sub-genomic sequences can be helpful to detect viral replication. Next slide.

So, by using the ISH technology, many groups now have been able to show that virus is in fact in the myocardium itself. And in our experience, most of the ISH-positive cells, which is usually a positive strand ISH, is detecting interstitial cells between the heart muscle cells, probably macrophages or or myeloid cells of some type. This can be associated with either myocarditis in a minority of cases, or some degree of inflammation not rising to the level of myocarditis in most cases. Next slide. These ISH-positive cells actually do correlate with the presence of markers of replication. In this particular study, looking at full transcript, full RNA profiling of the virus with NanoString, what you can see is that the positive strand, ISH-positive cells do correlate quite well with the presence of the reverse strand of the virus in the heart tissue indicating some degree of viral replication. Next slide.

Notice there is this one case where the ISH was negative, but there's a lot of positive signals for the positive strand but not for the reverse strand, and this is what you expect for circulating variant. So, when you're carefully looking at the tissue, you can distinguish between virus in the tissue versus virus in the blood, and it is making its way into the heart tissue. Next slide.

So, I told you most of the cells in the heart that are positive by ISH are these interstitial cells. You can occasionally see endothelial cells as you see in the top left. And in our patients who were heavily anticoagulated usually with therapeutic heparin levels, the presence of virus in the endothelial cells actually correlated with the presence of the CD61-positive platelet microthrombi. Also, in the bottom left we occasionally see virus within myocytes themselves. And when virus is present within the myocyte, it actually correlates with the presence of myocarditis. But again, myocarditis is an uncommon feature of this disease. Next slide.

So, inflammation in general is a pretty common feature in patients with both acute phases, and here we're going out almost to 50 days, so we're really talking about extended acute phase, both for lymphocytes and macrophages. There's a strong correlation with time from the onset of symptoms to death in these patients dying from a severe acute phase. And it turns out that this correlation is largely related to the presence of virus in the muscle. There's really not a correlation in the patients who did not have virus in the myocardium. So, virus in the myocardium seems to be driving this time-dependent increase in inflammation in the myocardium in this subset of patients.

Now, the question becomes what about patients who don't die of the acute phase? Some of these patients rarely will present heart failure with imaging findings of myocarditis. Some are not, most are not imaged. This was an example from a medical student at Indiana University who wrote up her own case report who was suffering from myocarditis. So even though you may not have severe acute phase disease, you can of course develop myocarditis subsequently after having SARS-CoV-2, but it is rare in this setting. But what we don't really know is for the vast majority of people who don't go into heart failure, what is happening to the inflammation within their myocardium after they recover from COVID-19, or when they're in the post-acute phase of COVID-19? We just don't know. Next slide.

So, moving on to the olfactory epithelium, there was a lot of questions of what was going on in this location given the anosmia that many people were experiencing. And it turns out that the virus appears to mostly be affecting the sustentacular cells, which you see on the left two panels, where the red virus is correlating with the blue. It does not tend to co-localize with the neurons, which explains why in some patients the anosmia is transient because the sustentacular cells can simply reproduce and replenish after they're injured. Next slide.

But this isn't the full story, this was a study out of Hopkins showing that there's other changes such as axonal injury in the olfactory tissue, and you can see the different stages of axonal injury in the top left. And the patients with COVID-19 not only have more axonal injury in the olfactory tissue than the controls, but those with abnormal sense of smell actually have more axonal injury than those with normal sense of smell even though they had COVID-19. And this unfortunately may not change or reverse rapidly, of course, it may even be permanent, which is why some people may not regain their sense of smell. Next slide.

So, in the brain there are changes during the acute phase of COVID-19. Again, micro-hemorrhages as you see in the top left, and these areas of axonal injury where you have increase in macrophages and loss of blue staining on Luxol fast blue. Unfortunately, these types of changes have not been correlated with virus and may simply represent changes secondary to the acute setting of the disease and the cytokine storm and microthrombi seen in the acute phase. Next slide.

However, virus can be detected in the brain, and this is in fact a study out of Banner Sun Health looking at different brain regions. And this was a very extensive study doing up to 80 PCR reactions per patient. And the key point to this study was that the most significant area containing virus, the area with the highest levels of virus, was

actually the olfactory bulb, which does seem to correlate with some of the other changes and symptoms that patients are having. Next slide. The peripheral nerves can also be involved. This was a study out of MGH showing that in patients presenting with clinical peripheral neuropathy on skin biopsy, the majority of them, 63% actually, had abnormally low levels of epidermal neurite density indicating that there are changes occurring in the peripheral nerves in the setting of Long COVID. Next slide.

So, this was a study out of Rockefeller showing that virus can be routinely detected in the GI tract. And these are examples from the small bowel, and if you look at the far right, the green dots are representing virus within the small bowel, and this is up to 173 days from the onset of infection. Interestingly though, this was not associated with any specific pathologic changes. Next slide. So, the pancreas is important because some of these patients develop new onset diabetes following the acute phase of SARS-CoV-2. This was a study out of Europe showing that the virus can actually localize within the islets of the pancreas and not only localized there, but inflammation is more intense in the islets of the pancreas compared with controls. But again, it's not a specific finding as you can see this inflammation in sepsis as well. Next slide.

So, a very important study that that did come out very recently was the study from NIH showing that you can detect virus by ddPCR in numerous organs throughout the body. And I've altered their figure a little, so you could easily look at different regions of the body, respiratory, cardiovascular, lymphoid, GI. One of the things you notice when you're looking at different timeframes, the acute phase, what these patients probably were in extended acute phase at 31 to 50 days, some of them may have been early post-acute phase, but then the true post-acute phase at 51 to 230 days, certainly you see the level of virus going down as the red color is more intense and the bluish colors are less intense.

But also, when you look at the post-acute phase patients, you don't see an obvious reservoir, you see a smattering of virus in a lot of different organs. And this is certainly one of the challenges that we face in trying to identify the reservoir. The vertical lines I should point out is the sub-genomic RNA, which initially is mostly in the lungs but also in other organs as well, and it does decrease in frequency as well over time. Next slide.

So, the viral load decreases in both the respiratory and non-respiratory tissues over time to the point that they basically equilibrate. And even though the lungs and the upper respiratory tract have high levels at the beginning, they end up equilibrating to the point that all organs are very similar once you get beyond six months. The question is what's happening after here? Because notice that both of these lines are intersecting those last group of patients at the bottom part. The question is, is this really flattening out or not? Next slide. So, is this going down or flattening out? And clearly more studies over longer periods of time are going to be important to understand how long virus is persisting within different organs in the body. Next slide.

So let me just wrap up with, at sites of high viral low, like the respiratory tract, there can be permanent tissue damage during the acute phase which may explain some forms of PASC. There's low level of extra-pulmonary organ involvement by SARS-CoV-2 in both acute and post-acute phases, but viral persistence in these

extra pulmonary sites in the post-acute phase may play a role in some forms of PASC, but the site and nature of the reservoir still is somewhat unclear and it's very important that we are able to have more studies correlating pathologic changes with both viral persistence and symptoms in the post-acute phase of COVID-19. All right, thank you.

Dr. Amy Proal

I will go next. I'm also very glad to be here. I'm going to give an overview of SARS-CoV-2 reservoir in Long COVID/PASC and follow up on some of what James just talked about. So, there's a variety, as Akiko emphasized, a variety of biological factors that can contribute to Long COVID/PASC symptoms in different patients. But several core biological trends have emerged as potential primary drivers of PASC pathology. And a growing body of evidence does suggest that a significant portion of PASC patients may not fully clear the SARS-CoV-2 virus after acute infection. And instead, a small amount of viral RNA potentially capable of translation, the production of viral proteins, may persist in patient tissue as a reservoir. And this persistence could modulate the local host immune response or lead to the ongoing or periodic release of viral antigen into the circulation.

So, there are a number of different studies now that show evidence of SARS-CoV-2 reservoir in samples collected from patients after acute COVID or in samples collected from patients with PASC. And the findings are compelling but of course all require further replication in additional cohorts. But nevertheless, evidence for viral reservoir in PACS so far comes from about three categories of research. And these are one, research demonstrating that SARS-CoV-2 RNA and protein are capable of persistence in a wide range of body and brain sites. Two, research has identified SARS-CoV-2 RNA or protein impacts samples or correlated identification of RNA and protein with PASC symptoms. And three, research on T or B-cell activity, the adaptive immune response that may reflect SARS-CoV-2 persistence in PASC.

Now, for the first category of research, studies that show SARS-CoV-2 capable of persistence in a wide range of body sites. The N NIH autopsy study that James just described in his talk is a prime example of this research. To recap, the team identified SARS-CoV-2 RNA and protein in various tissues of autopsy cases in which samples were obtained, in some instances, at least 31 days after acute COVID symptom onset. And 50% of these late cases had persistent RNA in the lymph nodes from the head and neck, from the thorax, in sciatic nerve, in ocular tissue, and in most sampled regions of the central nervous system including the cervical spinal cord and the basal ganglia. With sub-genomic messenger RNA, a potential marker of recent viral replication also identified in tissue obtained from some of these late cases indicating that SARS-CoV-2 replication may occur in non-respiratory tissues for several months at least after acute COVID.

And beyond this study, there are several other autopsy studies or studies where tissue samples were collected via biopsy or surgical procedures from patients that have also found SARS-CoV-2 RNA or protein in samples weeks or months after acute COVID. For example, this team found SARS-CoV-2 and nucleocapsid antigen

in olfactory mucosa samples collected from four patients with ongoing loss of smell after acute COVID, with samples collected between 110 days to 196 days after COVID onset. And a takeaway from this research is that studies of PASC blood alone are unlikely to reflect the extent of potential SARS-CoV-2 reservoir in the same patient's tissue.

For example, in the NIH autopsy study, SARS-CoV-2 RNA was detected in a perimortem plasma sample of only 12 of 44 cases. And negligible, if any RNA was detected in banked PBMCs from representative cases. And it's also important to consider that SARS-CoV-2 persistence in PASC made differ by cell type or body site due to differences in the local immune environment or the lifetime or turnover of infected cells. For example, viral persistence in long-lived cells, such as neurons or cardiac myocytes, may be different than viral persistence in gut epithelial cells that turn over more rapidly, so more research is needed.

And another thing to understand is that in nearly all studies where SARS-CoV-2 has been identified in acute COVID and tissue, the same subjects tested negative for SARS-Cov-2 via standard nasal PCR testing. In fact, in that olfactory study where subjects harbored persistent SARS-CoV-2 RNA and antigen in the olfactory and mucosa samples, the tissue of the nose, the same subjects tested negative for SARS-CoV-2 via routine nasal swab PCR. And here is another example. The team identified SARS-CoV-2 RNA and spike protein in the stool of 11 of 14 newborn babies born to mothers who had COVID that resolved 10 or more weeks prior to delivery. This suggests in-utero transmission of the virus an intestinal SARS-CoV-2 reservoirs in the newborns. And all newborns that harbor SARS-CoV-2 RNA in stool were negative for the virus via nasal swab PCR.

And will these newborns develop symptoms of PASC? We don't know, but still homogenates from 14 of the newborns elicited increased production of IL-6 and interferon-gamma from macrophage in vitro relative to non-COVID controls. So, it's a very concerning finding and it's just one of the reasons why the study of SARS-CoV-2 persistence is so important. Then we come to this second category of research, research that has identified SARS-CoV-2 RNA and protein in PASC samples are correlated, identification of RNA and protein with PASC symptoms. There are teams that have identified SARS-CoV-2 RNA and protein in PASC tissue acquired via biopsy procedures. For example, this team identified SARS-CoV-2 RNA and nucleocapsid protein in skin, appendix, and breast tissues of two patients who exhibited PASC symptoms 163 and 426 days after a symptom onset.

And this Stanford team tracked patients after COVID and found that four months after diagnosis, 12.7% still had SARS-CoV-2 RNA in stool. After seven months, 3.8% of subject stayed, and two individuals continued to shed RNA in stool at about 210 days post-infection. Importantly, PASC symptoms, including GI symptoms and systemic and respiratory symptoms, were associated with the presence of SARS-CoV-2 RNA in stool. And there are different ways to interpret these findings. You could say, "Oh good, persistent SARS-CoV-2 RNA seems to clear from most patients who've had COVID over time." Or you could say maybe over time persistent SARS-CoV-2 moves out of stool and deeper into the same patient's GI tissue where it can only be identified via tissue biopsy or

autopsy studies, which is why tissue biopsy studies are a very important focus of many PASC research teams that we are working with.

Now, this team used a convenient sample of inflammatory bowel disease patients undergoing routine colonoscopy or endoscopy to investigate the possibility that the gut may serve as a SARS-CoV-2 reservoir site. And despite mild acute cases, 77% of patients had SARS-CoV-2 RNA in gut mucosa about seven months after acute COVID. Viral nucleocapsid protein was also identified in gut epithelium and in CD8 T-cells in 52% of these patients. Viral RNA and protein persistence were unrelated to the severity of acute COVID or immunosuppressive therapy, but they did associate with PASC symptoms. In fact, only patients with detectable viral RNA in mucosal tissue reported symptoms compatible with PASC.

Now, a question with these findings is, can infectious virus be detected in samples? And that's because detection of infectious virus is gold standard for identification of replicating virus. And in the study I just showed you, the team did not succeed in culturing infectious virus from the intestinal or mucosal gut samples with persistent RNA and protein in their cohort, but there are considerations with culture when it comes to persistent RNA viruses. First, there are technical limitations. Culture usually requires co-cultivation with susceptible cells and may be influenced by the presence of neutralizing antibody and sample. But most importantly, mechanisms have been delineated whereby persistent RNA viruses deliberately suppress the production of infectious variants to facilitate the survival of the cells they've infected, and this facilitates persistence.

For example, they acquire mutations that decrease variant assembly or decrease RNA synthesis. Indeed, the acquisition of viral mutations is a well-established mechanism that facilitates the persistence of certain RNA viruses including measles and even other coronaviruses. In this review by Diane Griffin at Johns Hopkins called, "Why does viral RNA sometimes persist after recovery from acute infection?" Is critical to read to understand these mechanisms, it's a must-read if you're studying viral reservoir impacts. Another consideration when interpreting findings on viral reservoir in PACS is that overt inflammation does not have to be identified near persistent SARS-CoV-2 RNA protein for disease processes to occur.

For example, in that NIH autopsy study of SARS-CoV-2 persistence throughout the body and brain, the team observed little evidence of inflammation or direct viral cytopathology in much of the tissue they examined from cases after acute COVID, but persistent virus may down-regulate the immune response to drive disease as opposed to activating it. In fact, SARS-CoV-2 expresses several proteins that down-regulate the host immune response including host interferon signaling. Also, if a virus like SARS-CoV-2 is able to gain access to the nucleus to the center of the cells that it infects, it can directly interfere with human transcription, translation, DNA repair processes, and even the epigenetic environment. So, to better understand what is happening in PACS with viral reservoir, we need to use sequencing technologies on patient tissue samples to deeply characterize host immune and gene expression changes near-identified SARS-CoV-2 RNA or protein.

Now, returning to evidence for SARS-CoV-2 reservoir in PACS, other teams have identified SARS-CoV-2 protein in PACS plasma months after acute COVID. This German team measured circulating SARS-CoV-2 S1 protein in PACS subjects and controls, which were pride COVID individuals who did not develop PACS. And because S1 protein has been detected in plasma after vaccination, they restricted their analysis to individuals without prior vaccination. And in the PACS group, circulating S1 was detected in around 64% of individuals. And the past group also showed higher circulating S1 levels as compared to controls. However, about 35% of the controls did show measurable levels of circulating S1 protein.

Now, then this Harvard team used optimized ultra-sensitive single-molecule array Simoa assays to look for SARS-CoV-2 antigen in PACS plasma. And they did identify either spike, S1, or nucleocapsid protein in about 65% of plasma samples collected from PACS subjects several months after acute infection. Spike was detected most often in 60% of patients up to 12 months post-COVID with no spike detected in controls, which were again, COVID patients who did not develop PACS. In PACS, cases where the team obtained longitudinal samples, viral protein was detected at more than one sampling time point in 12 patients.

Now, how do we interpret these findings? Why is viral protein or antigen being identified alone in PACS plasma as opposed to the virus itself? Well, there's much more research needed on the topic, but one hypothesis is that SARS-CoV-2 itself may persist in PACS tissue reservoir sites, but full-length, uncleaved spike antigen may regularly butt off the virus or infected cells. And because spike is a transmembrane protein, it may circulate associated with a membrane in exosomes, small extracellular membrane vesicles, and enter the bloodstream via that transport. And indeed, this USF team did identify or higher mean levels of SARS-CoV-2 S1 in nucleocapsid protein, and in rich-plasma neuron, and astrocyte-derived vesicles, extracellular vesicles, impacts patients as compared to resolved acute COVID controls.

And I know that this team and the Harvard team are pursuing extended research on this topic of the antigen and exosomes. And it's worth noting that if this finding of SARS-CoV-2 antigen and plasma hold, they also strongly suggest that SARS-CoV-2 and RNA in patients with PACS is going through periods of replication. Otherwise, it is hard to explain why viral antigen would regularly be found in plasma and not cleared away.

Now three, we come to this third category of research on viral persistence impacts, research on tier B-cell activity that may reflect persistence of the virus. And this is important area of research because the adaptive immune response can be used as a very sensitive detector of viral persistence because with transcription-based technologies you can elucidate the specific stage of a tear B-cells lifespan, including a very recent detection of antigen. And it follows the studies of antibody secreting cells, T-cells, and B-cell, and related immune cell activity can be used to infer the presence of SARS-CoV-2 reservoir in patients with PACS.

Here's one study that went in that direction. The team found that in pulmonary PACS patients when compared to recovered controls had between six to 105-fold higher frequencies of interferon gamma and TNF producing SARS-CoV-2-specific CD4 and CD8 T-cells in peripheral blood using stimulation with peptide pools from

multiple SARS-CoV-2 proteins. And SARS-CoV-2-specific TNF producing CD8 T-cells had high Ki67, which is a marker of recent proliferation suggesting recent antigen stimulation in the PACS patients in the study with the antigen stimulation likely being tied to viral reservoir. More research needed.

Here is a study where B-cell activity strongly supports the finding that study subjects harbor a SARS-CoV-2 reservoir in tissue. The team collected pharyngeal lymphoid tissue, a mix of tonsil and adenoid tissue from non-vaccinated, COVID-convalescent children undergoing surgery. And the majority of tissue samples examined were positive for SARS-CoV-2 nucleocapsid RNA despite negative nasal swab PCRs in the same children at the time of surgery. And in fact, four of the children whose tissue was examined in the study had suffered from COVID at least 100 days before surgery, including one 303 days before surgery. Viral nucleocapsid RNA was not identified, by the way, in tissues from any infected controls, only in the children with evidence of COVID infection.

Now, coming back to the B-cell signaling, the team identified persistent expansion of germinal center, an antiviral lymphocyte populations associated with interferon gamma-type responses in the children's tissue. And single B-cell receptor sequencing indicated viral-specific, SARS-CoV-2-specific B-cell receptors were class-switched and somatically hypermutated. SARS-CoV-2 RNA copies in tissue significantly correlated with the percentages of S1 receptor binding domain positive B-cell among germinal center B-cell in lymphoid tonsil tissue. And this raises the strong possibility that it's SARS-CoV-2 antigen persistence as part of a viral reservoir in these children that contributed to the prolonged lymphoid and germinal cell responses in the study subjects.

And you can tell from this how you can then use this, the adaptive immune response, to infer the potential presence of antigen in the same study subject. Now, that study did not measure symptoms in the children, so we don't know if they had PACS symptoms, although they were undergoing surgery for sleep-disordered breathing or obstructive sleep apnea, which might have impacted the findings. But overall, it's important to note that multiple studies have identified SARS-CoV-2 RNA and protein in patients who were asymptomatic at the time of sample collection. So further research is needed to better understand factors that may lead to the development of specific symptoms in PACS patients harboring SARS-CoV-2 RNA and protein. And these factors include location of infection, transcriptional and translational activity of SARS-CoV-2 RNA and human genome mutations or HLA haplotypes that may predispose to differences in host innate or adaptive immune responses to persistent viral RNA or antigen that lead to symptoms in certain patients.

Now, in my last few minutes, I want to quickly discuss the relationship between SARS-CoV-2 reservoir and other biological abnormalities that have also been identified in PACS patients. For example, this South African team used florescence microscopy to demonstrate the presence of fibrin amyloid microclots resistant to fibrin lysis or breakdown in platelet-poor plasma samples collected from PACS subjects. And several teams that we work with at different institutions have preliminary data that replicates these findings. Now, it's possible that PACS patients who have microclots do not harbor SARS-CoV-2 reservoir, but the two factors can be connected.

In a separate study by the same South African team, the team added the SARS-CoV-2 S1 protein to healthy platelet-poor plasma. That resulted in structural changes to fibrinogen, including resistance to trypsinization that are very similar to the fibrin deposits identified in the PACS microclots. And other teams have also identified SARS-CoV-2 spike protein in COVID thrombi or reported that SARS-CoV-2 spike protein combined to fibrinogen and induced structurally abnormal blood clots with heightened pro-inflammatory activity. So it may be that SARS-CoV-2 reservoir and microclotting are connected. And remember that German team did find SARS-CoV-2 S1 protein in a high proportion of plasma samples in their PACS cohort.

Now, there are also studies that have found microbiome alterations or imbalances in patients with PACS or signs of intestinal permeability and the translocation of organisms from the gut into the bloodstream. For example, this team documented higher levels of fungal translocation as measured by beta-glucan, a fungal wall cell polysaccharide in the plasma of PACS patients as compared to controls. And they also found that PACS was associated in increased plasma levels of zonulin, which is a biomarker of intestinal permeability.

The translocation of organisms from gut to blood could happen in PACS patients without SARS-CoV-2 reservoir, but it is possible for the two factors to be connected. For example, this team studied children with MIS-C, a severe SARS-CoV-2-related inflammatory disorder with strong parallels to PACS. And they found that children with MIS-C harbor SARS-CoV-2 RNA in stool weeks after initial infection. And this persistence of SARS-CoV-2 RNA in stool was accompanied by significantly increased release of zonulin, a biomarker of intestinal permeability, and the identification of SARS-CoV-2 antigen in plasma. So, it may be possible that it is the inflammation associated with viral RNA persistence in the GI tract that is part of what facilitates breakdown of the mucosal barrier in these children and facilitates the translocation of viral antigen into the bloodstream leading to hyper inflammation in certain cases. In other words, the GI persistence of SARS-CoV-2 and other issues like intestinal permeability, leakage of antigen, or even microbiome issues, may be connected.

Another finding demonstrated in multiple PACS studies is reactivation of Epstein-Barr Virus in at least a subset of patients. Epstein-Barr Virus reactivation could happen in PACS patients without SARS-CoV-2 reservoir, but the two factors could also be connected. That's because SARS-CoV-2 expresses multiple proteins that allow it to counteract interferons. And interferons are the primary class of antiviral cytokine that play a central role in successful control of all viral infection including EBV infection and latency. So, a PACS patient with SARS-CoV-2 reservoir may be more likely to have EBV reactivate in the same immune environment.

And I could go on, I could talk about mechanisms by which SARS-CoV-2 reservoir could contribute to changes in neuroimmune signaling in patients with PACS or affect pathways that can contribute to neuroinflammation or even neuropathy. But overall, the takeaway is that we should study SARS-CoV-2 reservoir impacts in concert with other related biological factors in the disease. And I think that's how we will get the most rapid clarity on what is happening in PACS patients. Thank you.

Dr. Marrah Lachowicz-Scroggins

Thank you very much. So, I want to take this opportunity again to thank our speakers for their engaging presentations. And so, before we begin the panel discussion, I just want to give a brief recap on some of the main themes that were presented today as a primer for this year's RECOVER R3 series on the pathobiology and mechanisms of PASC. And so many hypotheses were raised today, and we hope that you will follow the series throughout the year as some of these pathways will be discussed in more detail and we hope that you'll participate in these future seminars.

So, we kicked off today's series with the foundational concept that post-acute viral syndromes are not new and can occur with many different pathogens. However, clinical presentations of these syndromes can vary widely. And while there's high value in shared learning across these syndromes, there's also strong rationale to do a deep dive into them individually. This was supported with data for the unique immunopathology of COVID and strategies using clinical data, machine learning, and immunoprofiling in the Long COVID clinic to systematically narrow down several hypotheses about contributing factors to PASC such as latent virus reactivation, residual tissue damage, and persistence of viral pathogens.

And next, we saw evidence on how acute infection can result in residual tissue damage in the heart, lungs, olfactory tissues, the brain, and GI tract, all of which can be linked to long-term symptoms in PASC. This was presented in well-documented, thorough, and rigorous autopsy and histopathology studies. Additionally, it was noted that tissue pathology after the post-acute phase can vary and understanding this variation may also help us understand how residual tissue damage may contribute to the long-term pathology seen in PASC. And this is an ongoing area of research. It was also noted that understanding key factors such as underlying comorbid conditions at the time of infection is also important.

Finally, the duration of local inflammation related to viral load and infection at extrapulmonary sites were noted as potential predictors of the onset and severity to PASC. Finally, across the presentations was the hypothesis of viral persistence, including persistence of virus or viral products as potential mediators to PASC. Limitations to prior research were noted, such as understanding what tissues are at the actual reservoirs and how long does a virus need to persist in that area to perpetuate immune inflammation and how to best sample these tissues, i.e., what tissues should be sampled and what techniques are needed to be employed, and when and how to sample. Current progress in these areas was presented, but open questions still remain.

The majority of questions of how the virus can cause such widespread and diverse pathology after acute infection is still a really big ongoing area of research, and additional focus on viral persistence will actually be the next topic for the R3 seminar series later this month.

So, with that, I'd like to try to get a couple of questions into the panel. The first one is related to a few of the audience questions that were submitted prior to the seminar today and also in the Q&A tool, is really how are we translating the information that was presented today into treatment and to care of those affected by PASC?

Given where we are and what we know now about risk factors and several solid plausible hypotheses that were presented for the mechanisms of PASC, including those today, how are we translating these into the PASC clinic ensuring that key specialties are represented, and the right testing is being done to identify the most likely pathways and helping to rapidly identify subsets of PASC patients and potential treatment options? So maybe I'll open that to Dr. Iwasaki first.

Dr. Akiko Iwasaki

Thank you so much. Oh, that was a great summary you just gave us. Thank you so much. So how are these basic insights leading to treatment options? That's a very important question, and I know so many people are waiting to be treated for their debilitating symptoms. That is why I emphasize the importance of trying to understand the endotypes of Long COVID. Let's say if the viral persistence was the main driver of Long COVID in a subset of patients, if we can find biomarkers to subset those patients, then we can start providing them with appropriate medicine like antivirals or monoclonal antibodies, or something to get rid of the viral reservoir. If it's just RNA alone, it might be a little bit difficult to get at that. But if there's some protein involvement, we can still use antibodies and other measures.

If it's a replicating virus, Paxlovid and other antivirals may be a viable way of treating these types of diseases. However, if it turns out that a subset part of patients is suffering from autoimmunity, antivirals are not going to work. So, there we might have to employ anti-inflammatories or immune antibodies against cytokines or JAK inhibitors, things to pump down the autoimmune components. And again, understanding what biomarkers might indicate people suffering from autoimmunity versus viral persistence would be very helpful in categorizing patients for treatment options.

And if let's say latent reactivation of EBV is contributing to the symptoms, there are antivirals that are potentially useful in that area as well against EBV and other DNA viruses. And so, understanding which type is present in which patients is a key first understanding to even suggesting what the appropriate treatments are. But at the same time, this is a very urgent issue. People are really desperate for therapy, and I think we should go ahead and do well-designed randomized clinical trials to see what patients are benefiting before and after and have biomarkers that correlate with that response. And that way, without knowing the molecular mechanism, we can start to understand by experiments and sort of measurements of these patients who might benefit and how that can inform future therapy options. So, I'm giving you two ways to approach this, but they should be done at the same time.

Dr. Marrah Lachowicz-Scroggins

Thank you. So, Dr. Stone, do you have anything to add related to how the tissue pathology studies are translating into potential clinical interventions?

Dr. James Stone

So, it's unfortunately a longtime course. It takes a while to develop therapies. I think the tissue pathology studies are supportive of the fact that they're for patients who are suffering, we're saying yes, there is indeed something going on. And we're seeing it, we are seeing viral persistence, we are seeing some changes in the tissue, but it's going to be difficult to really sort out what the proper target is and whether it's inflammation or whether it's virus without inflammation, that's the issue. And I know people really want us to be launching into therapies right away, but it does take time to figure out the right therapies so that we do more good than harm as we try to launch new therapeutic trials.

Dr. Marrah Lachowicz-Scroggins

So, in the interest of time, I'm going to close this question out with Dr. Proal and ask you to follow up on maybe what potential biomarkers seem most interesting and most valuable for us to translate into the clinic related to some of the things that we presented in your talk, and then I'll give it to Lisa to do the audience questions.

Dr. Amy Proal

Sure. So, I think that one of the top potential biomarkers would be those studies in which antigen was found in plasma, there's a decent hope there that those studies are... They have to be replicated in other cohorts; we have to still see. For example, I do think that the Harvard team that used the very sensitive Samoa assay to find spike in plasma, I do think they did work on a different cohort that had had more infection after omicron and didn't see the same level of spike. So, there's going to be a lot of considerations with these studies, and yet being able to identify antigen and plasma would be huge because plasma is regularly most easily collected from patients, because it is a big challenge here when we're thinking, when we're seeing a lot of tissue-based pathology happening impacts.

And so, we have the virus may be in someone's nerve or in someone's tissue sample, but of course biopsying, getting biopsy from patients can be difficult. That being said, our Long COVID research consortium is working on some tissue types that can be biopsied, for example, more easily at routine appointments than others. For example, there are punch biopsies that happen with teams we work at Harvard MGH regularly, and we are working those samples up in case there's signal in those tissue samples that are regularly connected at appointments from patients. Also, lymph node aspirate can sometimes be collected with a fairly routine procedure.

So, we're making sure that we analyze all these different types of samples to best understand what can be found where. And also, I think it's key that in the research studies that we're doing, we're combining patients who

have, for example, an intestinal tissue sample collected via colonoscopy with the analysis of blood in the same patient to figure out how much we can see and what correlates between what's found in the tissue and what we can pick up in blood, because the goal is to have a blood test that I can identify antigen or that.

So, I think that's the most hopeful biomarker. I actually do think that there is a Paxlovid clinical trial that we'll be using, or there's one or two Paxlovid clinical trials that we'll be using that testing for spike protein as one of the outcome markers. And I think also Akiko could speak to some of what she's been measuring being used as outcome measures in clinical trials. So that is moving forward, and so I think that while more research is needed, there are some leads especially there on what can be measured in blood from what Akiko's measuring and the spike in the antigen.

Dr. Marrah Lachowicz-Scroggins

All right. Thank you. We'll turn it over to Lisa to take some of the audience questions.

Lisa Newman

Okay, thank you. What do we know about the role mitochondrial dysfunction plays in Long COVID? Dr. Proal, do you want to take that one?

Dr. Amy Proal

Sure. I wrote a paper with my close colleague, Mike VanElzakker, called Pathogens Hijack Host Cell Metabolism. And it's a paper that actually walked through the mechanisms by which viral, bacterial, and fungal pathogens hijack the metabolism of the cells that they infect. And one thing to understand about viruses is their obligate intracellular pathogens. So, they require, when they replicate, they need to create another copy of their backbone and they must pull from substrates produced by the host cell mitochondria in order to proceed with that replication. And that undoubtedly will change the metabolism of the host cell in that infected cell. So, there is complete correlation between viral activity, viral replication in the metabolic profile of the cell.

So, it's a completely connected topic, and I think that it will be important to study changes in metabolism in concert with studies of the SARS-CoV-2 virus itself. In other words, not to approach the topics, that's two separate things and say, "Oh, this team's going to work on the virus and this team's going to work on metabolism." But the field of immunometabolism now is combining those topics. And if researchers in that field that's, for example, our articles published in the journal Immunometabolism, these research teams, these areas that are combining those areas of research are going to be important to pull into the PASC field.

Lisa Newman

Thank you. Dr. Iwasaki, this one is for you. Several epi studies are now pointing to an increased incidence of PASC in women. What do we know about mechanisms for this sex difference?

Dr. Akiko Iwasaki

That's a great question. We're actually preparing a paper on that as we speak. So, the MY-Long COVID study that I described to you today was not sex disaggregated but are now doing a study on that same subject to see whether there is any sex differences in immunity as well as symptoms. And there's actually a very distinct difference between male and female PASC patients with respect to symptoms, their immune responses, and the link between the immune response to the symptoms. So that's coming up. One particular interesting thing that we're seeing is that the EBV reactivation and the IL-6/IL-4 double positive CD4 T-cell, that link is particularly dominant in women compared to male patients. So, we will be learning a lot about that, but it's a great question.

Lisa Newman

Thank you. Dr. Stone, this one is for you. Are microthrombi in the lungs an acute infection the same as microclots detectable in Long COVID?

Dr. James Stone

An interesting question. Part of the problem in the acute phase is that the thrombi that we detect, particularly at autopsy, are heavily dependent on how the patient was treated. So even within deaths in the acute phase, thrombi can either be entirely fibrin thrombi or they can be platelet microthrombi. And it often determines how the patient was anticoagulated prior to death. There really haven't been, to my knowledge, systematic studies trying to compare microthrombi in Long COVID with acute COVID. Part of the issue is we don't tend to see as many microthrombi in the tissues of patients in the post-acute phase, so it's much harder to find those and to compare them. So, I don't know if there's a systematic difference or not at this point.

Lisa Newman

Thank you. And Dr. Iwasaki, given the intriguing finding of reduced cortisol levels in Long COVID, have HPA axis studies been conducted in these patients? And if so, what were the results?

Dr. Akiko Iwasaki

Yeah, another excellent question. So, as I mentioned, cortisol is regulated in diurnal fashion during the day. And so we're now collaborating with David Putrino's lab to collect diurnal levels of saliva cortisol from a number of his patient participants to see if we can see if there's any changes of their levels during the day. As I mentioned, we only collected one time point sample from these participants so far. So, we want to know that during the day if there's any difference in the pattern. And if so, if there's any difference in the ACTH level and other hypothalamic controls. And for that, we may need to do more MRI studies and others, which are also ongoing with David Putrino's group.

Lisa Newman

Great. And I think there's probably time for at least one last question. Again, for Dr. Iwasaki. Were you able to segment immunoprofiling for just brain fog sufferers? And was there any pattern of interleukin elevations of interest?

Dr. Akiko Iwasaki

Oh yeah, that's a great question once again. So, I mentioned the study that we published with Michelle Monje's group at Stanford. There we saw elevated CCL11, which is known as Eotaxin-1 as well, in circulation that correlated with the brain fog reporting by the same Mount Sinai participants that we're studying. And that tended to be elevated more in male than female. So that's interesting, that particular chemokine is correlating with brain fog, but more dominantly in male. So, there's a lot to study there.

Lisa Newman

A lot more to look at. All right. I think with that, we're at time, so I think we should close. But thank you, what an elucidating and intriguing set of presentations. Thank you to all of our panelists and thanks to the audience for joining us today. And as Dr. Lachowicz-Scroggins said, these presentations represented an overview of mechanistic pathways, and we will be deep diving on some of these pathways going forward.

An FAQ document for this webinar will be posted along with the recording of the webinar on recovercovid.org. It will include answers to all of the questions that were asked today and those that were submitted in advance. Questions about other scientific topics will be addressed in future webinars, and answers to broader questions about RECOVER will be available in the FAQs recovercovid.org. So, I encourage you to attend future R3 webinars that will deep dive into some of these broader topics. And again, thank you. Great presentations and great discussion and have a great afternoon and a good week.

Webinar Slides

To request a copy of the R3 Webinar slides, please email RECOVER_ACC@rti.org.

To Learn More

- Information about RECOVER research and to volunteer for studies: https://recovercovid.org/research
- Frequently Asked Questions about RECOVER and PASC: https://recovercovid.org/faqs
- CDC information: Information for the general public and for healthcare providers about post-Covid conditions: https://www.cdc.gov/coronavirus/2019-ncov/long-term-effects/